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Specific N-terminal cleavage of ribosomal protein L27 in *Staphylococcus aureus* and related bacteria

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Summary

Ribosomal protein L27 is a component of the eubacterial large ribosomal subunit that has been shown to play a critical role in substrate stabilization during protein synthesis. This function is mediated by the L27 N-terminus, which protrudes into the peptidyl transferase center. In this report we demonstrate that L27 in *Staphylococcus aureus* and other Firmicutes is encoded with an N-terminal extension that is not present in most Gram-negative organisms, and is absent from mature ribosomes. We have identified a cysteine protease, conserved among bacteria containing the L27 N-terminal extension, which performs post-translational cleavage of L27. Ribosomal biology in eubacteria has largely been studied in the Gram negative bacterium *Escherichia coli*; our findings indicate that there are aspects of the basic biology of the ribosome in *S. aureus* and other related bacteria that differ substantially from that of the *E. coli* ribosome. This research lays the foundation for the development of new therapeutic approaches that target this novel pathway.

Keywords

Ribosome assembly; Protease; Translation

Introduction

Ribosomes, the molecular machines that carry out protein biosynthesis, are ribozymes composed of ribosomal RNA (rRNA), which performs the primary catalytic function, as well as numerous proteins that are essential for ribosome assembly and that modulate ribosomal function. Ribosomal biology has been intensively studied in the Gram negative eubacterium *Escherichia coli*, but is less well understood in other bacteria. While ribosomal

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structure and function are generally conserved across all domains of life, there are differences in composition that impact ribosomal size and function between eubacteria, archaea and eukaryotes and that could potentially be exploited for therapeutic purposes.

Ribosomal protein L27, product of the *rpmA* gene, is a component of the large ribosomal subunit found only in eubacteria and in the ribosomes of mitochondria and chloroplasts. L27 is highly conserved, and although deletions can be tolerated to a limited extent in some bacterial species, *rpmA* is generally considered an essential gene. L27 consists of a C-terminal β -sandwich domain and a long N-terminal arm that extends into the region of the ribosome known as the peptidyl transferase center (PTC), where it plays a critical role in tRNA substrate stabilization during the peptidyl transfer reaction (Wang *et al.*, 2004a; Maguire *et al.*, 2005; Trobro *et al.*, 2008; Voorhees *et al.*, 2009). The 3' ends of both A- and P-site tRNAs in the PTC can be crosslinked to L27 in *E. coli* (Wower *et al.*, 1998), and structural studies place the N-terminus of L27 within hydrogen bond distance of both the A- and P-site tRNAs (Voorhees *et al.*, 2009). In a genetic complementation assay, deletion of the first three N-terminal amino acids (A2 H3 K4; the universal fMet is removed) from *E. coli* L27 resulted in a considerably decreased growth rate, loss of tRNA crosslinking and a defect in peptidyl transferase activity (Maguire *et al.*, 2005). FRET analysis of peptidyl-tRNA dynamics implicated L27 residue K4 in stabilization of the P-site tRNA (Wang *et al.*, 2012; Xiao *et al.*, 2012). Deletion of the entire *E. coli rpmA* gene led not only to a severe growth defect but also incomplete assembly of the 50S ribosomal subunit, indicating a role for L27 in ribosome assembly as well as catalysis (Wower *et al.*, 1998).

We previously noted that L27 proteins in *S. aureus* and other Gram positive bacteria have an N-terminal extension that is not present in *E. coli* L27 (Spilman *et al.*, 2012). Our discovery of this extended sequence had its genesis in our studies of capsid assembly in staphylococcal phage 80 α . The phage-encoded scaffold and capsid proteins are both subject to N-terminal processing, with cleavage within a conserved amino acid motif near their N-termini (Poliakov *et al.*, 2008) (Figure 1A). Subsequently, we found that this processing of capsid components was carried out not by a phage-encoded enzyme, as is usually the case, but by a host enzyme; expression of the full length capsid and/or scaffold genes in *S. aureus* yielded cleaved proteins whereas no such cleavage was observed when these genes were expressed in *E. coli* (Spilman *et al.*, 2012). We postulated that the protease responsible for this cleavage played a role in the normal biology of *S. aureus*, as it seemed unlikely that *S. aureus* encodes a protease for the purpose of bacteriophage capsid maturation. A database search for predicted *S. aureus* proteins containing the same conserved N-terminal sequence motif identified a single candidate protein, ribosomal protein L27 (Spilman *et al.*, 2012). This protein contained an N-terminal extension of nine residues compared to *E. coli* L27, with cleavage predicted to generate an N terminus identical to that found in *E. coli* L27. Several lines of evidence were consistent with this postulated cleavage. Based on the structure of L27 and its position in known ribosome structures (Voorhees *et al.*, 2009), the N-terminal extension would reach into the PTC and interfere with the peptidyl transfer reaction by occluding the residues responsible for tRNA stabilization. The N-terminal four residues of *E. coli* L27 constitute a highly conserved A(S/H)KK motif that is also found in *S. aureus* L27, immediately following the 9-residue N-terminal extension (Fig. 1A).

