Degradation of SsrA-tagged proteins in streptococci

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In prokaryotes, trans-translation is a process in which stalled ribosomes are rescued by tmRNA, a conserved specialized RNA with properties of both a tRNA and an mRNA. If a translating mRNA does not encode stop codons, or contains multiple rare codons, or is truncated, ribosomes are stalled at the 3’ end of the mRNA and form non-productive complexes containing peptidyl-tRNA. Eventually, protein synthesis ceases due to the failure of the recruitment of release factors needed for ribosome disassembly, and the stalled ribosomes are rescued by tmRNA by the process of trans-translation. A tmRNA charged with alanine enters the acceptor position of the translating ribosome and allows transfer of the alanine to the nascent polypeptide. Subsequently, the ribosome changes to the tmRNA to complete the translation process that leads to addition of a tag to the C-terminal polypeptide chain and proper disassembly of ribosomal subunits for recycling. This peptide tag, called the SsrA tag, serves as a target for degradation of the modified polypeptide, thereby assuring protein quality control in the cell by preventing accumulation of aberrant and incomplete proteins (Keiler et al., 1996).

In Escherichia coli, this tag consists of 11 residues (AANDENYALAA) with a conserved C-terminal LAA sequence. At least five proteases are involved in the degradation of SsrA-tagged protein in E. coli (Lies & Maurizi, 2008). Amongst them, two intracellular AAA+ proteases, ClpXP and ClpAP, degrade most of the SsrA-tagged peptides regardless of their inherent stability (Gottesman et al., 1998). The AAA+ component of the ClpXP or ClpAP complex (ClpX or ClpA) forms a hexameric structure that recognizes and unfolds the SsrA-tagged proteins using ATP hydrolysis. These unfolded polypeptides are then channelled into the proteolytic compartment of the complex consisting of two heptameric rings of ClpP protease (for a recent review, see Sauer & Baker, 2011). In E. coli, ClpXP degrades >90% of the SsrA-tagged peptides and the rest are degraded by other proteases, including ClpAP (Lies & Maurizi, 2008). Whilst both ClpXP and ClpAP can directly recognize the SsrA tag (Flynn et al., 2001), an adaptor protein named SspB significantly enhances ClpXP-mediated degradation (Levchenko et al., 2000).

Similar to Proteobacteria, SsrA tags in Gram-positive bacteria are also conserved except for the very last three terminal residues, LAA, which are sometimes replaced with VAA. In Gram-positive bacteria such as Bacillus subtilis and Streptococcus pneumoniae, ClpXP is the only known protease that degrades SsrA-tagged proteins (Ahlawat & Morrison, 2009; Wiegert & Schumann, 2001). Most of the Firmicutes, including Bacillus and Streptococcus, do not encode ClpA; rather, they encode two other AAA+ proteins, ClpC and ClpE. Similar to ClpA and ClpX, both ClpC and ClpE contain an IGF motif that allows them to interact with ClpP to form the protease complex (Kim et al., 2001). All the

INTRODUCTION

In prokaryotes, trans-translation is a process in which stalled ribosomes are rescued by tmRNA, a conserved specialized RNA with properties of both a tRNA and an mRNA. If a translating mRNA does not encode stop codons, or contains multiple rare codons, or is truncated, ribosomes are stalled at the 3’ end of the mRNA and form non-productive complexes containing peptidyl-tRNA. Eventually, protein synthesis ceases due to the failure of the recruitment of release factors needed for ribosome disassembly, and the stalled ribosomes are rescued by tmRNA by the process of trans-translation. A tmRNA charged with alanine enters the acceptor position of the translating ribosome and allows transfer of the alanine to the nascent polypeptide. Subsequently, the ribosome changes to the tmRNA to complete the translation process that leads to addition of a tag to the C-terminal polypeptide chain and proper disassembly of ribosomal subunits for recycling. This peptide tag, called the SsrA tag, serves as a target for degradation of the modified polypeptide, thereby assuring protein quality control in the cell by preventing accumulation of aberrant and incomplete proteins (Keiler et al., 1996).

