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Control of Natural Transformation in Salivarius Streptococci through Specific Degradation of σ^X by the MecA-ClpCP Protease Complex

Astrid Wahl,[a] Florence Servais,[a] Anne-Sophie Drucbert,[b] Catherine Foulon,[c] Laetitia Fontaine,[a] Pascal Hols[a]

Institut des Sciences de la Vie, Université Catholique de Louvain, Louvain-la-Neuve, Belgium[a]; Université Lille Nord de France and UDSL, Plate-Forme d'Interactions Moléculaires, IMPRT, IFR 114, Faculté de Médecine, Lille, France[b]; Université Lille Nord de France and UDSL, EA-4481, UFR Pharmacie, Lille, France[c]

Competence for natural DNA transformation is a tightly controlled developmental process in streptococci. In mutans and salivarius species, the abundance of the central competence regulator σ^X is regulated at two levels: transcriptional, by the ComRS signaling system via the σ^X/ComX/SigX-inducing peptide (XIP), and posttranscriptional, by the adaptor protein MecA and its associated Clp ATPase, ClpC. In this study, we further investigated the mechanism and function of the MecA-ClpC control system in the salivarius species *Streptococcus thermophilus*. Using *in vitro* approaches, we showed that MecA specifically interacts with both σ^X and ClpC, suggesting the formation of a ternary σ^X-MecA-ClpC complex. Moreover, we demonstrated that MecA ultimately targets σ^X for its degradation by the ClpCP protease in an ATP-dependent manner. We also identify a short sequence (18 amino acids) in the N-terminal domain of σ^X as essential for the interaction with MecA and subsequent σ^X degradation. Finally, increased transformability of a MecA-deficient strain in the presence of subinducing XIP concentrations suggests that the MecA-ClpCP proteolytic complex acts as an additional locking device to prevent competence under inappropriate conditions. A model of the interplay between ComRS and MecA-ClpCP in the control of σ^X activity is proposed.

Several bacteria are able to enter a transitory physiological state known as "competence for natural transformation," enabling the natural capture and chromosomal integration of exogenous naked DNA, a key process for genome plasticity and virulence (for a recent review, see reference 1). In Gram-positive bacteria, competence development is under the control of a master regulator that transcriptionally activates the so-called late competence (*com*) genes, encoding DNA uptake, protection, and homologous recombination machineries, among others (1). In bacilli, this master regulator is a transcriptional activator (ComK), while in streptococci, it is an alternative sigma factor (σ^X or ComX/SigX) that transiently associates with the core of the RNA polymerase. Both regulators were shown to specifically bind to specific DNA motifs located in the promoters of late *com* genes (1). Since DNA transformation is an energy-expensive process, with possible deleterious effects on genome integrity and cell division, a strict control of the production of the master regulator is essential to avoid its activation under inappropriate conditions (1). This control takes place during the so-called early phase of competence at two major levels: transcriptional, by a pheromone-based signaling system, and posttranslational, through the active degradation/stabilization of the master regulator (1).

In *Bacillus subtilis*, competence is activated at the entry into the stationary growth phase by the quorum-sensing peptide pheromone $ComX_{Bsu}$ (2). This pheromone is sensed by the ComAP phosphorelay system, which regulates the production of $ComS_{Bsu}$, a short peptide encoded within the surfactin A-encoding operon (3–5). $ComS_{Bsu}$ is necessary for ComK release from degradation. ComK then autoactivates its production and activates those of late *com* genes (3–5). The posttranslational control of ComK abundance has been investigated in detail and relies on the adaptor protein $MecA_{Bsu}$ (for a review, see reference 6). To prevent competence development under unfavorable conditions, ComK is sequestered by $MecA_{Bsu}$, which specifically interacts with ClpC, resulting in ComK degradation by the ClpCP proteolytic machinery (7–11). In contrast, under competence-inducing conditions, ComK is released from this inhibitory complex by the antiadaptor peptide $ComS_{Bsu}$ through a direct competitive interaction with $MecA_{Bsu}$ (9, 12). Short core sequences sharing some similarities were identified in ComK and $ComS_{Bsu}$ (FMLYPK and IILYPR, respectively) as important for their interaction with $MecA_{Bsu}$ (9). Thus, in *B. subtilis*, the pheromone-based signaling system indirectly controls the abundance of the master regulator ComK at the posttranslational level, a control level of key importance for competence development in this species (5, 6).

In streptococci, two alternative signaling systems, ComCDE and ComRS, directly control *comX* transcription via distinct activation mechanisms (for recent reviews, see references 1 and 13). Both systems activate competence transiently during the early logarithmic growth phase. The ComCDE system, present in the mitis and anginosus groups, has been extensively studied in *Streptococcus pneumoniae* (1, 14, 15). In this species, the extracellular pheromone competence-stimulating peptide (CSP) encoded by *comC* is sensed by the ComDE phosphorelay system, which in turn activates *comX* and *comCDE* (positive feedback loop) (1, 14) and other early *com* genes, including *comW* (16). *comW* encodes the small protein ComW, which stabilizes and activates σ^X at the posttranslational level (17). In addition, the abundance of σ^X is controlled by the ClpEP and ClpCP proteolytic machineries, which degrade σ^X and ComW, respectively (18). Thus, in *S. pneumoniae*,

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Address correspondence to Laetitia Fontaine, laetitia.fontaine@uclouvain.be, or Pascal Hols, pascal.hols@uclouvain.be.
L.F. and P.H. contributed equally to this article.
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the pheromone-based system controls the master regulator σ^X at both transcriptional and posttranslational levels. The recently discovered ComRS system, present in the salivarius (19–21), mutans (22–24), pyogenic (24, 25), and bovis (24, 26) groups, was experimentally shown to be the signaling system that directly controls *comX* transcription in at least one representative species of all the above-mentioned streptococcal groups. ComR is a transcriptional regulator of the Rgg family and ComS is the precursor of the secreted competence σ^X/ComX/SigX-inducing peptide (XIP) (19–21, 23, 24). The activation mechanism of the ComRS system was deeply investigated in *Streptococcus thermophilus* (19–21, 27) and *Streptococcus mutans* (22–24, 28). The extracellular pheromone XIP is not sensed by a phosphorelay system but is imported in the cytoplasm by the oligopeptide transporter Opp (Ami) (20, 24, 27), where it directly interacts with ComR (20). The XIP-ComR complex in turn directly activates the transcription of *comX* and *comS* by binding to the ComR box present in their promoter sequences (positive feedback loop) (20).

Besides the transcriptional control of *comX* by the ComRS signaling system, the activity of σ^X was also shown to be the target of a posttranslational negative control in one representative species of the pyogenic (25, 29), salivarius (30, 31), and mutans (32) groups. In these species, extensive *in silico* analyses did not allow the identification of a ComW homolog, suggesting that the regulatory system could be different from that in *S. pneumoniae*. We and others have shown that the adaptor protein MecA and the ClpC ATPase subunit act as negative regulators of competence development in both *S. thermophilus* (30, 31) and *S. mutans* (32). These studies revealed that the expression of late *com* genes and the abundance of σ^X were increased in MecA- and ClpC-depleted strains compared to the isogenic wild-type (WT) strains (30–32) In addition, bacterial two-hybrid (B2H) assays suggested that MecA could interact *in vivo* with both σ^X and ClpC (31, 32). By analogy to the posttranslational control of ComK in *B. subtilis*, we postulated that σ^X would be degraded by the MecA-ClpCP protease complex under nonpermissive conditions for competence development (31).