Antibiotics that inhibit the peptidyltransferase reaction by obstructing the PTC, such as oxazolidinone, crosslink to *S. aureus* L27 residues in the A(S/H)KK motif (Colca *et al.*, 2003; Leach *et al.*, 2007) and not to any residues N-terminal to those. These observations suggested that the N-terminal extension is not present in the mature, functioning ribosome. Consistent with this expectation, no tryptic peptide corresponding to the N-terminal extension was found in ribosomes isolated from *S. aureus* (Colca *et al.*, 2003). The *Bacillus subtilis rpmA* gene also encodes an L27 protein with a similar N-terminal extension (Fig 1A), and these extra amino acids are lacking in the L27 protein isolated from *B. subtilis* ribosomes; this discrepancy had been noted previously, but attributed to misannotation of the gene (Lauber *et al.*, 2009). Since L27 is positioned at the heart of the ribosomal active site, where it plays a key role in translation (Maguire *et al.*, 2005), we sought to elucidate further the role of this N-terminal extension in *S. aureus* biology.

In this report, we describe further the distribution of L27 proteins with this N-terminal extension motif, which immediately precedes the highly conserved A(S/H)KK motif that constitutes the N-terminus of L27 in *E. coli*. We demonstrate that this extension is cleaved post-translationally in *S. aureus*, prior to or concurrent with ribosome assembly. We have identified a cysteine protease, universally conserved among bacteria containing the L27 N-terminal extension, which performs this cleavage. Both L27 and the protease, which we have termed Prp, are essential in *S. aureus*, indicating that this cleavage event is essential as well. The recent emergence of virulent, antibiotic resistant strains of *S. aureus* that spread among otherwise healthy individuals is a considerable public health concern that necessitates the need for new antibiotics. Prp provides an attractive target for the development of antibiotics specific to *S. aureus* and other Gram-positive pathogens in which this specific L27 processing occurs.

Results

Cleavage of L27

A recent deep sequencing study of eubacterial ribosomal proteins placed Firmicutes, Tenericutes and Fusobacteria into one of three new “megaphyla” dubbed Megaphylum III (Yutin *et al.*, 2012). Our bioinformatic analyses revealed that all sequenced Firmicutes, Fusobacteria, and Synergistetes, as well as some Thermatogae and Tenericutes, encode an L27 containing an N-terminal extension with the conserved cleavage motif (Fig 1B). Taken together, these data support the existence of a group of evolutionarily related bacteria that exhibit a fundamental difference in the basic biology of the ribosome, involving a previously undescribed ribosomal processing event.

We tested the predicted N-terminal cleavage of *S. aureus* ribosomal protein L27 by comparing the products of the cloned full length gene in *S. aureus* and *E. coli*, as we had done for the phage 80 α capsid and scaffold proteins. To purify and identify *S. aureus* L27 in *E. coli*, we introduced C-terminal Myc and His₆ tags using vector pBADmycHis A (Invitrogen). The same tagged L27 cassette was introduced into the T7 expression plasmid pG164 (D’Elia *et al.*, 2006) for expression in *S. aureus*. The His-tagged proteins in clarified lysates from each overexpression strain were batch adsorbed to Nickel NTA resin (Clontech) and purified proteins were then examined on Western blots probed with anti-Myc

antibodies. L27 isolated from *S. aureus* is approximately 1 kDa smaller than the same protein isolated from *E. coli* (Figure 1C; lanes 1 and 2), consistent with the predicted N-terminal cleavage by a *S. aureus* protease that is absent from *E. coli*. The L27 protein expressed in *S. aureus* was extracted from the gel and subjected to N-terminal protein sequencing by Edman degradation. The first four residues were ASKK, confirming the predicted processing of L27 in *S. aureus* at the conserved cleavage motif. Apart from the common removal of the N-terminal formyl methionine, this is the first example of a specific N-terminal cleavage of a bacterial ribosomal protein precursor.

Identification of the protease

We expected the protease responsible for *S. aureus* L27 cleavage to be essential and conserved among bacteria with the L27 N-terminal cleavage motif. Our attention was drawn to an open reading frame of unknown function, first designated *ysxB* in *B. subtilis* (locus tag SAOUHSC_01756 in *S. aureus* NCTC8325), located between the genes encoding L21 (*rplU*) and L27 (*rpmA*). This open reading frame was an attractive candidate for the protease gene for several reasons: First, while *rplU* and *rpmA* are adjacent in most bacterial species (Garcia-Vallve *et al.*, 2002), all bacteria that encode an L27 with the N-terminal extension also carry a *ysxB* gene (Fig. 1B). Second, both *rpmA* and *ysxB* were classified as essential in *S. aureus* by saturation transposon mutagenesis (Chaudhuri *et al.*, 2009) and in an earlier antisense RNA study (Ji *et al.*, 2001). Third, the YsxB protein has features characteristic of a protease. The YsxB structure was solved in a structural survey of *S. aureus* proteins (PDB ID: 2P92) and was grouped with other similar structurally characterized proteins containing a common domain in NCBI's Conserved Domain Database (CDD) under the designation DUF464. The crystal structure of a DUF464 member from *Thermatoga maritima* (1S1L) was published in 2005, and the authors noted that the conserved residues could possibly constitute a catalytic center (Shin *et al.*, 2005). The structures of two additional family members from *Streptococcus mutans* (PDB ID: 2G0I) and *Streptococcus pneumoniae* (PDB ID: 2IDL) have also been solved. DUF464 proteins are distinguished by a pair of invariant histidine and cysteine residues with conserved spacing that form the classic catalytic dyad of a cysteine protease (Fig. 2A), and are clustered together in a cleft on observed DUF464 structures (Fig. 2B). In addition to the *ysxB* homologues present upstream of *rpmA*, there are a number of phages that encode a DUF464 family member (Fig. 2C). One member of this family has been shown to be a bona fide bacteriophage prohead protease, responsible for capsid protein cleavage in streptococcal phage Cp-1 (Martin *et al.*, 1998).

