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ClpXP Degrades SsrA-Tagged Proteins in *Streptococcus pneumoniae*[▽]

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Bacterial proteins that are abnormally truncated due to incomplete mRNA or the presence of rare codons are extended by an SsrA tag during ribosome rescue in a *trans*-translation process important for maintaining protein quality. In *Escherichia coli*, the SsrA-tagged proteins become the target of the Tsp, Lon, FtsH, ClpXP, and ClpAP proteases. Here we show that degradation of model SsrA-tagged proteins in *Streptococcus pneumoniae* depends primarily or exclusively on ClpXP in vivo. In addition, we show the *E. coli* SsrA tag is also a target of *S. pneumoniae* ClpXP in vivo, even though the N-terminal portions of the tags differ significantly between the two species, suggesting there may be no adaptor protein for SsrA in *S. pneumoniae*.

Efficient use of translational machinery is disrupted by stalled ribosomes. Causes of such stalling include the presence of a rare codon, broken message, scarcity of tRNA, and others (21). To rescue stalled ribosomes and to relieve the stress caused by accumulation of stalled ribosomes and incompletely synthesized proteins, a unique RNA known as transfer message RNA (tmRNA) is recruited to the stalled ribosome (12). The tmRNA, also known as the ssrA RNA or 10S RNA, has dual roles, in which it acts both as a tRNA and as an mRNA (12). The polypeptide chain is provided with alanine on the stalled ribosome by charged tmRNA; then, the ribosome resumes protein synthesis, now directed by a small internal open reading frame of the tmRNA (12, 26). The result is addition of a small SsrA peptide tag at the C terminus and eventual release of the newly synthesized, but likely defective, polypeptide (12, 26). The SsrA peptide tag added to the incomplete polypeptide serves as a recognition signal for proteolysis. This mechanism, designated *trans*-translation, maintains the quality of proteins by eliminating many aberrant ones. *ssrA* genes are widely conserved among all bacteria and most encode an SsrA sequence of approximately 10 amino acids (11).

TABLE 1. Bacterial strains used in this study

Strain	Description	Source or reference
CP1250	*hex- malM511 str-1 bgl-1*; Rx derivative, low β-galactosidase background	19
CP1343	CP1250, but Δ*clpL*::*erm*; Erm^r	17
CP1344	CP1250, but Δ*clpC*::*tet*; Tet^r	17
CP1359	CP1250, but Δ*clpP*::*tet*; Tet^r	17
CP1361	CP1250, but Δ*clpX*::*erm slx*; Erm^r	17
CP1363	CP1250, but Δ*ftsH*::*erm*; Erm^r	17
CP1851	CP1250, but Δ*clpE*::*erm*; Erm^r	25
CP1903	CP1250, but *aga*::*gfp-ssrA*::P*ckan*; Kan^r	CP1250 × *aga-gfp-ssrA*$_{Sp}$*-kan-rafE*
CP1904	CP1903, but Δ*clpP*::*tet*; Kan^r Tet^r	CP1359 × CP1903
CP1905	CP1903, but Δ*clpX*::*erm slx*; Kan^r Erm^r	CP1361 × CP1903
CP1906	CP1903, but Δ*clpC*::*tet*; Kan^r Tet^r	CP1344 × CP1903
CP1907	CP1903, but Δ*clpE*::*erm*; Kan^r Erm^r	CP1851 × CP1903
CP1903	CP1903, but Δ*clpL*::*erm*; Kan^r Erm^r	CP1343 × CP1903
CP1910	CP1903, but Δ*ftsH*::*erm*; Kan^r Erm^r	CP1363 × CP1903
CP1911	CP1250, but *aga*:: *gfp*::P*ckan*; Kan^r	CP1250 × *aga-gfp-kan-rafE*
CP1912	CP1911, but Δ*clpP*::*tet*; Kan^r Tet^r	CP1359 × CP1911
CP1913	CP1911, but Δ*clpX*::*erm slx*; Kan^r Erm^r	CP1361 × CP1911
CP1914	CP1911, but Δ*clpC*::*tet*; Kan^r Tet^r	CP1344 × CP1911
CP1915	CP1911, but Δ*clpE*::*erm*; Kan^r Erm^r	CP1851 × CP1911
CP1916	CP1911, but Δ*clpL*::*erm*; Kan^r Erm^r	CP1343 × CP1911
CP1954	CP1250, but *aga*::*cat-ssrA*::P*ckan*; Cm^r Kan^r	CP1250 × *aga-cat-ssrA-kan-rafE*
CP1957	CP2000, but *aga*::*gfp-ssrA*$_{Ec}$::CAT::P*ckan*; Cm^r Kan^r	CP1250 × *aga-gfp-ssrA*$_{Ec}$*-kan-rafE*
CP1958	CP1957, but Δ*clpP*::*tet*; Kan^r Cm^r Tet^r	CP1359 × CP1957
CP1959	CP1957, but Δ*clpX*::*erm slx*; Kan^r Erm^r	CP1361 × CP1957
CP1969	CP1954, but Δ*clpX*::*erm slx*; Kan^r Erm^r Cm^r	CP1359 × CP1954
CP1971	CP1954, but Δ*clpP*::*tet*; Kan^r Cm^r Tet^r	CP1359 × CP1954
CP2025	CP1250, but Δ*nagA*::*erm*; Erm^r	CP1250 × *adh-erm-spr1868*