The aim of this study was to further investigate the role of the putative MecA-ClpCP complex in the posttranslational control of σ^X activity and to reveal its interplay with the ComRS system in the control of competence development in *S. thermophilus*. On one hand, we show that MecA directly interacts *in vitro* with both σ^X and ClpC and demonstrate that σ^X is specifically degraded by the MecA-ClpCP proteolytic machinery in the presence of ATP. On the other hand, we show that at low noninducing XIP concentrations, DNA transformation is significantly increased in the absence of a functional MecA-ClpCP complex. Altogether, these results support our model of posttranslational control of σ^X in ComRS-containing streptococci where the adaptor protein MecA selects σ^X for specific degradation by ClpCP to avoid competence development under unfavorable conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* was grown with shaking at 37°C in lysogeny broth (LB) (33). *S. thermophilus* strains were grown anaerobically (BBL GasPak Systems, Becton, Dickinson, Franklin Lakes, NJ) at 37°C in M17 broth or Todd-Hewitt broth (THB) (Difco Laboratories Inc., Detroit, MI). Those media were supplemented with 1% (wt/vol) lactose or glucose (M17L or M17G and THBL or THBG, respectively). Solid agar plates were prepared by adding 2% (wt/vol) agar to the medium. When necessary, antibiotics were added to the media at the following concentrations: ampicillin at 250 μg ml^{-1} and kanamycin at 50 μg ml^{-1} for *E. coli* and erythromycin at 2.5 μg ml^{-1} for *S. thermophilus*.

Detection of absorbance and luminescence. Growth (optical density at 600 nm [OD_{600}]) and luciferase (Lux) activity (expressed in relative light units [RLU]) were monitored at 10-min intervals in a Varioskan Flash multimode reader (Thermo Fisher Scientific, Zellic, Belgium) as previously described (19).

Natural transformation experiments. Overnight cultures of *S. thermophilus* grown in THBG or -L at 37°C were diluted 30-fold in fresh medium and further grown at 37°C. Small volumes (300 μl) of the cultures were then supplemented with $ComS_{17-24}$ (purchased from Peptide 2.0, Chantilly, VA) at concentrations ranging from 0 to 40 nM. For kinetic transformation experiments, 1 μM $ComS_{17-24}$ was added to the culture, and then 2 μg of plasmid pGIUD0855ery (19) was added 0, 0.5, 1, 2, 3, or 4 h later. Samples (100 μl of serial dilutions in medium) were spread on M17 plates for the viability count and on M17 plates containing erythromycin for the selection of transformants. The transformation frequency was calculated after 30 h of anaerobic incubation at 37°C as the number of antibiotic-resistant CFU ml^{-1} divided by the total number of viable CFU ml^{-1}.

DNA techniques and electrotransformation. For general molecular biology techniques, the instructions given by Sambrook et al. (33) were followed. Electrotransformation of *E. coli* was performed as described by Dower et al. (34). The primers used in this study were purchased from Eurogentec (Seraing, Belgium) and are listed in Table S2 in the supplemental material. PCRs were performed with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA).

Construction of the pBADhisA-ComX, -MecA, -ClpC, -ClpE, and -ClpP expression vectors. The open reading frame (ORF) of each gene was amplified by PCR from the chromosome of strain LMD-9 with primers listed in Table S2 in the supplemental material. The PCR product restricted by XbaI and KpnI and pBADhisA restricted by NheI and KpnI were ligated and then transformed in *E. coli* TG1.

Construction of the pBAD-ComX-Strep expression vector. The *comX*::*strep* fusion (σ^X fused to the purification affinity tag StrepTagII at the C terminus) was amplified by PCR from the chromosome of strain CB0053 (31) with primers listed in Table S2 in the supplemental material. The PCR product and pBADhisA were restricted by NcoI and KpnI, ligated, and then transformed into *E. coli* TG1.

Bacterial two-hybrid assays and plasmid constructions. The method used was described by Karimova et al. (35). In order to obtain pUT18C-$MecA_{N1-103}$, pUT18C-MecA was digested by EcoRI and self-ligated. The partial coding sequences for the truncated versions of *comX* and *mecA* for all other constructs were amplified by PCR from *S. thermophilus* LMD-9 using primers reported in Table S2 in the supplemental material and inserted in plasmid pUT18, pUT18C, pKT25, or pKNT25. The complete list of recombinant plasmids used for B2H assays is reported in Table S3 in the supplemental material. B2H assays were performed as previously described (31). B2H assays were performed on MacConkey indicator plates supplemented with 1% maltose. Plates were incubated at 30°C for 36 h.

Purification of 6His-MecA, -ClpC, -ClpE, and -ClpP recombinant proteins. Precultures (20 ml) of strain TG1 transformed with pBAD6His-MecA, -ClpC, -ClpE, and -ClpP were diluted to an OD_{600} of 0.05 in 1 liter prewarmed LB (42°C) containing ampicillin and incubated at 42°C with continuous shaking according to the "thermal shift" procedure described before (36). At an OD_{600} of ~0.5, the culture was chilled on ice for 10 min with shaking every 2 min, and protein expression was then induced by adding 0.02% L-arabinose. After 4 h of induction at 28°C with continuous shaking, bacteria were centrifuged at 5,000 × *g* during 15 min. The pellet was washed once with cold 1× phosphate-buffered saline (PBS), frozen in liquid nitrogen, and kept at −80°C. The pellet was then resuspended in 40

ml lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol [DTT], 100 μg ml^{-1} of lysozyme, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cells were sonicated at 4°C (Bioruptor; Diagenode, Liège, Belgium), and the soluble fraction was collected after centrifugation (13,000 × *g* for 20 min at 4°C). The lysate was loaded on ProBond nickel-chelating resin (Life Technologies, Ghent, Belgium) in a 10-ml column. After washing with 20 mM imidazole, 6His-tagged proteins were eluted with 250 mM imidazole. The concentrations and purities of the fractions were estimated by SDS-PAGE and on a NanoDrop instrument (Thermo Scientific, Wilmington, DE). Glycerol was added to the different elution fractions at a final concentration of 25% (vol/vol). The purest fractions were used for further experiments. ComR-Strep was purified as previously described (20).

Purification of 6His-σ^X and σ^X-Strep recombinant proteins from inclusion bodies. For σ^X purifications, an overnight culture of *E. coli* TG1 cells carrying pBAD-ComX-Strep or pBADHisA-ComX was diluted 1:100 in 1 liter of LB containing ampicillin. Growth at 37°C with shaking was monitored until cells reached an OD_{600} of about 0.5 to 0.8. L-Arabinose was added to a final concentration of 0.02%. All further purification steps were carried out at 4°C unless otherwise specified. After an additional 4-h incubation at 28°C, the cells were harvested by centrifugation at 10,000 × *g* during 30 min and washed once with 1× PBS, and the pellet was frozen in liquid nitrogen and kept at −80°C. The pellet was then resuspended in 40 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM DTT, 100 μg ml^{-1} of lysozyme, 1 mM PMSF). Cell lysis was achieved by sonication for 10 min at 4°C (Bioruptor).