When *S. aureus* L27 Myc-His₆ and untagged YsxB were co-expressed in *E. coli*, the resulting tagged and purified protein was the same size as L27 produced in *S. aureus*; both are approximately 1 kDa smaller than L27 expressed in *E. coli* in the absence of YsxB (Figure 1C). Edman degradation confirmed that the N-terminus of *S. aureus* L27Myc-His₆ coexpressed with YsxB in *E. coli* was also ASKK. These data demonstrate that YsxB can perform specific N-terminal cleavage of *S. aureus* L27 in *E. coli* and confirm that this conserved gene of previously unknown function encodes the L27 protease. We have named this gene *prp*, for phage-related ribosomal protease. Further evidence to support the classification of this protein as a cysteine protease was provided by mutation of the predicted catalytic cysteine residue, C34, to alanine or serine, which resulted in the

inactivation of Prp (Fig 1C, lane 4; Figure S1A). Furthermore, when the uncleaved L27myc-His was purified from a strain co-expressing Prp C34A or C34S, the mutant Prp co-purified as an 11 kDa band on the gel accompanying the 13 kDa L27Myc-His₆ protein (Figure S1B). The identity of the 11 kDa co-purifying band was confirmed by mass spectrometry. These observations indicate that the mutant protease remains tightly bound to its substrate in the absence of catalysis. They also show that replacement of cysteine with serine in the catalytic site does not allow Prp to function as a serine protease, despite the presence of a conserved aspartic acid in the DUF464 motif.

The original identification of Prp was based on the cleavage of the major capsid and scaffolding proteins of bacteriophage 80α in *S. aureus*. We therefore confirmed that Prp was able to carry out the previously observed N-terminal cleavage of these two phage structural proteins upon co-expression in *E. coli* (Fig 2C). The small differences in electrophoretic mobility of these two proteins were shown to result from the expected sequence-specific cleavages by determining the total mass of the processed proteins by MALDI-MS and further confirmed by LC-MS of tryptic peptides. Prp thus serves an additional function in *S. aureus*, as the prohead protease for 80α and related staphylococcal phages whose capsid and scaffold proteins share the conserved N-terminal cleavage motif.

Effects of mutant Prp on *S. aureus*

Since the gene encoding Prp is essential in *S. aureus*, we investigated the effects of mutant Prp by examining the growth of strains overexpressing catalytically inactive Prp C34A compared to wild-type Prp, using a *S. aureus* T7 expression system (D'Elia *et al.*, 2006). Expression from the plasmids in *S. aureus* strain SA178RI was induced for 16 hours at 32° C and growth was compared to the same strain with empty vector. Overproduction of Prp C34A led to a marked growth defect compared to overproduction of the wild-type Prp (Fig 3A). (Note that the expression strain still contains native L27 and Prp.)

The role of Prp in *S. aureus* ribosome assembly was investigated by examining ribosomal profiles from cells overexpressing either wild-type Prp or inactive Prp C34A. Overexpression of Prp C34A had a very significant effect on ribosomal subunit profiles, resulting in a 50% decrease in mature 70S ribosomes. There was also an accumulation of what appeared to be pre-50S assembly intermediates (Fig. 3B), suggesting that proper ribosome assembly is dependent on L27 cleavage. However, the dominant negative phenotype of Prp C34A could have been a result of catalytically inactive Prp binding to L27 without cleaving, and thereby sequestering it from participation in ribosomal assembly. L27 has a known role in 50S subunit assembly, and the ribosomal subunit profile in the strain overexpressing Prp C34A resembled what has been observed with an intermediate depletion of L27 from the large subunit (Wower *et al.*, 1998).

