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Effects of CwlM on autolysis and biofilm formation in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

Chao Wang[a,1], Qiaoli Zhang[a,1], Xudong Tang[b,1], Yanan An[a], Shulin Li[a], Hongyue Xu[a], Yan Li[a], Xuefei Wang[a], Wenjing Luan[a], Yang Wang[a], Mingyuan Liu[a,c], Lu Yu[a,*]

[a] Key Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, First Hospital of Jilin University, College of Veterinary Medicine, Jilin University, Changchun, 130062, China
[b] Key Lab for New Drug Research of TCM, Research Institute of Tsinghua University in Shenzhen, Shenzhen, 518057, China
[c] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009, China

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ABSTRACT

Tuberculosis is a highly infectious disease and of high incidence in low-income countries that is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). *M. tuberculosis* can form biofilms *in vitro* and *in vivo*, and the cells in the biofilm can survive at high concentrations of antibiotics. CwlM is a peptidoglycan hydrolase (amidase) and can hydrolyze bacterial cell walls, and the effects of CwlM on autolysis and biofilms is worthy of in-depth study. In this study, we successfully constructed an *in vitro* biofilm model of *M. tuberculosis* and *Mycobacterium smegmatis* (*M. smegmatis*). Reverse transcription followed by real-time quantitative PCR (qPCR) revealed that the expression of *cwlM* in *M. tuberculosis* and *M. smegmatis* was significantly up-regulated during the middle stage of biofilm formation. Treatment with recombinant CwlM enhanced the autolytic ability of *M. tuberculosis* and *M. smegmatis* and reduced the formation of their biofilms. As *M. smegmatis* is a model bacterium of *M. tuberculosis*, we built the *M. smegmatis cwlM*-deletion strain MSΔ6935, whose autolytic ability, biofilm production, and eDNA and eRNA content were determined to be lower than those of its parental strain. In conclusion, the *cwlM* gene plays a key regulatory role in biofilm formation in *M. tuberculosis* and *M. smegmatis*. This study provided a theoretical basis for using peptidoglycan hydrolase as a target for the inhibition of biofilms.

1. Introduction

WHO data show that latent tuberculosis infections accounted for 1/3 of the world's population and the prevalence of *Mycobacterium tuberculosis* (*M. tuberculosis*) drug-resistant strains gradually increased; these strains are a cause of high mortality in *M. tuberculosis* infections (Bogale et al., 2017). *M. tuberculosis* is hard to clear from the human body, and the resistant strains can reactivate *in vivo*, leading to the recurrence of tuberculosis; these recurrences are closely related to the special cell wall of *M. tuberculosis* (Ojha et al., 2008). The cell wall of *M. tuberculosis* is mainly composed of a specific lipid-peptidoglycan complex, and previous reports demonstrated that *M. tuberculosis* in biofilms is surrounded by the lipid extracellular matrix, which includes mycolic acid (involved in cell wall synthesis) (Ojha et al., 2005). *Mycobacterium smegmatis* (*M. smegmatis*) is a model bacterium of *M. tuberculosis* and has a cell wall structure that is similar to that of *M. tuberculosis* but differs in that the *M. smegmatis* grows rapidly and has no pathogenicity.

With a deep understanding of the pathogenesis of bacteria, it was found that bacterial biofilms were resistant to antibiotics and had the ability to enable immune escape, leading to serious clinical problems (Paredes et al., 2014). In 2008, Ojha et al. demonstrated that *M. tuberculosis* forms biofilms *in vitro* and that the cells in the biofilm are still viable in high concentrations of antibiotics (Ojha et al., 2008). Bacterial biofilms are composed of self-secreted extracellular polymeric substances (EPS) and bacterial cells. Moreover, EPS are mainly composed of polysaccharides, DNA, proteins and other macromolecular compounds that stabilize the structure of the biofilm (Gilan and Sivan, 2013). Previous reports showed that the thickness of biofilms is not uniform and biofilms are heterogeneous in time and space; their gene expression and physiological activity are also heterogeneous (Donlan and Costerton, 2002). Zhiqiang Qin et al. demonstrated that eDNA, part of the extracellular matrix, plays an important role in the early adhesion of biofilms (Qin et al., 2007). In addition, biofilms that contain extracellular RNA (eRNA) have been reported (Trivedi et al., 2016).