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Similar to Proteobacteria, SsrA tags in Gram-positive bacteria are also conserved except for the very last three terminal residues, LAA, which are sometimes replaced with VAA. In Gram-positive bacteria such as Bacillus subtilis and Streptococcus pneumoniae, ClpXP is the only known protease that degrades SsrA-tagged proteins (Ahlawat & Morrison, 2009; Wiegert & Schumann, 2001). Most of the Firmicutes, including Bacillus and Streptococcus, do not encode ClpA; rather, they encode two other AAA+ proteins, ClpC and ClpE. Similar to ClpA and ClpX, both ClpC and ClpE contain an IGF motif that allows them to interact with ClpP to form the protease complex (Kim et al., 2001). All the

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known proteolytic activities of ClpCP require adaptor proteins. For example, in *B. subtilis* and *Streptococcus thermo-
philus*, MeCA acts as an adaptor protein for ClpC, and is required for hexamerization and complex formation with
ClpP (Boutry et al., 2012; Mei et al., 2009; Wang et al., 2011). The substrates and the adaptor proteins for ClpEP-mediated
degradation are unknown, and the exact role of ClpEP in protein quality control remains to be explored (Kirstein
et al., 2009).

In this study, we used *Streptococcus mutans*, an oral pathogen that causes human dental caries, as a model *Streptococcus*
organism to investigate the degradation of SsrA-tagged pro-
teins. We observed that ClpXP was the major protease for
SsrA-tagged substrate and did not require any adaptor
protein for the degradation. Surprisingly, we also found that
both ClpCP and ClpEP degraded SsrA-tagged proteins in the
absence of ClpX. We also found that ClpCP- and ClpEP-
m ediated proteolysis of SsrA-tagged substrates was induced by
heat stress.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *S. mutans*
strains and plasmids used in this study are listed in Tables 1 and 2. *E.
coli* strains were routinely grown in Luria–Bertani medium supple-
mented with 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 100 µg
spectinomycin ml⁻¹ or 300 µg erythromycin ml⁻¹. *S. mutans* strains
were grown at 37 °C in Todd Hewitt medium (BBL; BD) sup-
plemented with 0.2 % yeast extract (THY medium). When necessary,
5 µg erythromycin ml⁻¹, 400 µg kanamycin ml⁻¹ or 300 µg
spectinomycin ml⁻¹ was included in THY medium. A previously
described protocol was used for *S. mutans* transformation by natural
competence (Biswas et al., 2007).

**Mutants construction.** A markerless gene replacement method that
utilized the Cre-loxP-based recombination system was used for
deleting *clpC, clpE* and *clpX* genes as described previously (Banerjee &
Biswas, 2008; Biswas et al., 2007; Tao et al., 2012). The mutant
constructs were verified by PCR and sequencing.

**Construction of chromosomally integrated gfp reporter strains.** A DNA fragment containing the *gfpmut3a* gene (*gfp*) was PCR
amplified from pAD123 (Dunn & Handelsman, 1999). Various C-
terminal tags (SsrA tag and its derivatives) were added to the gfp gene
by fusion PCR. These PCR products were cloned under the P23
promoter (a strong lactococcal phage promoter) in plasmid pLB184 Km
for expression of GFPs in *S. mutans* (Biswas et al., 2008; Que et al.,
2000). All the constructs were sequenced. For chromosomal expres-
sion of GFP or its derivatives, DNA fragments carrying gfp genes with the
P23 promoter were amplified from pLB184 derivatives and cloned into
pLB107, a plasmid used for integration at the SMU.1405 locus (Biswas &
Biswas, 2006). The resulting plasmids were linearized by BglII and
transformed into *S. mutans* to obtain the desired strains.

**Protein extraction and Western blot analysis.** Unless otherwise
stated, overnight cultures were reinoculated in THY medium and
grown to exponential phase (OD600 0.5). A 10 ml aliquot was harvested
by centrifugation, resuspended in 500 µl PBS and homogenized with a
bead beater (MP Biomedicals). Cell lysate was centrifuged at 18000 *g*
for 5 min; the supernatant was carefully transferred into a new
microcentrifuge tube and stored as cell extract sample at −20 °C with
protease inhibitors. Protein concentration was determined by
A²₆₀ using a NanoDrop 2000c spectrophotometer (Thermo Scientific).
Approximately 100 µg protein was loaded onto each well in the gel and
separated by SDS-PAGE. The gel was blotted onto a PVDF membrane
for Western blot analysis. Western blot assays were carried out using
standard techniques. An anti-GFP N-terminal antibody (Sigma) was
used as the primary antibody to detect GFP. His-tagged ClpX and
ClpXP<sub>EG16W</sub> were probed with an anti-polyhistidine mAb (Sigma). To
detect *S. mutans* ClpE, a polyclonal antibody against *B. subtilis* ClpE
produced in rabbits was employed (kindly donated by Dr Ulf Gerth,
Ernst Moritz Arndt University Greifswald, Greifswald, Germany). The
abundance of cellular enolase was chosen as an internal control and
was probed by an anti-*S. mutans* enolase rabbit polyclonal antibody
(Genscript; I. Biswas, unpublished). Western blots were developed with
Pierce ECL Plus reagents (Thermo Scientific). A Typhoon FLA9000
imager (GE Healthcare) was used for fluorescent signal detection. All
Western blot experiments were repeated at least twice to confirm the
results.