In order to investigate further the effects of Prp C34A overexpression on ribosome assembly, we analyzed the composition of both wild type and aberrant 50S particles by mass spectrometry (LC-MS/MS). We chose this approach because these fractions are a complex mixture of both ribosomal proteins and co-migrating cellular protein complexes with a similar sedimentation coefficient. Furthermore, these cells also contain wild-type ribosomes. LC-MS/MS of tryptic peptides allowed us to confirm the presence of specific ribosomal

proteins in these fractions, while examination of the MS1 spectra of the peptides from individual ribosomal proteins allowed us to determine the relative amounts of individual 50S subunit proteins within each sample. Chromatograms for the precursor masses of tryptic peptides from all large subunit ribosomal proteins detected were generated from both particle types. Peptide peak areas for identical peptides in both samples were calculated using conventional tryptic digest mass spectrometry MS 1 spectra, and we compared peptide abundances for each of the large subunit proteins to those of two proteins, L3 and L4, that bind directly to the 23S rRNA early in 50S subunit assembly (Herold *et al.*, 1987; Chen *et al.*, 2013). The ratio of L3 and L4 to each other and to the majority of other ribosomal proteins detected remained close to one when comparing the wild-type 50S and aberrant particles, consistent with the inherent stoichiometry of ribosomal proteins (Fig. 3C; Table S3). However, the internal ratios of several proteins, L18, L20, L16, L27 and L25, were noticeably diminished compared to early assembly proteins L3 and L4 in the aberrant particles. The altered, substoichiometric distribution of these ribosomal proteins in the aberrant particles suggests that incomplete assemblies of the 50S subunit accumulated following overexpression of PrpC34A. Proteins L9, L28, L34 and L35 were not detected in either sample. Conventional tryptic digest LC-MS/MS is particularly problematic for small, highly negatively or positively charged proteins due to their distribution of trypsin cleavage sites. There are very few predicted detectable tryptic peptides from L28, L34, and L35. The same is true for Prp, which was also not detected in these particles by mass spectrometry. Only one peptide was found from each of L24, L32, L33 and L36 (Table S3), so they are not included in Figure 3C. However, based on these limited data, L33 and L36 also appear to be present in sub-stoichiometric amounts in the aberrant particles, consistent with a late assembly defect (Li *et al.*, 2013; Jomaa *et al.*, 2014).

The presence of L27 and Prp in the aberrant 50S particles was also examined by Western blot, using polyclonal antibodies (Fig 3D). L27 was detected in aberrant particles as a doublet that appears to contain large amounts of uncleaved L27 and only a small amount of the cleaved form, while wild type 50S subunits only contained the smaller cleaved form of L27. In addition, Prp was detected by immunoblot in the aberrant 50S particles, but not in normal 50S subunits.

Variant forms of L27 were also overexpressed to determine their effects on cell growth and ribosomal subunit distribution. Pre-cleaved (deletion of L27 residues 2-9) and uncleavable mutants (L27 F8A-F9A; Fig S2A) were compared to wild-type L27. In contrast to the strong dominant negative effect of Prp C34A, overexpression of the mutant forms of L27 showed little effect on cell growth (a mild defect was seen with the uncleavable mutant; Fig S2B) and yielded ribosomal subunit profiles similar to cells expressing empty vector (Fig S2C). We also investigated the effect on *S. aureus* growth of overexpressing the L27 N-terminal peptide that would be released upon cleavage (MLKLNLQFF). The growth curve was qualitatively and quantitatively different from that of L27 overexpression, exhibiting a pronounced lag in growth in late exponential phase, although ultimately reaching the same cell density as the other strains (Fig. S2D). The ribosomal subunit profile of this strain showed no anomalies (data not shown). How the overexpressed peptide alone is able to exert this striking effect on *S. aureus* growth remains an open question.

Discussion

This study has identified a highly conserved essential cysteine protease that performs a novel, site-specific cleavage of an essential ribosomal protein in *S. aureus*. It is striking that the conservation of Prp and the extended form of L27 are found within a subset of bacteria that had been grouped together previously based on other characteristics of their ribosomal proteins (Yutin *et al.*, 2012). The fact that this group of bacteria has retained Prp and the extended form of L27, rather than just expressing the shorter form of L27 found in most other bacterial species, suggests an important function for this cleavage. There is little known yet about regulation of *prp* expression to provide clues as to its possible role. In *S. aureus* and almost all other bacteria carrying this gene along with the extended form of L27, *prp* lies between the gene for L27 and the gene for another ribosomal large subunit protein, L21, and these three genes are predicted to be co-transcribed as a single operon (Wang *et al.*, 2004b). Consistent with this prediction, *S. aureus* microarray data show similar trends in transcript levels for *prp* and the two flanking ribosomal protein genes under a variety of conditions (Scherl *et al.*, 2005; Anderson *et al.*, 2006; Fuchs *et al.*, 2007; Anderson *et al.*, 2010). As an enzyme, Prp would not be expected to be required in stoichiometric amounts relative to these two ribosomal proteins, unless the gene product has an additional function. There are no available data about Prp protein levels in the cell. L21 and L27, while abundant, are not readily detected by standard 2D gel analysis due to their small size and alkaline pI; multidimensional liquid chromatography and mass spectrometry have been used to detect these proteins in *S. aureus* and to correlate changes in protein levels with that of their mRNA (Scherl *et al.*, 2005; Becher *et al.*, 2009). The small, acidic Prp protein has never been detected in any of these studies, suggesting that it is much less abundant than the other two proteins expressed from the operon. Further studies will be needed to evaluate Prp levels and their regulation in the cell.