The insoluble fraction containing 6His-σ^X or σ^X-Strep was collected, washed, and then solubilized with Sarkosyl (*N*-lauroylsarcosine sodium salt solution, 30% aqueous solution; Sigma-Aldrich, Diegem, Belgium) as previously described (37). The centrifugation-clarified dialyzed sample was applied to a 10-ml Ni-nitrilotriacetic acid (NTA) agarose column in the case of 6His-σ^X and to a 1-ml Strep-Tactin Superflow column (IBA, Göttingen, Germany) in the case of the σ^X-Strep. 6His-σ^X was purified as described above. σ^X-Strep was purified according to the manufacturer's instructions for Strep-Tactin Superflow columns (IBA) using buffer W (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA). The purest fractions containing σ^X (identified by SDS-PAGE) were then combined, dialyzed against storage buffer (50 mM Tris-HCl [pH 8.0], 50% glycerol, 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT), and stored at −80°C.

SPR experiments. Surface plasmon resonance (SPR) experiments were performed on a Biacore 3000 instrument using a research-grade CM5 sensor chip (GE Healthcare, Vélizy-Villacoublay, France). For all experiments, the temperature was set at 25°C and a continuous flow of running buffer (1× HBS-EP, consisting of 10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) was maintained over the sensor surface. 6His-MecA protein was immobilized on one flow cell of the sensor chip by amine coupling according to manufacturer's instructions (GE Healthcare). After activation of the carboxymethyl groups of the dextran surface with an *N*-hydroxysuccinimide (NHS)–1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) mixture, a 15 μg ml^{-1} MecA solution prepared in 10 mM sodium acetate buffer (pH 4.0) was injected during 1 min on the surface at 5 μl min^{-1}. Finally, the surface was deactivated by an injection of ethanolamine (1 M). The level of covalently bound MecA protein was 1,150 resonance units (RU). Similarly, a reference flow cell was prepared (by activation and deactivation of the surface without injection of protein). All SPR analyses were performed at 20 μl min^{-1} by passing samples prepared in the running buffer for 120 s over the active and the reference flow cells. Return to baseline between each injection, i.e., removal of noncovalently bound analytes, was achieved by extended washing with the running buffer.

The sensorgrams, expressed as the difference between the active cell and the reference cell, were analyzed using BIAevaluation software version 4.0.1. Subtraction of a blank sensorgram, corresponding to a buffer injection at the beginning of each set of experiments, was performed in order to correct bulk effect and systematic artifacts. To determine the affinity of σ^X for MecA, a dose-response curve (steady-state response versus σ^X concentration) was constructed and fitted using the BIAevaluation software, considering a 1:1 stoichiometry. The equilibrium dissociation constant (K_D) for the MecA-σ^X complex was calculated with the following equation: $K_D = [\text{MecA}][\sigma^X]/[\text{MecA} - \sigma^X]$.

***In vitro* degradation assay.** Purified σ^X-Strep or ComR-Strep (0.6 μM) was incubated at 37°C in KTME buffer (100 mM KCl, 25 mM Tris-HCl [pH 8], 5 mM $MgCl_2$, 0.1 mM EDTA) with equimolar concentrations of 6His-MecA, -ClpC (or -ClpE), -ClpP, and ATP (5 mM) in a final volume of 40 μl. An ATP regeneration system consisting of pyruvate kinase (PK) (1.17 μM) and phosphoenolpyruvate (PEP) (2 mM) (Sigma-Aldrich) was added to ensure that ATP was not limiting the ATPase activity of ClpC or ClpE. At 30 min after addition of ATP, 10 μl of Laemmli buffer (5×) was added to each sample and heated for 5 min at 96°C. SDS-PAGE, electrotransfer onto nitrocellulose membranes, and Western blot analyses were performed as previously described (31). Whole protein content was analyzed by SDS-PAGE with Coomassie blue staining. σ^X-Strep and the negative control ComR-Strep were detected using monoclonal antibody against StrepTagII (StrepMAB-Classic) as the primary antibody and a horseradish peroxidase-conjugated rabbit anti-mouse polyclonal antibody as a secondary antibody according to the manufacturer's instructions (IBA). Quantifications were performed by densitometric analyses (Kodak 1DV.3.5.3 software) of the intensities of the different bands.

RESULTS

MecA interacts *in vitro* with both σ^X and ClpC. Previous *in vivo* bacterial two-hybrid (B2H) assays strongly suggested that MecA from *S. thermophilus* interacts with both ClpC and σ^X (31). To validate the MecA-σ^X and MecA-ClpC interactions in this species, real-time interactions were tested *in vitro* using surface plasmon resonance (SPR). For this purpose, His-tagged MecA, ClpC, and σ^X (N-terminal fusions) were purified to homogeneity. His-tagged ClpE, an alternative Clp ATPase subunit (47% of identity with ClpC), was also used as control in MecA interaction experiments since it has a molecular weight similar to that of ClpC. The SPR sensor chip was functionalized with 6His-MecA.

In a first set of experiments, the specificity of the MecA-ClpC interaction was investigated by injecting (in duplicates) 0.1 μM pyruvate kinase (PK) (negative control), 6His-CplC, or 6His-ClpE on the sensor chip (Fig. 1A). As expected, no increase in the response was observed during PK injection on the surface (Fig. 1A). Regarding Clp ATPases, the response obtained at the end of the association phase for 6His-ClpC was ~4-fold higher than that for 6His-ClpE, indicating that ClpC interacts more strongly with MecA than ClpE.

In a second set of SPR experiments, the MecA-σ^X interaction was investigated by injecting 0.1 μM 6His-σ^X (in duplicates). The σ^X response obtained at the end of the association phase (27 RU) reflected a strong and direct interaction (Fig. 1A). Increasing concentrations of σ^X (from 0.003 to 1 μM) were next injected on the biochip, and an extrapolated dose-response curve was constructed to determine the affinity constants as described in Materials and Methods (38). As expected, the SPR signal was proportional to the 6His-σ^X concentration (from 0.003 to 1 μM) (Fig. 1B). The MecA-σ^X stoichiometry was set to 1:1 as proposed for the MecA-ComK complex (9). An equilibrium dissociation constant (K_D) equal to 0.87 ± 0.2 μM was calculated for the MecA-σ^X complex.

Altogether, these results validate the specificity of the interaction of MecA with both ClpC and σ^X, reinforcing the hypothesis that these three proteins from a ternary complex *in vivo*.