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In *Escherichia coli*, five proteases (Tsp, FtsH, Lon, ClpXP, and ClpAP) recognize the SsrA sequence and can participate to some extent in degrading polypeptide chains attached to it (3, 6, 9, 24), but ClpXP is thought to be the major protease responsible for the degradation of SsrA-tagged proteins in vivo under normal conditions in two gram-negative bacterial species (*E. coli* and *Caulobacter crescentus*) and in the gram-positive *Bacillus subtilis* (2, 6, 27). An adaptor protein, SspB, in *E. coli* and its ortholog (SspBα) in *C. crescentus* promote degradation of SsrA-tagged proteins by ClpXP, while no similar adaptors are known in gram-positive bacteria (2, 7, 14, 15). For most other bacterial species, SsrA-tagged proteins are speculated to be substrates of ClpXP (10). However, ClpX is not conserved in all bacteria, while FtsH is, and for bacteria lacking ClpX, FtsH or Lon is considered to be the likely protease for SsrA-tagged proteins (8, 10). The mycoplasmas, for example, which lack ClpX and ClpP orthologs, possess a Lon protease with exceptional activity on SsrA-tagged substrates (8).

An SsrA tagging system is active in *Streptococcus pneumoniae* (pneumococcus), but the specific proteases responsible for the recognition and degradation of its SsrA-tagged proteins are unknown (18). The pneumococcal SsrA sequence (AKNN TSYALAA) resembles the one in *E. coli* (AANDENYALAA) in length and also in the sequence recognized directly by ClpX (underlined) (5). There are fewer cellular proteases in *S. pneumoniae* than in *E. coli* or in *B. subtilis.* As it has just five putative *clp* genes (*clpC*, *clpE*, *clpL*, *clpP*, and *clpX*) and carries *ftsH*, encoding a well-characterized membrane-bound protease, *S. pneumoniae* lacks genes for proteases of the Lon, Tsp, ClpA, and ClpYQ classes (20). Thus, ClpXP and FtsH are both good candidates for SsrA-specific proteases; however, it should be noted that while degradation by ClpXP in vivo requires the presence of the adaptor protein SspB in *E. coli* (4), no apparent pneumococcal homologs of SspB are known.

To identify pneumococcal cytoplasmic proteases that are involved in degrading SsrA-tagged proteins in vivo, we constructed synthetic genes for model SsrA-tagged proteins and determined the effects of individual protease mutations on the level of the tagged proteins. The results showed that proteins tagged with SsrA at their C terminus are strongly stabilized by mutations of *clpX* and *clpP* but not by mutations affecting any other Clp protease or FtsH and suggest that ClpXP recognition of SsrA tags may not require an adaptor protein.