Ribosome assembly in Firmicutes has only recently been investigated in any detail, largely in *Bacillus subtilis*. Structures of late stage assembly intermediates and their ribosomal protein compositions have indicated that the final region built during the maturation of the 50S subunit is the central protuberance (CP) and the functional region beneath it. 45S particles that accumulated in *B. subtilis* cells depleted of the assembly-related GTPase RbgA carried sub-stoichiometric amounts of L16, L27, L28, L33, L35 and L36 (Li *et al.*, 2013; Jomaa *et al.*, 2014). We were not able to detect L28 or L35 in this study, but L16, L27, L33 and L36 all appeared to be significantly depleted in the aberrant large subunit assemblies that were formed upon overexpression of the catalytically inactive Prp C34A. Furthermore, the remaining L27 that was found in these particles was largely uncleaved, and appeared to be incorporated along with bound Prp. Prp incorporation is presumably an artifact of the tight interaction between the mutant PrpC34A and L27 in the absence of catalysis. We cannot distinguish at this point whether the observed reduction in L27 incorporation and accompanying reduction of other proteins in the CP is due to lack of L27 cleavage or to steric hindrance by the tightly associated PrpC34A. Whatever the reason for the reduced efficiency, it does appear that uncleaved L27 can be incorporated into the pre-50S particles – cleavage is therefore not a prerequisite to assembly. We also observed a reduction in L18, which is thought to be incorporated earlier into the CP than L27 (Li *et al.*, 2013; Jomaa *et*

Prp was detected in neither ribosome sample by mass spectrometry, but was clearly present in the aberrant particles when assayed by immunoblot. Trypsin digestion of Prp yielded only one peptide that was detected by mass spectrometry, even in the analysis of the co-purified PrpC34A band (Fig. S1), and this peptide was only detected in 3 scans, although its identity in the purified sample was unambiguous. Thus, the failure to detect it in the aberrant pre-50S assemblies by mass spectrometry is not surprising.

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L27(2-9). Cleavage of L27 may serve as a regulatory switch, in which Prp activity affects formation of new functional ribosomes. The N-terminal extension of L27 may have a direct role in this process – perhaps the small peptide released by Prp cleavage acts as an environmental sensor to regulate ribosome processing and assembly – or some other event in the life cycle of the cell. The unusual lag in the growth curve following overexpression of the N-terminal peptide alone might suggest a regulatory role. Further studies of the role of L27 cleavage in ribosome assembly and function will require development of a complementation system in which expression of the wild type *prp* and *rpmA* genes can be regulated in the presence of mutant alleles.

Our discovery of Prp originated in its exploitation as a bacteriophage prohead protease. About one-third of the staphylococcal *Siphoviridae* with reference genomes in Genbank encode major capsid and/or scaffold proteins with the N-terminal Prp cleavage motif, suggesting that in *S. aureus* this is one widely used mechanism for capsid protein maturation. In some phages of other bacterial genera, Prp homologues were found to be encoded in the capsid gene cluster, the expected location for a phage-encoded prohead protease. The phage-encoded Prp proteins appear to be more closely related to each other than they are to the chromosomal Prp proteins encoded by their bacterial hosts (Fig. S3). We speculate that phage acquisition of *prp* was a rare event followed by horizontal transfer to other phages. One interesting case is that of *Lactobacillus johnsonii* NCC 533, which encodes an L27 protein with the extended N-terminal motif but lacks the upstream *prp* gene. This strain does still retain a copy of *prp*, but it is carried on a prophage, Lj965, which would predict that the cell must retain this prophage for viability.

Ribosomal biology in eubacteria has largely been studied in the Gram-negative bacterium *E. coli*. Our findings indicate that there are aspects of the basic biology of the ribosome in *S. aureus* and other related bacteria that differ substantially from that of the *E. coli* ribosome. Ribosomal protein processing is an unexplored mechanism that may provide new insights into the regulation of protein synthesis. Furthermore, the eubacterial ribosome has been a prime target for numerous antibiotics, including macrolides (e.g. erythromycin), lincosamides, oxazolidinones and tetracyclines, which work by blocking aminoacyl-tRNA binding, preventing peptidyl transfer or causing premature peptidyl-tRNA release (Tejedor *et al.*, 1986; Auerbach *et al.*, 2002; Colca *et al.*, 2003; Yonath, 2005). In light of the rise in antibiotic resistance in *S. aureus* and other Gram-positive pathogens, such as *Clostridium difficile* and *Enterococcus faecium*, and the paucity of novel antibiotics in the development pipeline (Gould, 2009; Hughes *et al.*, 2014), the findings reported here lay the foundation for the development of new therapeutic approaches that interfere with protein synthesis by targeting the processing of L27.