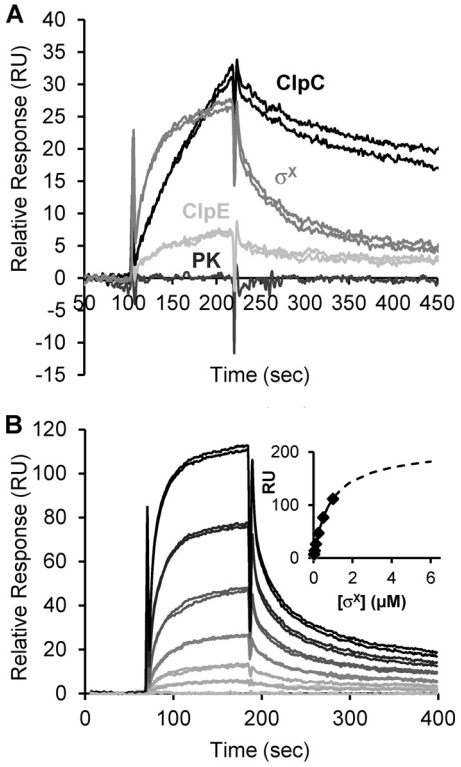

FIG 1 Interactions of MecA with σ^X, ClpC, or ClpE, evaluated by SPR. (A) Sensorgrams depicting the *in vitro* interactions of pyruvate kinase (PK) and 6His-ClpE, -ClpC, and -σ^X (in duplicates) with 6His-MecA immobilized on the sensor chip. (B) Sensorgrams showing the real-time interaction of 6His-σ^X with 6His-MecA. The response curve values, in resonance units (RU), were recorded for 6His-σ^X solutions injected in duplicates at the following concentrations: 0.03, 0.06, 0.12, 0.25, 0.5, and 1 μM. RU signals were then normalized as described in Materials and Methods. The inset shows the normalized RU response as a function of σ^X concentration (binding isotherm) as calculated at the end of the injection. Diamonds represent experimental points, and the dotted line represent the extrapolated curve.

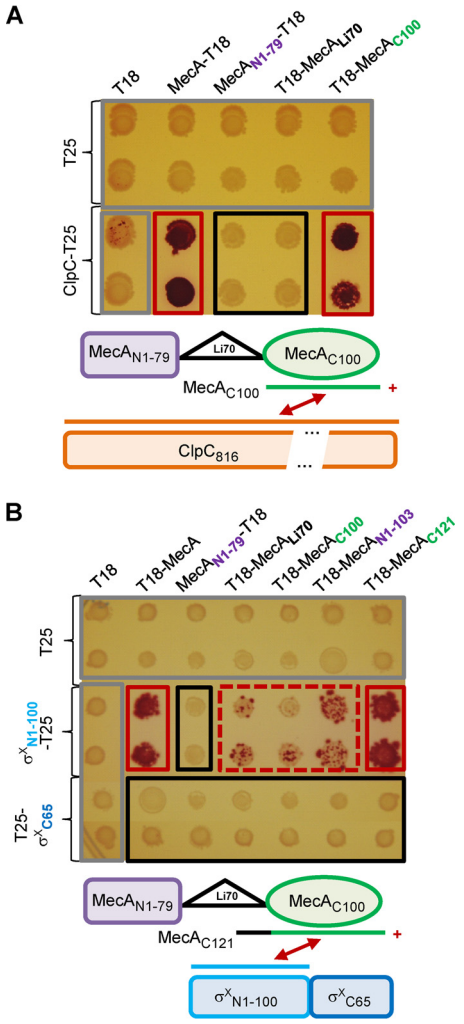

FIG 2 Subdomain interactions of MecA with σ^X or ClpC, evaluated by B2H assays. (A) Top, matrices of B2H interactions between MecA domains and full-length ClpC on MacConkey plates. Bottom, MecA domain organization and positive (+) $MecA_{C100}$-ClpC interaction (red arrow). (B) Top, matrices of B2H interactions between MecA domains and σ^X domains on MacConkey plates. Bottom, organization of MecA and σ^X domains and positive (+) $MecA_{C121}$-$\sigma^X{}_{N1-100}$ interaction (red arrow). In both panels, controls are surrounded by a gray rectangle, and T25 and T18 correspond to the empty vectors pKT25 and pUT18, respectively. Red, red dashed, and black rectangles highlight positive, weak, and negative interactions, respectively.

MecA interacts with a predicted surface-exposed region of the N-terminal domain of σ^X. The B2H assay was used to further map the different MecA subdomains interacting with ClpC and σ^X. In B2H, interacting T18 and T25 fusion proteins lead to functional α-complementation of the adenylate cyclase activity and thus enable maltose utilization (colonies display a red coloration on MacConkey agar-based medium supplemented with 1% maltose) (35). Although less quantitative, this method seems reliable to study MecA interactants. Indeed, interactions observed using SPR were in good agreement with those deduced from our previous B2H results (31). Regions of the *mecA* and *comX* or *clpC* ORFs were fused to the T25- and T18-encoding fragments of *cyaA*, respectively, and the resulting plasmids were cotransformed in the appropriate *E. coli* strain. In most cases, hybrid proteins were designed so that the native orientation of the protein domains was respected. The T18-X or T25-Y and X-T18 or Y-T25 nomenclatures indicate that protein X (or a truncated domain thereof) is fused to the C terminus and N terminus of T18 or T25, respectively.

MecA from *B. subtilis* is organized in three macrodomains (8). Multiple-sequence alignment with streptococcal orthologs (31) (see Fig. S1 in the supplemental material) was used to design three T18 fusion proteins for our B2H assays, i.e., $MecA_{N1-79}$-T18, T18-$MecA_{C100}$, and $MecA_{Li70}$-T18, which correspond to the first 79 amino acids (aa) (N), to the last 100 aa (C), and to a 70-aa linker region (Li) between domains N and C of MecA, respectively (Fig. 2A; see Fig. S1 in the supplemental material). It is noteworthy that the MecA Li region is much less conserved among orthologs than domains N and C (31) (see Fig. S1 in the supplemental material). First, we showed that the full-length ClpC interacts solely with the C domain of MecA (Fig. 2A). Then, we investigated whether specific domains of MecA and σ^X were interacting. Based on the three-dimensional (3D) structural prediction for σ^X (see Fig. S2A in the supplemental material), the sigma factor was divided into two macrodomains (see Fig. S2B in the supplemental material): the first 100 aa (N, $\sigma^X{}_{N1-100}$-T25), which corresponds to region 2

of σ^{70} (Pfam domain 04542), and the last 65 aa (C, T25-σ^X_{C65}), corresponding to a domain of unknown function (see Fig. S2A in the supplemental material). The B2H results presented in Fig. 2B suggest that the domain N of σ^X (first 100 aa) is able to interact with full-length MecA but that it is not able to react or reacts extremely weakly with either of its macrodomains. Two additional MecA-T18 derivative fusions, T18-MecA$_{N1-103}$ and T18-MecA$_{C121}$, were thus constructed to expand domains N and C, respectively, in order to include part of the linker domain (see Fig. S1 in the supplemental material). Interaction between domain N of σ^X and the extended domain C of MecA (T18-MecA$_{C121}$) was found to be similar to that between domain N of σ^X and full-length MecA (T18-MecA) (Fig. 2B). Based on all these B2H assays, we propose that both domains Li and C of MecA are important for the interaction with the domain N of σ^X.