**ELISA.** ELISAs were performed with conventional methods in 96-well
round-bottom ELISA plates (Nunc MaxiSorp). Briefly, 50 µg cell
extract was added to each well with a buffer containing 50 mM
NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> (pH 9.6) and incubated overnight at 4 °C. The
anti-GFP antibody was used as primary antibody to monitor the
amount of GFP. Colorimetric signals were developed by adding
150 µl of 1-Step Turbo TMB-ELISA solution (Thermo Scientific) and
incubated at 37 °C for 30 min. The reaction was then stopped by
the addition of 150 µl 1 M HCl. A<sub>450</sub> readings were obtained using a
microplate reader (BioTek).

**Protein purification.** DNA fragments containing the ORFs of *clpX*,
gfp and its derivatives were amplified by PCR, digested with PstI
and EcoRI (the primers contain the restriction sites), and cloned into a
pASK-IBA43 + vector. Similarly, *clpP* and *clpE* fragments were
amplified by PCR, restricted with BamHI and PstI, and cloned into a
pASK-IBA43 + vector. A DNA fragment carrying the *clpP*-coding
region with an additional six histidine codons (His tag) at the C
terminus was amplified by PCR, restricted with Ndel and Xhol, and
then cloned into a PETDuet plasmid. A DNA fragment carrying a
meca gene was also PCR amplified, digested with BamHI and SalI,
and cloned into a PETDuet plasmid.

His-GFP, His-GFPalg, His-gfpX, His-gfpE, His-gfpC, His-MecA
and ClpP-His were expressed in *E. coli* strain BL21(DE3). His-
GFPssrA, His-GFPavaa and His-GFPavaaNL were expressed in *E. coli*
strain JW0427-1, a strain in which *clpP* had been inactivated (Baba
et al., 2006). For expression of proteins from pASK-IBA43 +, derived
plasmids, *E. coli* cells were induced with 200 µg anhydrotetracycline
1⁻¹. To express His-gfpE, *E. coli* cells carrying pBlJS4 were induced
with 1 mM IPTG. His-tagged proteins were purified by Ni-NTA resin
(Novagen) according to the manufacturer’s instructions. Proteins
were dialysed thoroughly against a buffer containing 20 mM Tris/His
(pH 7.4), 200 mM KCl, 25 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 % glycerol.
The purity of the proteins was >95 %, as determined by
SDS-PAGE. Protein concentration was determined by A<sub>280</sub> using a
NanoDrop 2000c spectrophotometer (Thermo Scientific).

**In vitro degradation assay.** In vitro degradation assays were
performed as described previously (Hersch et al., 2004; Kim et al.,
2000). Briefly, 0.3 µM His-ClpX, 0.3 µM His-ClpE, 0.3 µM His-ClpC,
0.1 µM MecA, 0.8 µM ClpP-His and/or 0.1 µM substrate GFPs were
added to a 100 µl reaction system. The reactions were carried out at
30 °C for 30 min in a buffer containing 25 mM HEPEs/NaOH
(pH 7.6), 5 mM MgCl<sub>2</sub>, 200 mM KCl, 0.052 % NP-40, 10 % glycerol
and an ATP regeneration system (4 mM ATP, 16 mM creatine
phosphate, 0.032 mg creatine kinase ml⁻¹). Fluorescent signals were
detected with a Typhoon FLA9000 imager (GE Healthcare).
**RESULTS**

**ClpXP degrades SsrA-tagged proteins in S. mutans, and the complex formation between ClpX and ClpP is essential for the degradation**

It was previously reported that ClpXP was responsible for SsrA-tagged protein degradation in Gram-positive bacteria such as *B. subtilis* and *S. pneumoniae* (Ahlawat & Morrison, 2009; Wiegert & Schumann, 2001). To determine whether ClpXP also degraded SsrA-tagged proteins in *S. mutans*, a sequence encoding *S. mutans* SsrA tag was added to the C-terminal of the GFP. The fusion construct (gfp-ssrA) was integrated into the genome of either the WT or clpX-deleted *S. mutans* strain. Chromosomally expressed GFPssrA was thoroughly degraded in the WT strain, but not in the ΔclpX strain. Complementation of His-tagged ClpX protein expressed from a plasmid restored the cellular proteolysis of SsrA-tagged GFP (Fig. 1a). We also built a ClpX derivative (ClpX_{EGW}) with the tripeptide motif IGF, which is essential for ClpXP complex formation (Kim et al., 2001; Martin et al., 2007), mutated to the triplet EGW. Expression of ClpX_{EGW} in the ΔclpX strain did not complement the proteolytic activity and the amount of GFPssrA remained the same as in the ΔclpX strain (Fig. 1a). This suggested that interaction between ClpX and ClpP for complex formation was required for SsrA-tagged protein degradation in *S. mutans*.