Experimental Procedures

Strains and Plasmids

All *S. aureus* strains used in this work are derivatives of the phage-cured, restriction-defective strain RN4220 (Kreiswirth *et al.*, 1983). The *S. aureus* expression strain SA178RI carries a T7 RNA polymerase expression cassette under *lac* operator control (D'Elia *et al.*, 2006). The plasmid used for expression in SA178RI was pG164, an *E. coli*-*S. aureus* shuttle

vector carrying the T7 late promoter into which a *lac* operator, a multiple cloning site, an optimized gram-positive ribosome binding site, and a constitutively expressed copy of the *lac* repressor gene were introduced (D'Elia *et al.*, 2006). Growth of *E. coli* and *S. aureus* strains and general DNA manipulations were performed using standard methods (Sambrook *et al.*, 1989) and manufacturer's protocols.

Plasmids used in this study are listed in Supplementary Information (SI) Table S1 and were constructed by Gibson assembly (34). Genes were amplified by PCR using Pfu Ultra II Fusion polymerase (Agilent) with the primers listed in Table S2. PCR products and linearized vectors were gel purified (Nucleospin Kit, Macherey Nagel) and assembled using an In-Fusion Kit (Clontech). Assembled plasmids were introduced initially into *E. coli* Stellar® Competent cells (Clontech) and verified by sequence analysis (MWG Biotech). Expression was carried out in *E. coli* BL21 (DE3) RIL (Invitrogen) or *S. aureus* SA178RI.

Cell growth was routinely measured with a Klett-Summerson colorimeter. *E. coli* strains were grown in LB medium with appropriate antibiotics at 37°C at 200rpm to Klett of 80, then shifted to 32°C at 200rpm for protein induction. *E. coli* strains containing a plasmid with the P_{BAD} promoter were induced using 0.2% arabinose. All strains containing IPTG-inducible promoters were induced with 1mM IPTG.

S. aureus strains were grown in Trypticase Soy Broth (TSB) at 32°C unless otherwise specified. All protein expression strains for Western blot analysis were induced at a Klett of 90 (OD₆₀₀ of 0.6) and subsequently grown for 4 hours. For growth curve analysis, triplicate cultures of each strain were grown overnight, then inoculated into a 96-well plate to an initial OD of 0.1. The plate was placed into a BioTek Microplate Reader and grown under inducing conditions at 32°C for 16 hours with shaking. Optical density at 600nm was measured every 15 minutes.

The 80α scaffolding and capsid proteins were expressed in *E. coli* from the pET21-derived plasmid pPD2 as described previously (Poliakov *et al.*, 2008), except that growth was carried out at 30°C. For co-expression with Prp, pPD2 and pEW15 were both introduced into *E. coli* BL21 (DE3), grown in LB at 30°C and induced with 0.5 mM IPTG. The resulting capsid-related structures were pelleted by centrifugation at 160,000 g for 1 hr, resuspended in procapsid storage buffer (50mM Tris-HCl pH 8.0, 100mM NaCl, 10mM MgCl₂), and purified on 10–40% sucrose gradients in the same buffer. Sucrose was removed by dialysis prior to SDS-PAGE and mass spec analysis.

Edman Degradation

Ni-NTA purified L27 Myc-His₆ protein samples were loaded onto a 16.5% Tris-tricine precast gel (Biorad) and run until the 10kDa band of the Precision Plus Protein Prestained Standards (Biorad) approached the bottom of the gel. These proteins were electroblotted to a 0.45 μm PVDF membrane for 4 hours at 300mA in a Criterion Tank Blotter (Biorad). The membrane was then stained with Coomassie and the appropriate bands were marked and coded and sent to the Iowa State University Protein Facility for Edman degradation. There the protein samples on the membrane were washed six times with deionized water and loaded onto a 494 Procise Protein Sequencer/140C Analyzer (Applied Biosystems, Inc).

Polysome analysis

Overnight cultures of strains for polysome analysis were inoculated into a flask of media containing inducer and appropriate antibiotics (TSB with 2µg/mL tetracycline, 30 µg/mL chloramphenicol and 1mM IPTG) beginning at a 1:100 dilution and grown until they reached a Klett reading of 90 or for 4 hours. As noted above, *S. aureus* expressing Prp C34A will not grow above Klett 50 for over 8 hours, so this strain was grown at double volume compared to the others. Cell pellets from at least 100mL of culture were resuspended in buffer PA (20mM Tris pH 7.8, 100 mM NH₄Cl, 10 mM MgCl₂, 6mM beta mercaptoethanol), broken by bead beating and then cleared by centrifugation for 10 minutes at 13000 rpm at 4C. The clarified cell lysates were applied to the top of 10-40% sucrose gradients in buffer PA. The gradients were centrifuged for 17 hours at 19000 rpm on an SW-28 rotor at 4C. Gradients were analyzed for absorbance at 254 nm using a Biocomp Piston Gradient Fractionator with a BIORAD Econo UV Monitor with a Full Scale of 1.0. Data were recorded using DataQ DI-158-UP data acquisition software, as has been previously reported (O'Farrell *et al.*, 2012). Areas under each peak were calculated using the trapezoid method.