To further delimit a region in domain N of σ^X necessary for MecA interaction, truncated derivatives of the σ^X_{N1-100}-T25 fusion, encompassing either the first 75 (σ^X_{N1-75}-T25) or 50 (σ^X_{N1-50}-T25) residues of σ^X, were tested. Only σ^X_{N1-75}-T25 still interacted with MecA (both T18-MecA and MecA-T18) (Fig. 3A), indicating that the 25-aa region between positions 50 and 75 of σ^X is involved in this interaction. Interestingly, fragment σ^X_{50-75} was sufficient to complement the adenylate cyclase activity (fusion σ^X_{N50-75}-T25 in Fig. 3A). This region is predicted to include a surface-exposed loop (F_{49} to K_{58}) which is located between α-helices H2 and H3 of the N domain (Fig. 3B; see Fig. S2A in the supplemental material) and could therefore constitute a preferential domain of interaction with MecA. However, B2H results indicate that residues located downstream of this predicted loop, i.e., downstream of residue 58, were also involved in σ^X-MecA interaction, since fusion σ^X_{N1-58}-T25 was affected in its ability to interact with both T18-MecA fusions, in contrast to σ^X_{N1-68}-T25, which behaved similarly to the σ^X_{N1-75}-T25 fusion (Fig. 3A). Altogether, these results suggest that a short region of the domain N of σ^X, located between residues 50 and 68 and encompassing a putative surface-exposed loop, has a major contribution in the formation of the σ^X-MecA complex (Fig. 3B).

σ^X is degraded *in vitro* by the MecA-ClpCP complex. Previous studies have shown that MecA of *B. subtilis* can either sequester a regulatory protein (i.e., Spo0A) (39) or address it to the ClpCP proteolytic machinery (i.e., ComK, ComS) (9, 10). From our previous *in vivo* results, we have proposed that MecA not only sequesters σ^X but is responsible for its degradation by ClpCP (31). To test this hypothesis, an *in vitro* degradation assay was set up.

Purified samples of σ^X fused to a C-terminal StrepII tag (σ^X-Strep) were incubated for 30 min at 37°C in absence or presence of equimolar preparations of purified 6His-MecA, -ClpC, and/or -ClpP. The assay was started by adding ATP and/or phosphoenolpyruvate (PEP)/PK in order to ensure energy regeneration in the reaction mixture (the activity of Clp proteases is ATP dependent) (Fig. 4A). At the end of the assay, Western blotting using a monoclonal anti-StrepTagII antibody was performed (Fig. 4B), and relative amounts of σ^X-Strep were determined by densitometry (using lane 1, i.e., σ^X alone in the reaction, as the 100% value) (Fig. 4D). The results indicate that σ^X-Strep degradation is strictly dependent on 6His-MecA, -ClpC, and -ClpP and ATP, with a higher degradation observed in the presence of the ATP regeneration system (Fig. 4, compare lanes 2 and 6). Importantly, Clp-dependent degradation is specific to σ^X-Strep, since ComR-Strep, which was used instead of σ^X-Strep in parallel reactions, was stable under

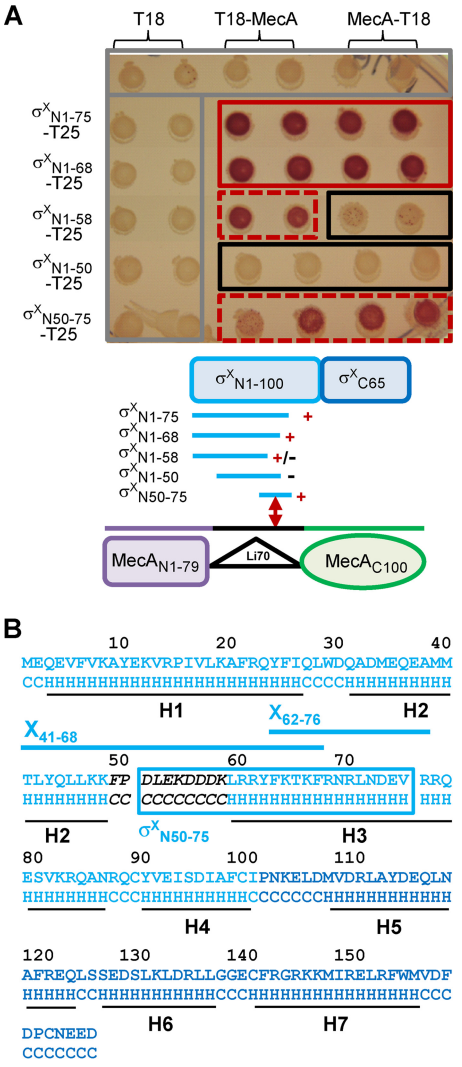

FIG 3 Interactions between MecA and truncated N-domain variants of σ^X, evaluated by B2H assays. (A) Top, matrices of B2H interactions between full-length MecA and truncated N-domain variants of σ^X on MacConkey plates. The color code for rectangles is the same as in Fig. 2. Bottom, organization of MecA and σ^X domains and summary of interactions. +, +/−, and − indicate positive, weak, and negative interactions, respectively. (B) σ^X sequence and predicted secondary structure (LOMETS server [http://zhanglab.ccmb.med.umich.edu/LOMETS/]) with α-helixes H1 to H7 (black lines). The predicted secondary structure is indicated below the protein sequence. H, α-helix; C, coil. Light and dark blue sequences correspond to the N and C domains of σ^X, respectively. X_{41-68} and X_{62-76} peptides and their position are mapped with blue lines. σ^X_{N50-75}, which interacts with MecA in B2H assays, is indicated by a blue box. The sequence of the predicted surface-exposed loop is in black italic.

all conditions tested (Fig. 4C). In addition, 6His-ClpE was found to be ~ 20-fold less efficient than 6His-ClpC in these *in vitro* assays (compare conditions 2 and 5), which corroborates the SPR results where weak MecA-ClpE interactions were detected (Fig. 1A). Interestingly, the abundance of 6His-MecA was also found to decline in the presence of ClpCP, although more slowly than that of σ^X, which may indicate a sequential degradation of σ^X and MecA by the ClpCP protease complex (Fig. 4A; see Fig. S3 in the supplemental material). These *in vitro* experiments strengthen a model where σ^X is degraded *in vivo* in a MecA- and ATP-dependent manner by the protease complex ClpCP.

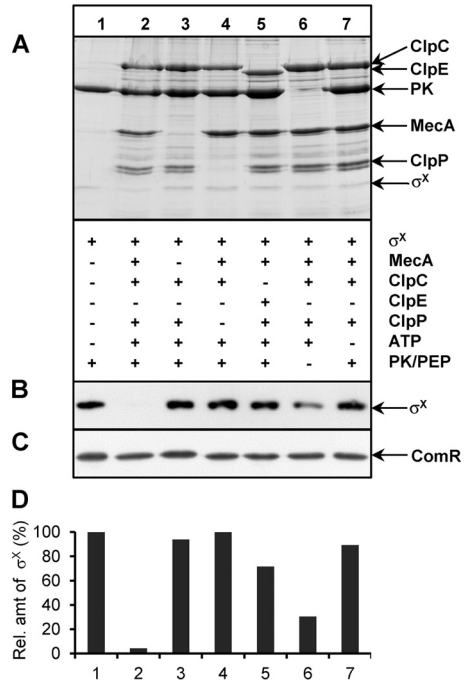

FIG 4 *In vitro* degradation of σ^X. (A) Top, SDS-PAGE with Coomassie blue staining of various combinations of σ^X-Strep, 6His-MecA, 6His-ClpC, 6His-ClpE, 6His-ClpP, ATP, and the pyruvate kinase/phosphoenolpyruvate (PK/PEP) ATP regeneration system. Bottom, presence (+) or absence (−) of a specific compound in the reaction mixture. (B) σ^X-Strep detection by Western blotting. (C) ComR-Strep detection by Western blotting. ComR-Strep was used as negative control and substitute for σ^X-Strep in a parallel experiment performed under the same conditions. (D) Quantifications by densitometry of the relative amount of σ^X-Strep detected by Western blotting, using control lane 1 as 100%. The specific *in vitro* degradation of σ^X in the presence of MecA-ClpCP and ATP has been reproduced with 4 independent batches of purified proteins. The presented experiment with all the controls on one gel was performed in duplicates, which showed similar results.