**SsrA-tagged protein degradation does not require adaptors in S. mutans**

The homologue of SspB was not found in any Gram-positive bacteria, including *S. mutans*. To verify the potential of other SspB-like tethering roles in *S. mutans*, we replaced the last three residues, VAA, in the *S. mutans* SsrA tag to LGG. A four-residue linker, SENY, was added before the last four residues of the SsrA tag to enhance the tethering role, as reported previously (Hersch et al., 2004; McGinness et al., 2006, 2007). The modified SsrA tags, SENY-VAAG, SENY-ALGG and AVAA, were then added to the C terminus of GFP; the resulting GFP constructs (GFP-ssrA, GFPssrA*, GFPavaa, GFPalgg and GFPavaaNl) were cloned into the shuttle vector pIB184Km for expression in the *S. mutans* strains. Plasmid-expressed GFP without any tag was steady and stable in the WT, ΔclpP

### Table 1. *S. mutans* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>UA159</td>
<td>WT, serotype c</td>
<td>Ađić et al. (2002)</td>
</tr>
<tr>
<td>IBS12</td>
<td>UA159 derivative, ΔclpP</td>
<td>Zhang et al. (2009a)</td>
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<td>UA159 derivative, ΔclpC</td>
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<td>UA159 derivative, ΔclpX</td>
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</tr>
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<td>UA159 derivative, ΔclpE</td>
<td>Tao et al. (2012)</td>
</tr>
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<td>UA159 derivative, ΔclpX, ΔclpE</td>
<td>Tao et al. (2012)</td>
</tr>
<tr>
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<td>UA159 derivative, ΔclpX, ΔclpC</td>
<td>Tao et al. (2012)</td>
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<td>UA159 derivative, Smu1405::pIBJ71, Km'</td>
<td>Tao et al. (2012)</td>
</tr>
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<td>Tao et al. (2012)</td>
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</tr>
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<td>IBS19</td>
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<td>Tao et al. (2012)</td>
</tr>
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<td>IBS29</td>
<td>UA159 derivative, ΔclpX, ΔclpE, ΔclpC, Smu1405::pIBJ71, Km'</td>
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<td>Tao et al. (2012)</td>
</tr>
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</table>

Heat stress assay. Various *S. mutans* strains expressing GFP and its derivatives were grown at 37 °C and allowed to reach exponential phase (OD_{600} 0.3). The cultures were then either incubated further at 37 °C or shifted to 44 °C. A 10 ml aliquot of culture was removed at different time points to prepare cell extracts. OD_{600} was measured before protein extraction to ensure equal amounts of bacteria.
and ΔclpX strains (data not shown). As expected, GFP with the SsrA or AVAA tag was degraded rapidly in the WT S. mutans as observed by Western blot analysis (Fig. 1b). However, no significant differences were observed amongst the bands corresponding to GFP, GFPssrA* and GFPalg as judged by Western blot analyses (Fig. 1b); similar results were also obtained with the ELISA analyses (data not shown). These results suggested that tethering played no significant role in the degradation of SsrA-tagged proteins in S. mutans.

**GFPssrA is also degraded in a ClpP-dependent but ClpX-independent manner**

To further identify the pattern of the degradation of SsrA-tagged proteins, we determined the stability of GFP, GFPssrA*, GFPavaa or GFPalg in the ΔclpP and ΔclpX strains. The Western blot analyses showed that the stability of the WT GFP and all other tagged GFP derivatives was maintained in the ΔclpP strain (Fig. 2a, b), indicating that ClpP played a core role in the proteolysis of SsrA-tagged proteins. However, we expected that a similar pattern would be observed in the ΔclpX strain, as ClpA was absent and ClpXP was the only known proteolytic complex for the degradation of SsrA-tagged proteins in streptococci (Ahlawat & Morrison, 2009). Surprisingly, although significant amounts of GFPssrA and GFPavaa proteins were still present in the ΔclpX strain, they were drastically reduced (Fig. 2c). Quantitative data obtained with ELISA analysis also confirmed that the levels of GFPssrA and GFPavaa were significantly different, as compared with GFP or GFP with the ALGG tag (Fig. 2d). These data indicated that there were as yet unknown pathways for the degradation of SsrA-tagged proteins in this organism and these pathways were ClpP dependent but ClpX independent. Furthermore, we found no significant differences between the level of GFPssrA and GFPavaa, demonstrating that the first eight residues of the SsrA tag (AAKNTNSY) played no role in ClpX-dependent or ClpX-independent proteolysis of the SsrA-tagged proteins.