GFP-SsrA is unstable in *S. pneumoniae*. To determine the fate of GFP-SsrA in vivo, we constructed strains in which genes encoding GFP-SsrA or green fluorescent protein (GFP) were inserted in the chromosome of *S. pneumoniae* downstream of the native site of the gene *aga*, creating a transcriptional fusion dependent on the raffinose-inducible *aga* promoter (Table 1; Fig. 1). The resulting expression cassette, *aga-gfp-ssrA-aphA*, also includes a constitutive promoter for the kanamycin resistance marker, *aphA*. To ensure maximal expression of GFP-SsrA, a synthetic ribosome binding site, 5′-AGGAGGTA, was positioned 6 bases upstream of the start codon of GFP. This sequence is found at pneumococcal ribosomal protein genes and is predicted to be a strong ribosome binding site (23). The constructs were confirmed by PCR analysis using primers binding outside and within the insertions and by sequencing each entire insert (Table 2). As *aga* encodes the enzyme α-galactosidase, which hydrolyzes *p*-nitrophenyl-D-galactopyranoside to the yellow product *p*-nitrophenol, the expression of the synthetic operon can be readily assayed spectrophotometrically (22).

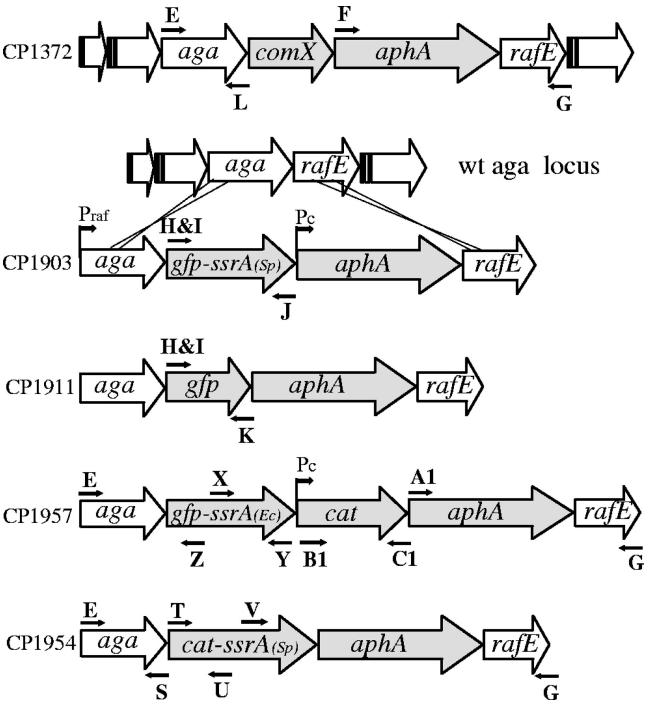

FIG. 1. Organization of the *aga* locus and construction of cassettes for regulated synthesis of GFP- and SrA-tagged proteins. Fragments containing a truncated *aga* gene (′*aga*) or the *aphA* (kanamycin resistance) gene and a truncated *rafE* gene (*rafE*′) were amplified with primer pairs E-L and F-G, respectively, from the CP1372 DNA template. *gfp-mut2* was first cloned into pMAL-c2X after amplification from plasmid pKL147, kindly provided by Adam Driks (Loyola University), using primers A and B. *ssrA* was amplified from CP1250 with primers C and D and inserted downstream of *gfp-mut2*. A heterologous fragment with *gfp-ssrA* was amplified in two steps: one to add a synthetic ribosome binding site (primers H and J) using plasmid pMAL-c2X (*gfp-ssrA*) as a template and the second to add a restriction site to the first product (primers I and J). After digestion at restriction sites incorporated by the PCR primers, the second product was ligated with the targeting *aga* and *aphArafE* fragments as indicated. After transformation into CP1250 with Kan selection, the structure of the new synthetic locus in CP1903 was verified by sequencing. Filled arrows, genes manipulated during the construction; open arrows, conserved genes of the raffinose locus and its flanking genes. Promoters: Praf, *aga* promoter; Pc, artificial constitutive promoter derived from *amiA*. CP1911, CP1957, and CP1954 were constructed similarly but by using primer pairs H-K/I-K, E-Z/X-Y/B1-C1/A1-G, and E-S/T-U/V-G.