Mass Spectrometry

Ni-NTA purified L27 Myc-His₆ protein fractions were separated on a 16.5% Tris-Tricine precast gel (Biorad) and stained with Coomassie Blue. The co-purifying protein was excised from the gel and the gel band was subjected to trypsin digestion and LC-MS analysis of tryptic peptides using a Thermo Electron hybrid LTQ-Orbitrap mass spectrometer.

Pooled sucrose gradient fractions containing the 50S ribosomal assembly intermediates were concentrated and de-salted prior to trypsin digestion and LC-MS/MS. The solutions were diluted with 50 µL 100 mM ammonium bicarbonate. The samples were reduced with 5 µL of 10 mM dithiothreitol in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. Then they were alkylated with 5 µL 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The samples were digested with 1 µg trypsin overnight and then quenched with 5% (v:v) glacial acetic acid. 3 µL of the final solutions were injected for analysis.

The LC-MS system consisted of a Thermo Electron hybrid LTQ-Orbitrap mass spectrometer system with a nanospray ion source interfaced to a Waters nanoAcquity UPLC system equipped with a Waters NanoAcquity C18 column. 5 µL of the extract was injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.4 µL/min over 60 minutes. The nanospray ion source was operated at 3.5 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 10000 CAD spectra of ions ranging in abundance over several orders of magnitude. Not all CAD spectra are derived from peptides.

Capsid protein fractions were desalted using a C4 ziptip and spotted on a sinapinic acid (SA) matrix for determination of full-length protein masses by MALDI-MS, using a Bruker

Ultraflex III spectrometer. Samples containing capsid and scaffold proteins were also separated by electrophoresis on a 10% Bis-Tris gel, excised, digested with trypsin, and subjected to LC-MS analysis, using a Thermo Finnigan LTQ XL spectrometer and a Mascot search of the UniRef100 database.

Calculation of the relative abundance of 50S ribosomal proteins

Individual peptides were assigned to specific ribosomal proteins following LC-MS/MS by database searching against NCBI's non-redundant database using the Mascot search algorithm. The precursor ion (MS1) spectra from primary mass spectrometry were used to determine the amount of each tryptic peptide detected in the sample, by measuring the areas under the peaks of individual peptides. The ratio of the amount of each peptide from each ribosomal protein to the amount of each peptide from L3 or L4 from the same sample was calculated (i.e. peptides from wild type 50S were compared to wild type L3 and L4; peptides from aberrant PrpC34A 50S were compared to aberrant L3 and L4) and then the ratio of aberrant/wild type for each peptide was calculated and averaged. The MS1 areas for each peptide detected for each of the different ribosomal proteins and the calculated ratios are tabulated in Table S3.

Polyclonal antibodies

Custom rabbit polyclonal antibodies were raised to purified N-terminal 6x histidine-tagged L27 2-9 (New England Peptide) and to purified Prp with a C-terminal 6x histidine tag (Thermo Fisher Custom Antibody Group).

Western blotting

Protein samples were loaded onto 16.5% Tris-Tricine precast gels (Biorad) for SDS PAGE and run until the 10kDa ladder band of the Precision Plus Protein Prestained Standards (Biorad) approached the bottom centimeter of the gel. For detection of L27, gels were electroblotted to a 0.45 micron PVDF membrane for 3 hours at 300mA in pH 8.3 Tris-Glycine transfer buffer containing 0.05% SDS, to ensure movement of the small, positively charged L27 protein. For detection of the small, acidic Prp protein, gels were electroblotted in Towbin buffer (Tris-Glycine pH 8.3 20% methanol) for 1 hour 45 minutes at 200mA. The membranes were blocked with phosphate-buffered saline/tween-20 (PBST) containing 5% skim milk for one hour at room temperature and then rocked overnight at 4C with a 1:5000 dilution of primary antibody. L27myc-His6 was detected with monoclonal mouse anti-myc antibody (gift from Dr. William Barton) while untagged L27 and Prp were detected with polyclonal rabbit antibodies raised to each protein (New England Peptide; Thermo Fisher). All antibody dilutions were made in 5% skim milk PBST. The membrane was then washed thoroughly with PBST and probed with a 1:10000 dilution of Protein A conjugated to Horseradish Peroxidase (Invitrogen) in 5% skim milk PBST and protected from light for one hour at room temperature. The membrane was protected from light, washed thoroughly with PBST and then Pierce ECL Western Blotting substrate was added for 5 minutes. The blots were then exposed to x-ray film (Bioexpress) and developed.

Bioinformatics

The L27 protein alignment (Figure 1A) was generated through the Mobyle Portal of the Pasteur institute using BOXSHADE v3.31 C (beta, 970507 <http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::boxshade>) (Neron *et al.*, 2009). A MUSCLE alignment of the five proteins was generated using the CLUSTALW algorithm and default preset values in the bioinformatics software package Geneious (Kearse *et al.*, 2012). This alignment file was submitted to the Mobyle Pasteur boxshade server. The L27 and Prp phylogenetic trees (Figures 1B and S3, respectively) were generated in Geneious using the BLOSUM80 cost matrix. The L27 tree is derived from representative L27 protein sequences from each bacterial phylum. The Prp tree was derived from selected Prp homolog sequences. These sequence data were subjected to global alignment with free end gaps (gap open penalty set to 12, gap extension penalty set to 3). The genetic distance model utilized was Jukes Cantor and the tree build method was neighbor-joining with no outgroup.