**S. mutans ClpP alone does not degrade GFPssrA in vitro**

In E. coli, several proteases, including ClpXP, ClpAP, Lon and FtsH, are able to degrade SsrA-tagged proteins (Choy...
et al., 2007; Gottesman et al., 1998; Herman et al., 1998; Spiers et al., 2002). In addition to ClpXP, only an orthologue of FtsH is found in S. mutans. Therefore, FtsH might be a possible protease to degrade SsrA-tagged proteins in S. mutans. Unfortunately, after multiple attempts we were unable to delete ftsH in S. mutans and we presumed that ftsH might be an essential gene in this bacterium. Thus, we overexpressed the cytosolic domain of FtsH (FtsHs) in both the ΔclpP and ΔclpX strains carrying the chromosomally integrated gfp-srrA gene. Comparisons of Western blot analyses showed that the amounts of GFPssrA between the control and FtsHs-overexpressing strains were similar (data not shown), indicating that FtsH might not be important for SsrA-tagged protein degradation in S. mutans.

Albeit at a greatly reduced rate, in vitro E. coli ClpP alone could degrade certain substrates (Jennings et al., 2008a,b). However, the degradation of SsrA-tagged proteins was not observed in E. coli when ClpA and ClpX were absent (Farrell et al., 2005). In addition, the N terminus of E. coli ClpP formed a flexible axial loop and was important for complex formation with ClpA or ClpX. When the associated Clp ATPases were absent, the N-terminal loop of ClpP was crucial and controlled the proteolytic activity by preventing the substrates from entering into the central chamber. Deletion of certain N-terminal residues allowed ClpP to degrade unfolded substrate without the ATPase subunits (Bewley et al., 2009; Gribun et al., 2005; Jennings et al., 2008a). We compared the N-terminal region of E. coli and S. mutans ClpP proteins (Maurizi et al., 1990), and found that S. mutans ClpP did not have the N-terminal loop. However, the rest of the N-terminal regions of the mature peptides were extremely conserved between these two species. We also tested the proteolytic activity of S. mutans ClpXP or ClpP alone towards GFP, GFPssrA or GFPavaa in vitro. As expected, ClpXP showed a distinct proteolytic activity for the degradation of GFPssrA or GFPavaa (Fig. 3a). However, no obvious degradation of GFPssrA or GFPavaa could be observed with ClpP alone (Fig. 3b). Thus, the S. mutans ClpP itself could not degrade SsrA-tagged proteins.

**ClpCP and ClpEP both degrade SsrA-tagged proteins**

We observed the ClpP-dependent but ClpX-independent degradation of SsrA-tagged proteins. We also found that ClpP alone could not degrade SsrA-tagged GFP. Therefore, we speculated that either ClpC or ClpE, which are the two other AAA + proteins present in Gram-positive that form a complex with ClpP, could play a role in SsrA tag degradation. We first measured the stability of plasmid-expressed GFP, GFPssrA, GFPssrA*, GFPavaa and GFPalgg in the clpE or clpC mutant strains. The Western blot analysis was similar to the WT strain (data not shown). However, this could have been due to the presence of both ClpX and ClpP, as ClpXP degraded SsrA-tagged proteins both in vivo and in vitro (Figs 2 and 3). Therefore, we constructed two double-mutant strains, ΔclpXΔclpE and ΔclpXΔclpC, to evaluate the stability of SsrA-tagged GFP. As only the AVAA sequence was needed for the degradation, we used GFPavaa as substrate instead of GFPssrA to investigate the ClpX-independent degradation in S. mutans. Chromosomally expressed GFP, used as a control, showed that GFP expression was stable in those strains (data not shown). The level of GFPavaa was slightly elevated in both the double-mutant strains as compared with the clpX single mutant, but the level was significantly lower than the ΔclpP strain (Fig. 4a and data not shown). We conjectured that both ClpCP and ClpEP might have contributed to the degradation of SsrA-tagged proteins, although the proteolytic activities were much lower than ClpXP.