For induction of the *aga* locus with raffinose, cultures were grown in medium containing 0.1% raffinose and expression of the compound *aga* locus was monitored by determining α-galactosidase activity. Lysates of the wild type and of strains carrying GFP-SsrA or GFP constructs all displayed 10-fold induction of *aga* expression compared to controls without raffinose, indicating comparable expression levels for both *gfp* constructs (data not shown). Strains CP1903 and CP1911, expressing the GFP-SsrA or GFP genes, respectively, were examined for accumulation of GFP by a Western blotting assay using anti-GFP antibody. GFP-SsrA was not detectable, whereas untagged GFP was readily detected as a band at a

TABLE 2. Oligonucleotides used in this study

Primer	Lab name	Sequence[a]	Location
A	SA 47	5′-ggttgccggcATGAGTAAAGGAGAAGAACT	*gfp*
B	SA 48	5′-ggttggatccTTTGTATAGTTCATCCATGC	*gfp*
C	SA 51	5′-ggttgtcgacGCAAAAAATAACACTTCTTA	$ssrA_{Sp}$
D	SA 52	5′-ggttaagcttTGGAGCCGGTGGGAGTCGAA	*ssrA*
E	SA 81	5′-ATATTCTCTTTGAGTCCTGCTCTGG	*aga*
F	SA 85	5′-ggttgccggcGGATCCGTTTGATTTTTAATGG	*aphA*
G	SA 86	5′-caacgatatcTTTGACTAACTGT	*rafE*
H	SA 90	5′-aggaggtaaatctaATGAGTAAAGGAGAAGAAC	*gfp*
I	SA 92	5′-ggttggtaccAGGAGGTAAATCTAATGAGT	*gfp*
J	SA 93	5′-ggttgccggcCAAACACCTGCCAACATATT	*gfp-ssrA*$_{Sp}$
K	SA 94	5′-ggttgccggcCGACTCTAGAGGATCCTTATTT	*gfp*
L	SA 96	5′-ggttggtaccTCATAGTTTTCTAAAAATATACT	*aga*
M	SA 98	5′-ggttgccggcTTTGTATAGTTCATCCATGC	*gfp*
N	SA 100	5′-TGGCGAAGTTTACTCAGGTG	*aga*
O	SA 101	5′-TTTCCCGTTCCACATCATAGG	*aphA*
P	SA 102	5′-GCATGGCACTCTTGAAAAAGTC	*gfp*
Q	SA 103	5′-GTAACAGCTGCTGGGATTACAC	*gfp*
R	SA 104	5′-ATATAATGGTTCGGGGAAATTG	*cat*
S	SA 105	5′-ggttggatcCTCATAGTTTTCTAAAAATATACTG	*aga*
T	SA 106	5′-ggttggatccGCTTGATGAAAATTTGTTTGAT	*cat*
U	SA 107	5′-ggttctcgagTAAAAGCCAGTCATTAGGCC	*cat*
V	SA 108	5′-ggttctcgagGTAACAGCTGCTGGGATTAC	*gfp*
W	SA 109	5′-AGGAGTCCAAATACCAGAGAATG	*cat*
X	SA 120	5′-ggttggatccGCAAACGACGAAAACTACGC	$ssrA_{Ec}$
Y	SA 121	5′-ggttgtcgacCGTCCGAAATTCCTACATCC	$ssrA_{Ec}$
Z	SA 122	5′-ACTCTAGAGGATCCTTTGTATAG	*gfp*
A1	SA 123	5′-ggtttctagaCGGTGGATCCGTTTGATTTT	*aphA*
B1	SA 124	5′-ggttgtcgacCGGTATCGATAAGCTTGATGA	*cat*
C1	SA 125	5′-ggtttctagaGGTTAGTGACATTAGAAAACCG	*cat*
D1	SA 130	5′-GATCTGTCAATGGTTCAGATAC	*spc*
E1	SA 131	5′-AAAGATATTGCGGGAAATGC	*spc*
F1	SA 153	5′-ggttctcgagCAAAATTTGTTTGATTTGTATCT	*erm*
G1	SA 154	5′-ggttggatccGTCGGCAGCGACTCATAG	*erm*
H1	SA 157	5′-ggccggatccAGAGAGAAAGCAGAAGTTAGAG	*adh*A
I1	SA 158	5′-GCCAGACAAGGAAAGAAATATG	*adh*A
J1	SA 159	5′-TAATTGGAGTTTGGCAGTTG	SPR1868
K1	SA 160	5′-ggccctcgagTAGGCATAATGTTAACCTCCTT	SPR1868