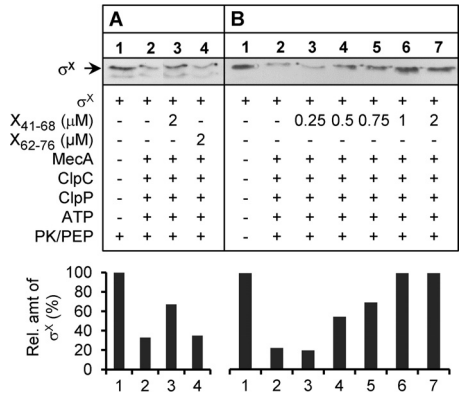

FIG 5 Competition of synthetic peptides for *in vitro* σ^X degradation. (A) Addition of synthetic peptides X_{41-68} and X_{62-76} (2 μM) to the degradation assay mixture. (B) Addition of X_{41-68} at increasing concentrations (0 to 2 μM) to the degradation assay mixture. In both experiments, σ^X-Strep (0.6 μM) was incubated with 6His-MecA, -ClpC, and -ClpP, ATP, and the pyruvate kinase/phosphoenolpyruvate (PK/PEP) ATP regeneration system. + or −, presence or absence of a specific compound in the reaction mixture, respectively. σ^X-Strep was detected by Western blotting. Quantifications by densitometry of the relative amount of σ^X-Strep using control lane 1 as 100% are shown under the respective Western blots.

σ^X degradation is inhibited by competition with a peptide encompassing the surface-exposed region of its N-terminal domain. An interesting result of the B2H assays reported above is the identification of a short region (18 aa) encompassing a putative surface-exposed loop in domain N of σ^X as required for the interaction with MecA (Fig. 3A and B). To evaluate the ability of this region to interfere *in vitro* with σ^X degradation, two artificial peptides, X_{41-68} and X_{62-76},were synthesized and tested in the *in vitro* degradation assay (Fig. 3B). Peptide X_{41-68} was designed such as to cover the surface-exposed loop with adjacent residues of α-helices H2 and H3. In contrast, peptide X_{62-76} excludes the predicted loop and corresponds to the N-terminal part of α-helix H3 (Fig. 3A). In a first set of experiments, an ~3-fold molar excess of X_{41-68} or X_{62-76} (2 μM) was mixed with equimolar concentrations of the other partners, σ^X-Strep, 6His-MecA, -ClpC, and -ClpP (0.6 μM), and incubated in the presence of ATP and phosphoenolpyruvate (PEP)/PK (30 min at 37°C). Interestingly, σ^X-Strep degradation, as deduced from its quantification after Western blotting, was ~3-fold less efficient in the presence than in the absence of X_{41-68} (Fig. 5A, compare lanes 2 and 3). In contrast, X_{62-76} had no inhibitory effect (Fig. 5A, compare lanes 2 and 4). In a second set of experiments, increasing concentrations of X_{41-68} (from 0 to 2 μM) were mixed with σ^X-Strep under the same incubation conditions (Fig. 5B). Notably, the amount of remaining σ^X-Strep was proportional to the concentration of X_{41-68} (Fig. 5B, compare lanes 2 to 6), with a complete relief of σ^X-Strep degradation observed at a 2:1 molar ratio of X_{41-68} and σ^X-Strep. These data strongly suggests that residues 41 to 68 of σ^X interact with an essential σ^X-binding region in MecA.

σ^X degradation is a locking mechanism that prevents natural transformation at low ComS concentrations. In *S. thermophilus* LMD-9, MecA and/or ClpC depletion activates the expression of late *com* genes under nonpermissive competence conditions (i.e., complex THBG medium) but not under permissive conditions (i.e., CDM medium) (31). However, we were not able to show any difference in transformation efficiency between MecA- or ClpC-depleted and WT strains (31). Since production of XIP is probably the limiting factor in THB, in contrast to CDM, we hypothesized that intermediate XIP concentrations could be required to observe differences in transformation efficiencies.

We thus compared the effects of low nonsaturating $ComS_{17-24}$ (synthetic XIP) concentrations (from 0 to 40 nM) on competence development of WT and MecA-depleted strains under THBG growth conditions. Both natural transformation frequencies and expression of the late *com* gene *comGA* (P_{comGA}::*luxAB* transcriptional fusion) were monitored. The results presented in Fig. 6 show that MecA depletion improved the transformation efficiency at all low $ComS_{17-24}$ concentrations tested (Fig. 6A) and increased P_{comGA} activity compared to that of the WT strain (Fig. 6B). However, differences were attenuated as the $ComS_{17-24}$ concentration increased. Indeed, the ratios of transformation frequencies between the strains were ~10 to 15-fold from 5 to 20 nM $ComS_{17-24}$ but were reduced to only ~1.5-fold at 40 nM (Fig. 6B). In accordance, the ratios between maximal luciferase activities driven by P_{comGA} also became smaller with increasing $ComS_{17-24}$ concentrations (from ~100 to ~5 between 0 and 40 nM ComS). At saturating $ComS_{17-24}$ concentrations (1 μM), both strains were similarly competent (data not shown). This strongly suggests that (i) MecA plays a negative-control role in natural transformation at