To verify that both ClpCP and ClpEP contributed to SsrA-tagged protein degradation, we constructed a triple-mutant strain of ΔclpXΔclpPCΔclpE. In this mutant strain, the gfp-avaa fusion was integrated into the genome for expression from the chromosome. The growth kinetics of this triple-mutant strain were similar to the ΔclpP and ΔclpX strains (Fig. 4b). We found that proteolysis of AVAA-tagged GFP in the triple-mutant strain was greatly inhibited as compared with the ΔclpX strain. The difference was observed in all growth stages and was distinct after the late exponential phase (Fig. 4c). To confirm that ClpE indeed played a role in SsrA degradation, we first restored the ClpE activity by expressing it from a plasmid in the triple-mutant strain (Fig. 4d). We found that GFPavaa was efficiently degraded when ClpE was overexpressed in the...
triple-mutant strain. We also restored ClpC activity by overexpressing it from a plasmid. We found that degradation of GFPavaa was increased when ClpC was overexpressed in the triple-mutant strain (Fig. 4e). Taken together, these data indicated that both ClpE and ClpC were responsible for the degradation of SsrA-tagged substrates in vivo.

To verify whether ClpCP- and ClpEP-mediated SsrA tag degradation required a tethering linker sequence, we analysed the stability of GFPavaaNL (without the SENY linker). This construct was expressed in the WT, ΔclpP, ΔclpX, ΔclpXΔclpE, ΔclpXΔclpC and ΔclpXΔclpCΔclpE strains. We found that the degradation pattern of GFPavaaNL was

**Fig. 2.** Degradation of GFP with the SsrA tag by a ClpP-dependent but ClpX-independent pathway. (a) Western blot analysis of GFP with or without different degradation tags expressed from a plasmid in the clpP mutant strain. (b) Relative abundance of GFP or its derivatives in the clpP mutant strain as determined by ELISA. (c) Western blot analysis of GFP with or without different degradation tags expressed from a plasmid in the clpX mutant strain. (d) Relative abundance of GFP or its derivatives in the clpX mutant strain as determined by ELISA. Endogenic enolase (Eno) was employed as an internal control in (a, c). Proteins expressed from the plasmids are denoted by (p). n=3; *P<0.05, NS, not significant.

**Fig. 3.** S. mutans ClpP is unable to degrade SsrA- or AVAA-tagged GFP in vitro. (a) In vitro degradation of GFP, GFPssrA or GFPavaa by (a) ClpXP or (b) ClpP was monitored by measuring green fluorescence in the reaction mixture. The relative value of the initial GFP fluorescence was set as 1.
similar to the pattern obtained with GFPavaa that contained the linker (Fig. 5a). Thus, similar to ClpXP, ClpCP and ClpEP did not require the linker sequence for degradation.

Degradation of SsrA-tagged proteins by ClpEP and ClpCP was also tested in vitro. Four substrates, GFP, GFPalgg, GFPavaa and GFPavaaNL, were used for this purpose. We observed that ClpEP efficiently degraded both GFPavaa and GFPavaaNL. The extent of degradation was comparable with ClpXP-mediated degradation (Fig. 5b). However, when we used ClpCP, we did not observe degradation of any of the substrates. As MecA is an adaptor protein for ClpCP, we also added MecA to the in vitro reaction. We found that ClpCP failed to degrade any of the substrates even after the addition of the MecA protein.

**ClpEP- and ClpCP-mediated proteolysis of SsrA-tagged peptides is induced by heat stress**

CtsR is a regulator of stress and heat shock response, and controls the expression of clpE, clpC and clpP (Frees et al., 2007). Therefore, proteolytic degradation mediated by ClpEP and ClpCP might be associated with the stress response. To investigate further, first we inactivated the ctsR gene in the DclpX DclpE strain by the insertion of a spectinomycin resistance gene cassette. As expected, the expression of ClpE was upregulated and the degradation of GFPavaa was increased (Fig. 6). We also determined ClpEP/ClpCP-dependent proteolysis of SsrA-tagged proteins under the heat stress condition. Western blotting showed that the cellular protein amount of GFPavaa gradually decreased after the cells were exposed to a high temperature, indicating a faster degradation rate under heat stress. At normal growth temperature, however, the protein level of GFPavaa was maintained steadily during the exponential phase (Fig. 6). Taken together, the proteolytic activity of ClpCP and ClpEP, which were regulated by CtsR, was controlled by the heat stress response.