[a] Restriction nuclease recognition sites are underlined and nucleotides matching templates are in uppercase letters.

position corresponding to the size of purified GFP (Fig. 2). We conclude that GFP-SsrA is degraded in vivo in the wild-type background but the untagged version of GFP is more stable, implying that the SsrA tag renders GFP a substrate of cytoplasmic proteases.

GFP-SsrA is stable in ClpP- and ClpX-deficient cells but unstable in all other protease mutants. Since *S. pneumoniae* carries genes for ClpX, ClpP, and other Clp protease subunits, we asked whether ClpXP or another protease might participate in the degradation of SsrA-tagged GFP in pneumococcus by determining the accumulation of GFP-SsrA in *clpX*, *clpP*, *clpC*, *clpE*, *clpL*, and *ftsH* mutant strains. Although *clpX* was reported to be essential in *S. pneumoniae* (20), P. Luo obtained a deletion of *clpX* in the presence of an unlinked suppressor of this lethality (*slx*) (16; A. Piotrowski, personal communication), allowing the examination of *clpX* mutants reported here.

The level of untagged GFP was not elevated in any of these protease-deficient backgrounds, suggesting that GFP itself is not a substrate of any of the corresponding proteases (Fig. 2). GFP-SsrA was detected in *clpX* and *clpP* mutants but not in the wild-type background or in the other mutant strains (Fig. 2). The parallel effects of *clpP* and *clpX* mutations strongly suggest that the observed stabilizing effect of these mutations on GFP-SsrA arises from disruption of ClpXP protease activity and not from any polar effects of the two mutations. We conclude that ClpXP is the major pneumococcal Clp protease that degrades SsrA-tagged GFP. However, the possibility that FtsH or another protease has some minor role in degradation of SsrA-tagged proteins cannot be ruled out, as ClpX is present in the other protease mutants and could mask minor additional activities.

GFP tagged with *E. coli* SsrA is degraded in *S. pneumoniae*. The sequence of the *E. coli* SsrA tag ($SsrA_{Ec}$), AANDENYA LAA, is similar to that of *S. pneumoniae* ($SsrA_{Sp}$), AKNNTS YALAA. The first 7 amino acids, AANDENY of the *E. coli* SsrA tag contain the binding site for its adaptor protein, SspB, while the terminal 3 amino acids (LAA) interact with ClpX (5). In vitro, SspB enhances the degradation rate of proteins tagged with SsrA by at least 10-fold and is required for their degradation by ClpXP in vivo (4). The three terminal residues of the pneumococcal SsrA tag are identical to those of the *E. coli* tag; the tags only differ in the portion specific for the adaptor protein. We reasoned that if *S. pneumoniae* requires an adaptor protein for ClpXP specific for AKNNTSY, then proteins tagged with $SsrA_{Ec}$ would not be degraded, as the AANDENY portion of the tag recognized by the *E. coli* adaptor protein is