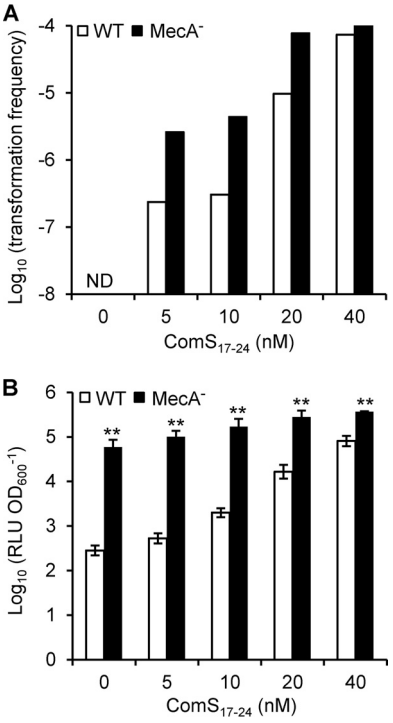

FIG 6 Effect of MecA depletion on DNA transformation frequencies and P_{comGA} activity. (A) Transformation frequencies (expressed in $\log_{10}$) of strains CB007 (WT, white bars) and CB0072 (MecA$^-$, black bars). Cultures in THBG medium (OD_{600} = 0.05) were supplemented with $ComS_{17-24}$ at concentrations ranging from 0 to 40 nM. ND, below the detection limit of 10^{-8}. (B) Maximum specific luciferase activities (RLU OD_{600}^{-1}) of P_{comGA}-*luxAB* fusions of strains CB007 (WT, white bars) and CB0072 (MecA$^-$, black bars) at each $ComS_{17-24}$ concentration reported in panel A. The presented results are from one representative experiment from two independent experiments showing similar results. Each experiment was performed with triplicate cultures of each strain. The transformation frequency was calculated from one randomly selected culture. Specific luciferase activities are expressed as geometric means ± standard deviations from triplicate cultures (expressed in $\log_{10}$). Significant difference between WT and MecA$^-$ is based on Student's *t* test performed on $\log_{10}$-transformed data; **, $P < 0.01$.

limiting concentrations of $ComS_{17-24}$ and (ii) above a certain threshold of $ComS_{17-24}$ concentration in the medium, activation of the ComRS system overcomes the locking mechanism mediated by MecA-ClpCP.

DISCUSSION

The importance of the MecA-ClpCP complex in the negative posttranslational control of the central competence regulator σ^X has recently been highlighted in two ComRS-containing streptococci, *S. thermophilus* (30, 31) and *S. mutans* (32). Based on genetic evidence and B2H assays, it was proposed that the adaptor protein MecA interacts with both σ^X and ClpC (31, 32). By analogy with the posttranslational control of ComK by the MecA-ClpCP complex in *B. subtilis*, it was hypothesized that MecA directly targets σ^X for degradation by the protease complex ClpCP (31, 32). In this work, we strengthened this model through complementary *in vitro* experiments. First, MecA-σ^X and MecA-ClpC interactions were confirmed by SPR experiments with purified proteins (Fig. 1). Interestingly, the K_D values for $MecA_{Sth}$-σ^X and $MecA_{Bsu}$-ComK complexes extrapolated from SPR experiments are in the same range (~1 μM), suggesting that the adaptor protein MecA interacts with similar strengths with both regulators. Second, we were able to show that the *in vitro* degradation of σ^X is fully dependent on the presence of MecA and requires ATP (Fig. 4). In addition, $MecA_{Sth}$ is also degraded by ClpCP, as reported for MecA from *B. subtilis* (see Fig. S3 in the supplemental material) (11, 40). Intriguingly, the $ClpE_{Sth}$ protein was able to partially substitute for $ClpC_{Sth}$ in the *in vitro* assays (Fig. 4), while we previously reported an absence of interaction between MecA and ClpE using B2H assays and no σ^X accumulation in a ClpE-deficient strain (31). Interestingly, independent results obtained with another *S. thermophilus* strain (LMG18311) grown under different conditions also suggested that ClpE could participate in the posttranslational control of σ^X, but with a lower contribution than ClpC (30). Thus, it remains unclear if ClpE could be a partner of MecA for σ^X degradation *in vivo*.

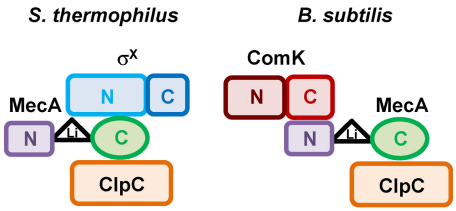

FIG 7 Model of subdomain interactions in the putative σ^X-MecA-ClpC ternary complex of *S. thermophilus* (left) compared to the *B. subtilis* ComK-MecA-ClpC complex (right). N, C, and Li correspond to domain N, domain C, and the linker region, respectively.

During this work, we also explored the MecA-σ^X and MecA-ClpC complexes in order to identify subdomain interactions. We found that domain C of $MecA_{Sth}$ was responsible for the MecA-ClpC interaction (Fig. 2A). This is fully consistent with data obtained for *B. subtilis*, where two regions of domain C of $MecA_{Bsu}$, around E_{184} and E_{198}, were shown to be important for interaction with $ClpC_{Bsu}$ (41, 42). Interestingly, these two glutamate residues are conserved in all MecA proteins from streptococci (see Fig. S1 in the supplemental material and data not shown). This suggests that evolution has maintained a similar MecA-ClpC interaction to selectively address specific target proteins to degradation by the serine protease ClpP. More interesting is the dissection of the MecA-σ^X interaction, which shows that the interacting domains are different from those between MecA and ComK (Fig. 7). Concerning $MecA_{Sth}$, it appears that domain N is not involved, while it is essential for the MecA-ComK interaction (8). Conversely, the C-terminal region (last 121 aa) encompassing domain C and part of the interdomain linker seems to interact with σ^X. In *B. subtilis*, MecA was shown to be flexible in terms of interactions with different partners. For instance, it could sequester the Spo0A regulator of *B. subtilis* based on interactions with both domains N and C while keeping its ability to bind ComK (39). Concerning σ^X, B2H and competitive degradation assays point toward a short region (18 aa) encompassing a predicted surface-exposed loop (F_{49} to K_{58}) of its domain N as playing a preponderant role in its interaction with MecA (Fig. 3 and 5). This contrasts with the major interacting region of ComK, which consists of a hexameric peptide localized close to the C terminus (9). The remarkable fact from all these results is that MecA has maintained an adaptor role between bacilli and ComRS-containing streptococci to address the central competence regulator to ClpCP degradation by interacting differently with proteins that do not display any primary sequence identity but perform similar functions.