**DISCUSSION**

Although tmRNA is present in all bacterial genomes, the proteolysis systems for SsrA-mediated degradation vary...
In E. coli, ClpXp is the major proteolytic complex that recognizes and degrades >90% of SsrA-tagged protein substrates in vivo (Lies & Maurizi, 2008). In addition, other proteases, such as ClpAP, Lon, FtsH and Tsp, also degrade SsrA-tagged proteins in E. coli (Choy et al., 2007; Gottesman et al., 1998; Herman et al., 1998; Spiers et al., 2002). As the orthologues of many of these proteases are not ubiquitously present in other organisms, the pathways for SsrA-tagged protein degradation are not the same. For instance, Mycoplasma does not encode ClpX, ClpA and FtsH proteases; rather, it encodes only Lon protease. Lon is the sole protease that is responsible for degradation of SsrA-tagged substrate in this organism (Ge & Karzai, 2009; Gur & Sauer, 2008). Similarly, the genus Streptococcus does not encode ClpA, Lon and Tsp proteases. A previous study showed that only ClpXP degrades SsrA-tagged proteins in S. pneumoniae (Ahlawat & Morrison, 2009). Although FtsH is present in S. pneumoniae, this protease does not play a role in the degradation of SsrA-tagged proteins in this organism (Ahlawat & Morrison, 2009). In the present study, we also found that ClpXp is the major protease complex responsible for the degradation of SsrA-tagged proteins in S. mutans. Based on our preliminary estimation, it appears that >50% of the SsrA-tagged GFP substrate is degraded by ClpXp and the extent of the degradation is dependent on the tag. Whilst the SsrA- and SENYAVAA-containing substrates are degraded efficiently, it appears that substrates with just four residues, AVAA, are processed better (Fig. 5b). In this study, surprisingly, we also discovered that ClpCP and ClpEP both degraded SsrA-tagged substrates.

ClpC is an important AAA+ protein that is present in a wide range of bacteria. This ATPase is involved in stress response as well as required for virulence for certain pathogens, such as Listeria and Staphylococcus (Chatterjee et al., 2009; Rouquette et al., 1998). ClpC is also involved in the regulation of cellular metabolism, degradation of toxin-antitoxin systems, stationary-phase survival and other cellular processes (Chatterjee et al., 2005, 2010). A recent study in S. aureus identified >100 proteins belonging to various functional categories, including proteins involved in stress response, as potential ClpC substrates (Graham et al., 2013). Whilst this work was in preparation, Donegan et al. (2014) reported that ClpCP is the major complex that degrades SsrA-tagged proteins in S. aureus. Surprisingly, these authors found that ClpXp is not involved in the degradation of SsrA-tagged protein. However, the pathways for SsrA-tagged protein degradation are different in S. mutans as compared with S. aureus. In S. mutans, ClpCP is not the major complex that degrades SsrA-tagged protein. The contribution of ClpCP, although significant, is much lower than ClpXp.

We found that ClpEP also degrades SsrA-tagged proteins. The extent is similar to ClpCP-mediated degradation. ClpE is also an important AAA+ protein that is uniquely present in Gram-positive bacteria (Nair et al., 1999). This protein is also involved in the stress response and pathogenesis (Miethke et al., 2006; Zhang et al., 2009b). Unlike ClpC, substrates for ClpE-mediated degradation were not identified. To the best of our knowledge, the present study is the first study to identify that ClpEP could degrade SsrA-tagged substrates. At present, we do not know whether ClpEP-mediated SsrA tag degradation occurs in other organisms. As ClpE is highly conserved amongst Gram-positive bacteria, we speculate that ClpE-mediated degradation of the SsrA tag is not unique to S. mutans.

In E. coli, adaptor proteins play a significant role in regulating SsrA-mediated degradation. The adaptor protein SspB generally recognizes and binds to the same SsrA motif as ClpA, thereby blocking the access to SsrA-tagged substrates (Flynn et al., 2001). During heat stress, the level of SspB is increased, which in turn redirects ClpAP to degrade other substrates, including denatured proteins. Gram-positive bacteria such as S. aureus or B. subtilis do not encode homologues of SspB, although they contain ClpX. However, several adaptor proteins for ClpC have...
been identified in Gram-positive bacteria, including MecA, McsB, NbIA, YphH and TrfA (Andersson et al., 2006; Donegan et al., 2014; Karradt et al., 2008; Kirstein et al., 2007; Persuh et al., 2002; Turgay et al., 1998). All known ClpC activities require the participation of an adaptor protein (Kirstein et al., 2007, 2009; Schlothauer et al., 2003; Turgay et al., 1998; Wang et al., 2011). For example, TrfA is required for the degradation of SsrA-tagged proteins in S. aureus (Donegan et al., 2014). It is currently unknown whether an adaptor is required for the recognition of SsrA tag by ClpCP in S. mutans. As MecA is an important adaptor for ClpC function, we used purified MecA in our in vitro studies. However, we found that addition of MecA did not change ClpCP-mediated degradation, suggesting that MecA probably is not the adaptor for ClpCP for SsrA-mediated degradation. It is important to note that streptococci, including S. mutans, do not encode homologues of other known adaptors. Therefore, one needs to identify the adaptor protein for ClpC experimentally. Similarly, no adaptor proteins have been reported for ClpE activities (Kirstein et al., 2009). As our in vitro studies suggest that the ClpEP complex can degrade SsrA-tagged substrates without any accessory proteins, it is possible that ClpE may directly recognize the C-terminal sequence of the SsrA tag. This sequence may be the same or overlapping with the ClpX binding sequence. In this respect, the function of ClpE is analogous to ClpA of E. coli.