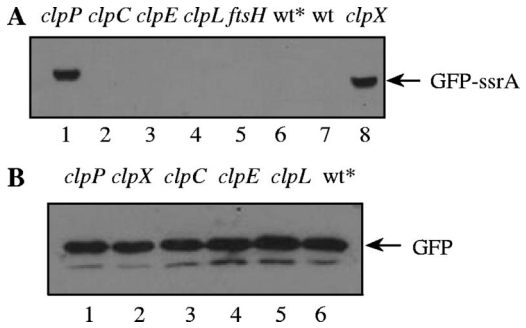

FIG. 2. Stabilization of GFP-SsrA in *clpX* and *clpP* mutants. (A) Strains expressing *gfp-ssrA* in *clpP* (CP1904), *clpC* (CP1906), *clpE* (CP1907), *clpL* (CP1908), *ftsH* (CP1910), or *clpX* (CP1905) mutant backgrounds were harvested, lysed, and loaded in lanes 1 to 5 and 8 of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. CP1903, expressing *gfp-ssrA* in the wild-type background (wt*), and the wild-type with no *gfp* insertion at the *aga* locus (wt) were loaded in lanes 6 and 7, respectively. (B) Strains expressing untagged *gfp* in *clpP* (CP1912), *clpX* (CP1913), *clpC* (CP1914), *clpE* (CP1915), or *clpL* (CP1916) mutant backgrounds or in the wild-type background (wt*; CP1911) were harvested, lysed, and loaded in lanes 1 to 6. Strains expressing *gfp-ssrA* or untagged *gfp* in protease-proficient backgrounds are represented as wt*. Cultures were grown to an optical density at 550 nm of 0.25 in a casein hydrolysate yeast extract medium (13) supplemented with raffinose (1 g/liter) for maximal induction of the *aga* locus. Cell pellets were resuspended and heated in a 1/100 volume of lysis buffer (100 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, and 200 mM dithiothreitol) for determination of GFP or GFP-SsrA by Western blotting with anti-GFP antibody (Roche). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to polyvinylidene difluoride membranes, the membranes were probed with a mouse anti-GFP primary antibody (1:1,000; Roche) and with an anti-mouse immunoglobulin G secondary antibody conjugated to horseradish peroxidase (1:10,000; GE). Detection was performed with an enhanced chemiluminescence substrate (ECL Plus; GE) and Hyblot CL film (Denville Scientific) with exposures between 10 and 600 s.

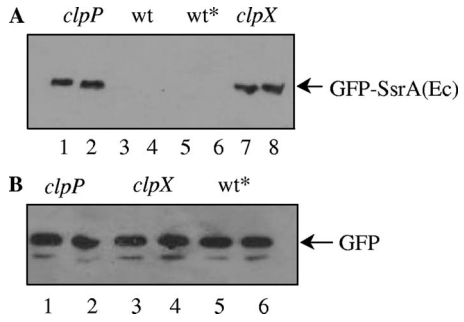

FIG. 3. Targeting of *E. coli* SsrA by ClpXP in *S. pneumoniae*. (A) Strains expressing *gfp-ssrA*$_{Ec}$ in *clpP* (CP1958) and *clpX* (CP1959) null mutant backgrounds were harvested, lysed, and loaded in lanes 1 and 2 and 7 and 8, respectively. The wild type with no *gfp* insertion at the *aga* locus (CP1250) and a strain expressing *gfp-ssrA*$_{Ec}$ in the wild-type background (wt*; CP1957) were loaded in lanes 3 and 4 and lanes 5 and 6, respectively. (B) Strains expressing *gfp* in *clpP* (CP1912), *clpX* (CP1913), or wt (wt*; CP1911) backgrounds were loaded in lanes 1 and 2, 3 and 4, or 5 and 6, respectively. Cultures were induced with raffinose and prepared for Western analysis as described for Fig. 2, but all the samples were loaded in duplicate.