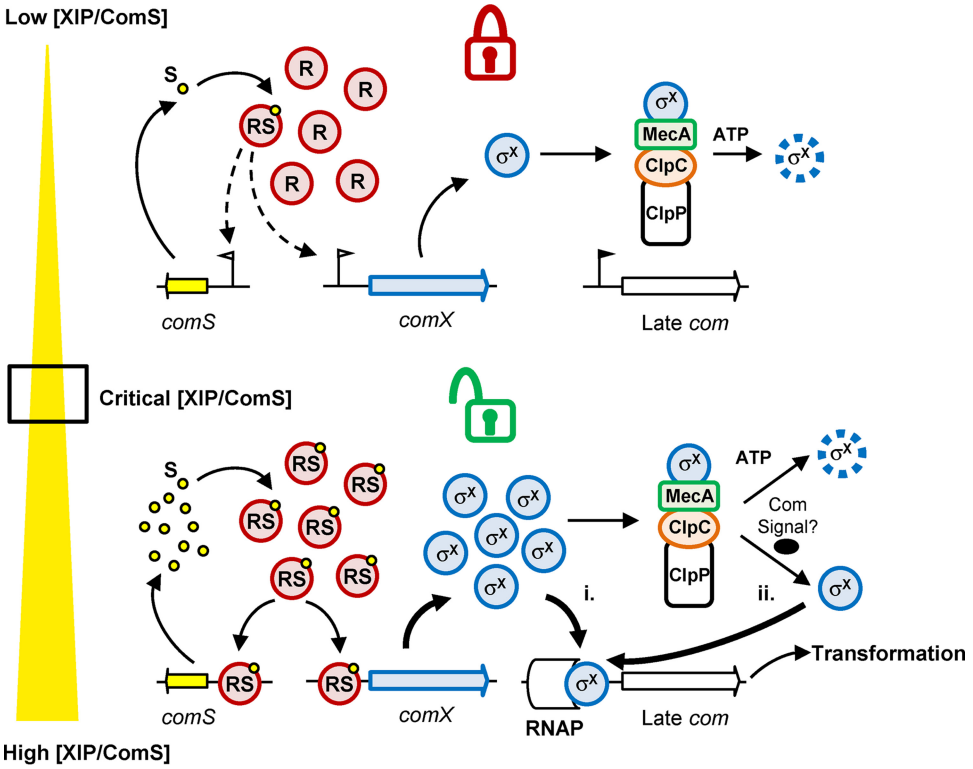

FIG 8 Model of the interplay between the ComRS and MecA-ClpCP systems for competence regulation in *S. thermophilus*. (Top) Locked competence. At very low XIP/ComS (S) concentrations, free ComR (R) or a small amount of ComRS (RS) complexes is present, which results in a low level of σ^X production. Under these conditions, adaptor protein MecA represses the onset of competence by selecting σ^X for degradation by the protease complex MecA-ClpCP. (Bottom) Unlocked competence. Above a critical XIP/ComS concentration, ComRS complexes are formed in large amounts, which results in a high production of σ^X. Either the high and abrupt σ^X production saturates the proteolytic machinery (i) or a so-far-unidentified competence signal (Com signal) releases σ^X from the MecA-ClpCP degradation complex (ii). In both scenarios, free σ^X molecules interact as an alternative sigma factor with the RNA polymerase (RNAP) and induce late *com* genes and DNA transformation. Degradation of σ^X is indicated by a dashed line.

An important question is the role of MecA in posttranslational control of competence development in other streptococci, containing either the ComRS or the ComCDE signaling system. Notably, the MecA protein is ubiquitous among streptococci and could be found in the complete genomes of all species sequenced so far (data not shown). Concerning ComRS-containing streptococci, the role of the MecA-ClpCP complex could easily be extrapolated to *S. mutans* based on previous results (32) and eventually to *S. pyogenes* based on the central role played by ClpP in σ^X degradation and the absence of a ComW protein (25, 29). Concerning ComCDE-containing streptococci, with *S. pneumoniae* as a model species, the situation is less clear, since σ^X is preferentially degraded by ClpEP and the ComW protein, which stabilized and activated σ^X, is degraded by ClpCP (17, 18). The possibility that MecA from *S. pneumoniae* could interact with its cognate σ^X protein was quickly evaluated by B2H assays. In addition, we also included cognate pairs of *S. thermophilus* and *S. mutans* to check cross-interactions between MecA and σ^X (see Fig. S4 in the supplemental material). From these assays, it appears that the three MecA proteins are flexible to recognize σ^X from *S. thermophilus* and *S. mutans* but do not or very weakly recognize σ^X from *S. pneumoniae* (see Fig. S4 in the supplemental material). This may indicate that σ^X from *S. pneumoniae* has diverged during evolution in such a way that it lacks recognition by MecA. Interestingly, the predicted loop proposed to be a key determinant in the MecA-σ^X interaction in *S. thermophilus* is poorly conserved in *S. pneumoniae*. While *S. thermophilus* and *S. mutants* have 7 out of 10 similar residues, *S. pneumoniae* shares only 3 similar residues with the loop of *S. thermophilus* (see Fig. S2B in the supplemental material). Corroborating these B2H assays, the inactivation of MecA in *S. pneumoniae* has no major impact on the *in vivo* degradation of σ^X but dramatically stabilizes ComW (43). This makes sense since ClpCP degrades ComW and ClpC has been considered until now a required partner of MecA (7, 42). Notably, this shows again a high flexibility of the adaptor protein MecA to potentially recognize an important player in competence activation that displays no sequence similarity with either σ^X or ComK. However, the role of this specific degradation of ComW by MecA-ClpCP in the posttranslational control of competence in *S. pneumoniae* remains to be investigated.

The next essential question is the importance of the posttranslational control of σ^X for competence development in ComRS-containing streptococci. In *B. subtilis*, the posttranslational control of ComK exerted by the MecA-ClpCP machinery is of key importance, since the release of a small amount of ComK dramatically affects its abundance by activating the autoamplification loop (5). The inactivation of MecA in this species resulted in DNA transformation which became independent of growth conditions (44). In ComRS-containing streptococci, previous results showed that the inactivation of one or more members of the MecA-ClpCP

complex has an impact on the activation of the late competence phase but not on DNA transformation efficiency (25, 29, 31, 32), except for the inactivation of ClpC in *S. thermophilus* LMG18311, where a moderate improvement of DNA transformation was observed in a very specific growth medium (30). In addition, the competence state in MecA- and ClpC-deficient strains of *S. mutans* was recently shown to be prolonged in rich medium supplemented with XIP, suggesting a role of the posttranslational control in competence shutoff (32). However, our investigations showed that the kinetics of the activation of late genes and DNA transformation in *S. thermophilus* is unaffected by MecA inactivation under similar growth conditions, indicating that this role could not be extended to *S. thermophilus* (reference 31 and data not shown). By using subinducing concentrations of XIP, we show here that the MecA-ClpCP degradation machinery not only restricts the transcriptional activation of the late phase but also abolishes DNA transformation under unfavorable conditions in *S. thermophilus* (Fig. 6). Conversely to the case for ComK of *B. subtilis*, σ^X production is not autoregulated in streptococci, and the key control checkpoint of competence development is the signaling system where the positive feedback loop takes place (1). Thus, the MecA-ClpCP degradation machinery acts downstream of ComRS regulation in *S. thermophilus* as a complementary locking device to tightly control competence activation when XIP availability is too low. Moreover, in the absence of the degradation machinery, activation of late genes seems much less responsive to increasing XIP concentrations than the classical nonlinear dose response observed in the wild type (Fig. 6B). This suggests that σ^X degradation by MecA-ClpCP participates in the dynamics of the response performed by the ComRS system on the activation of the late phase. A model of the interplay between ComRS and MecA-ClpCP to control competence in *S. thermophilus* is proposed in Fig. 8. Below a critical XIP concentration, σ^X that is present at a basal level would be degraded by MecA-ClpCP, and the cell remains in a noncompetent state. When a critical concentration of XIP is reached, the positive feedback loop is activated, resulting in XIP autoproduction, which in turn leads to an increase of σ^X and possibly a not-yet-identified interfering early gene product. Two nonexclusive scenarios then could be envisaged: (i) the increase of σ^X alone is sufficient to overcome the MecA-ClpCP degradation machinery, which would be the limiting factor, and free σ^X could then induce competence, and/or (ii) an interfering early gene product that would act as anti-anti-sigma factor might release σ^X from the complex, similarly to ComS of *B. subtilis*, which compete with ComK for MecA binding (9, 10). Future work will aim to investigate these two scenarios in order to unravel the unlocking mechanism.

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