Amongst the Gram-positive organisms, the degradation of SsrA-tagged proteins seems to be highly diverse. Whilst ClpXP is the major proteolytic complex responsible for degradation of SsrA-tagged proteins in most, ClpXP does not play a role in S. aureus. We found that, in addition to ClpXP, both ClpCP and ClpEP degrade SsrA-tagged proteins in S. mutans. This function of ClpCP and ClpEP appears not to be present in all streptococci because these two protease complexes are not involved in SsrA tag degradation in S. pneumoniae (Ahlawat & Morrison, 2009).

As ClpX is not under CtsR regulation, we speculate that ClpXP normally degrades SsrA-tagged peptides under non-stressed conditions. Under heat shock or other stresses, the induced proteolytic activity of ClpEP and ClpCP is required for the degradation of additional SsrA-tagged substrates that are generated during the stress. Thus, we speculate that in streptococci that often encounter various stresses, the ClpCP and ClpEP complexes have evolved to participate in the additional degradation SsrA-tagged proteins. Future studies are necessary to identify the ClpC or ClpE alleles from various streptococci that take part in SsrA tag degradation.

Although tmRNA is universally present in bacteria, the length and the sequence can vary greatly. When we searched the tmRNA database (tmRDB; Zwieb et al., 2003), we found that the majority of SsrA sequences are ~11 residues. However, we found that the Mollicutes (Firmicutes) group is an exception in which the sequences vary greatly. For example, whilst the SsrA tag of Mycoplasma mycoides is 12 residues (ADSHQRDYALAA), the tag for Phytoplasma asteris is 35 residues. We also used a separate database to analyse the SsrA sequences of streptococci (http://bioinformatics.sandia.gov/tmRNA/index.html). Our search returned some key attributes of the SsrA tags in this genus. First, most of the streptococci encode an 11-residue; however, the last three residues are two different types. About two-thirds of the streptococci encode LAA, whilst the rest, including S. mutans, encode VAA. Second, we found

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**Fig. 6.** ClpCP- and ClpEP-mediated degradation is induced by heat stress. (a) Western blot analysis of GFPavaa in the ΔclpXΔclpC and ΔclpXΔclpCΔctsR mutants. Cultures at both exponential phase (E) and late exponential phase (LE) were investigated. (b) Kinetics of SsrA degradation during heat stress. Total proteins were extracted from S. mutans cells grown either at 37 or 44 °C at the indicated time points t. (c) Level of GFPavaa in cell extracts was determined by Western blot analysis using an anti-GFP antibody. Enolase was used as an internal control.
that the SsrA tag in *Streptococcus salivarius* is 17 residues – the longest amongst all the streptococci. Surprisingly, we also found that *Streptococcus infantarius* encodes the shortest SsrA tag with only the YAVAA sequence. In *E. coli*, the last three residues of the SsrA tags are recognized completely by ClpX and partially by ClpA (Flynn et al., 2001). Binding of SspB is not necessary for the degradation of SsrA-tagged proteins, but it enhances the ClpXP-dependent degradation rate (Flynn et al., 2001). Moreover, the presence of SspB would allow ClpXP to degrade SsrA-tagged proteins with the last three residues mutated to certain other residues, such as DAS or LGG (Griffith & Grossman, 2008; McGinness et al., 2006). *S. mutans* and other streptococci do not encode SspB-like adaptor protein and, unlike *E. coli*, mutating the last three residues of the *mutans* SsrA tag to LGG completely inhibits its degradation, suggesting that the SspB-like tethering role is absent in this genus. Therefore, the exact role of the N-terminal residues of SsrA tags in degradation remains to be explored.

As mentioned above, we noticed that the last three residues of the SsrA tag are either VAA or LAA; the exact reason for this difference is currently unknown. Interestingly, Ahlawat & Morrison (2009) reported that in *S. pneumoniae*, which encodes the SsrA tag with LAA, both ClpCP and ClpEP apparently do not play a role in SsrA-mediated degradation. Therefore, we speculate that for ClpCP- and ClpEP-mediated degradation, the last three residues are important. We are currently in the process of understanding the role of the C-terminal residues in SsrA tag degradation in streptococci.

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