considerably different in sequence and overall charge. To seek an indication of a pneumococcal adaptor with a different specificity for SsrA, we constructed a strain containing *gfp* fused to the *ssrA*$_{Ec}$ tag and determined the levels of GFP-SsrA$_{Ec}$ and GFP in vivo by Western blotting. Surprisingly, GFP-SsrA$_{Ec}$ was completely absent from wild-type cells, while untagged GFP was readily detected in parallel controls, as expected. Suspecting that ClpXP may be responsible for the absence of GFP-SsrA$_{Ec}$, as it is for GFP-SsrA$_{Sp}$, we transformed the SsrA$_{Ec}$ construct into *clpX* and *clpP* mutants. GFP-SsrA$_{Ec}$ was readily detectable in the *clpX* and *clpP* mutants at levels comparable to GFP-SsrA$_{Sp}$ in the same backgrounds (Fig. 3), strongly suggesting that ClpXP degrades GFP tagged with SsrA$_{Ec}$, just as it degrades GFP-SsrA$_{Sp}$. This result implies that ClpXP likely does not require an adaptor to degrade SsrA-tagged protein in *S. pneumoniae*.

Degradation of SsrA-tagged CAT by ClpXP. To test the generality of SsrA targeting by ClpXP and to seek an adaptor for SsrA by an independent method, we constructed strains expressing chloramphenicol (Cm) acetyltransferase (CAT) fused to a terminal SsrA tag. If CAT-SsrA were also targeted by ClpXP, Cm resistance could depend on loss of *clpX*, *clpP*, or loss of an adaptor for ClpXP. In the new cassette, *cat-ssrA* was expressed constitutively and was linked to a Kan (*aphA*) marker to allow selection independent of accumulation of CAT-SsrA. The structure of the new cassette (*cat-ssrA-aphA*) was confirmed by sequencing. In a *clpX* or *clpP* mutant background, the synthetic *cat-ssrA* gene conferred resistance to Cm comparable to that provided by the untagged CAT gene (Table 3), while the *clpX*$^+$ *clpP*$^+$ CAT-SsrA strain CP1954 was as sensitive to Cm as its wild-type parent, CP1250. Thus, CAT-SsrA was stabilized in *clpX* and *clpP* mutants, indicating that ClpXP degrades a second SsrA-tagged protein.

Transformation of the Cm-sensitive *cat-ssrA* strain with a complex random *spc* insertion library (1) failed to reveal any insertions outside of *clpX* and *clpP* that stabilized the model SsrA-tagged CAT (data not shown). While this result is consistent with the conclusion that pneumococcal ClpXP acts without an adaptor to recognize SsrA, other explanations are also possible, including incomplete saturation by the *mariner* transposition reactions or essentiality of the putative adaptor. Degradation of SsrA-tagged proteins in vivo by ClpXP without the participation of an adaptor protein may not be exceptional; while this study was under way it was reported that degradation of SsrA-tagged proteins in *B. subtilis* similarly appears to lack an adaptor for ClpXP (7). Perhaps the amino-proximal portion of the gram-positive SsrA tag acts primarily as a spacer be-

TABLE 3. Stabilization of CAT-SsrA by *clpP* or *clpX* mutations

Strain	CAT allele	Genotype		Cm MIC[a] (μg/ml)
		clpX	*clpP*	
CP1250		+	+	8
CP1359		+	−	4
CP1954	*cat-ssrA*	+	+	8
CP1972	*cat-ssrA*	+	−	32
CP1969	*cat-ssrA*	−	+	32
CPM7	pEVP3 *cat*	+	+	32

[a] The lowest level of Cm in a twofold dilution series at which colonies failed to appear during 20 h at 37°C.

tween its C-terminal ClpX-interacting portion and the body of the protein linked to it.

In summary, ClpXP appears to be the primary protease of *S. pneumoniae* that degrades proteins tagged with SsrA signals of *S. pneumoniae* or *E. coli* and it appears to act without the participation of an adaptor protein.

Nucleotide sequence accession numbers. Nucleotide sequences of the new cassettes reported here have been deposited as GenBank accession numbers FJ495555 to FJ495558.

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