The existing crystal structure of *S. aureus* Prp (PDB ID: 2P92) lacks the loop that includes the active site residues H22 and C34. We built the loop in I-TASSER (Roy *et al.*, 2010) using 2P92 and the structures of Prp from *Streptococcus pneumoniae* (PDB ID: 2IDL) and *Streptococcus mutans* (PDB ID: 2G0J) as templates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(C) Western blot analysis of L27 N-terminal cleavage. Plasmid-encoded *S. aureus* L27 carried C-terminal Myc and His₆ tags; the blot was probed with anti-Myc antibodies. Lane 1: L27 produced from pEW5 in *E. coli* BL21; lane 2: L27 produced from pEW7 in *S. aureus* SA178RI; lane 3: L27 co-expressed with Prp in *E. coli* BL21 (pEW5 + pEW15); lane 4: L27 co-expressed with inactive Prp mutant C34A in *E. coli* BL21 (pEW5 + pEW28). L27 in lanes 2 and 3 has undergone specific cleavage at the conserved motif to yield the N-terminus ASKK, confirmed by Edman degradation.

Figure 2.

Prp structure and conservation of catalytic residues

(A) ClustalW alignment of Prp sequences from *S. aureus* (Sau), *B. subtilis* (Bsu), *Streptococcus pneumoniae* (Spn), *Streptococcus mutans* (Smu), *Aminobacterium colombiense* (Aco), *Fusobacterium nucleatum* (Fnu), *Thermotoga lettingae* (Tle) and *Mycoplasma pneumoniae* (Mpn). Identical residues are highlighted in solid red, conserved residues in yellow. Asterisks designate the proposed active site residues. The secondary structure elements from the crystal structure of *S. aureus* Prp (PDB ID: 2P92) are shown above the alignment. Figure made with ESPript 2.2.

(B) Molecular surface of the complete *S. aureus* Prp model generated by I-TASSER (Roy *et al.*, 2010) using the partial crystal structure of the *S. aureus* protein (PDB ID: 2P92) and the complete structures of *S. pneumoniae* (PDB ID: 2IDL) and *S. mutans* (PDB ID: 2G0J) as templates. Residues are colored by conservation from white (non-conserved) to yellow (partially conserved) and then red (completely conserved) based on the alignment in (A). The active site Cys and His residues are indicated by arrowheads. Figure created in UCSF Chimera (Pettersen *et al.*, 2004).

(C) Prp cleavage of bacteriophage 80α scaffold and major capsid proteins. Capsid-like structures formed upon expression of gp46 and gp47 from plasmid pPD2 in the absence (lane 2) and presence (lane 1) of pEW15, expressing Prp, were purified on sucrose gradients and resolved by SDS-PAGE. Lane 3 shows the two samples mixed together, in order to better illustrate the small shift in electrophoretic mobilities of the two proteins following cleavage. M is the BenchMark protein ladder (Life Technologies), with selected sizes indicated next to the gel. A MALDI spectrum of the purified capsid fraction run in lane 1 showed major peaks at 21,704 Da and 35,042 Da, corresponding to the masses of cleaved

gp46 (theoretical mass 21,701 Da) and cleaved gp47 (35,063 Da), respectively. Tryptic fragments of these two protein bands were also analyzed by LC-MS to confirm that they corresponded to the expected cleaved proteins.

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Figure 3.

Dominant negative effects of catalytically inactive Prp on *S. aureus* growth and ribosome assembly.

(A) Growth curves of SA178RI containing empty T7 expression vector pG164 (WT), or derivatives expressing wild-type Prp (pEW25) or Prp C34A (pEW26). Bacteria were grown in the presence of inducer and appropriate antibiotics in 96-well format at 32° C. Starting culture density was between 0.12 and 0.15 absorbance at 600nm, and absorbance was measured every 15 minutes for 16 hours using a BioTek Microplate reader. Error bars represent a 95% CI, n=3.

(B) Ribosomal subunit profiles of the strains grown in (A). Typical results are shown.

(C) Relative abundance of ribosomal proteins in aberrant 50S particles, as determined from MS1 spectra of individual peptides. Only those proteins for which at least two tryptic peptides were detected are shown in this figure; data for all proteins detected are shown in Table S3. Peptides from each ribosomal protein were normalized separately to peptides from ribosomal proteins L3 and L4, as indicated. Error bars indicate one standard deviation above and below the mean. Proteins for which the relative abundance is the same in wild type and mutant particles should have a value of 1. Not detected: L9, L28, L34 and L35.

(D) Western blot analysis of L27 (left blot) and Prp (right blot). In each blot, wild type 50S subunits are in the left lane and aberrant 50S subunits from PrpC34A overexpression are in the right lane.