* Corresponding author.
E-mail address: yu_lu@jlu.edu.cn (L. Yu).
[1] These authors contributed equally to this work.

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Autolysis is a form of cellular self-destruction in which bacterial own hydrolase degrade their own cells. Under the conditions that are not suitable for growth and anabolism, bacteria will be autolyzed. Autolysis is not just an extension of bacterial growth and stability but is also associated with several biological functions of bacteria. In cases such as that of *M. tuberculosis*, autolysis may lead to protein release, and these proteins are important virulence factors such as isocitrate dehydrogenase (ICD) and superoxide dismutase (SOD) proteins for the host (Andersen et al., 1991). Our previous studies have shown that *Staphylococcus aureus* can form biofilms by autolysis and then release eDNA or extracellular matrix components (Liu et al., 2011; Wang et al., 2011). Previous reports confirmed that bacterial autolysin LytA can hydrolyze the nascent peptidoglycan of the cell wall and peptidoglycan hydrolase deficiency affects the bacterial adhesion of host cells (Li et al., 2015). Additionally, *Bacillus cereus* autolysin CwlB plays an important role in bacterial cell lysis; if the autolysin gene is deleted, bacterial fragmentation and the delayed release of virulence proteins occur (Yang et al., 2013). Previous studies showed that the CwlM protein is encoded by the *Rv3915* gene in *M. tuberculosis* and by the *MSMEG_6935* gene in *M. smegmatis* (Deng et al., 2005). However, the study of the *Rv3915* and *MSMEG_6935* genes has not been comprehensive and deep.

In this study, the expression of the *M. tuberculosis Rv3915* and *M. smegmatis MSMEG_6935* genes during different periods of biofilm formation was studied, and the relationship between the CwlM protein and the phenomena of autolysis and biofilm formation was discussed. These results provide a theoretical reference for the targeting of peptidoglycan hydrolase as a biofilm inhibitor.

2. Materials and methods

2.1. Bacterial strains and culture conditions

M. tuberculosis H37Rv (ATCC 27294) and *M. smegmatis* Mc^2155 were purchased from the China Pharmaceutical Biology Products Identification Institute. *E. coli* strains DH5a and BL21 (DE3) were purchased from Beijing Tiangen. *M. tuberculosis, M. smegmatis* and MSΔ6935 strains were grown in 7H9B media that contained OADC and 0.05% Tween 80. *E. coli* strains DH5a and BL21 (DE3) were grown in LB broth or on LB agar plates. All of the experiments that were associated with H37Rv in this study were performed in a P3 Biosafety Laboratory.

2.2. Biofilm culture conditions

Firstly, *M. tuberculosis* H37Rv and *M. smegmatis* Mc^2155 were grown in a 7H9B media that contained OADC and 0.05% Tween 80 liquid medium at 37 °C in an Erlenmeyer flask to an optical density at 600 nm (OD_{600}) of 0.7–1.0. The culture was used as an inoculum at a 1:100 dilution. In total, 15 ml of a modified Sauton medium (0.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 4 g L-aspartic acid, 2 g Citric acid, 0.05 g Ammonium ferric citrate dissolved in 900 ml of deionized water, add 60 ml of glycerin, adjust the pH to 6.8, and dilute to 1000 ml.) was dispensed into a 50 ml serum bottle. Next, 500 µl of the inoculum was added to the medium and the bottle was capped very tightly and placed undisturbed in a humidified 37 °C incubator for 3 or 28 days.

2.3. Plasmids and DNA manipulation

Restriction enzymes, T4 DNA ligase and Phusion DNA polymerase were purchased from New England Biolabs and Takara. All vectors, along with their antibiotic resistance markers, are listed in Table S1. The expression vectors pCold I (Hayashi and Kojima, 2008) and pPR27 were provided by our laboratory. The vectors pcold-6935-plys and pcold-3915-plys, which contain the *MSMEG_6935* and *Rv3915* genes, respectively, were created by amplifying the genes using the primers that are listed in Table S2 and then ligating the amplified genes into vector pCold I that had been restricted with HindIII and NdeI. Construction of pPR27_6935 and pVV16_6935 (Dhouib et al., 2010) were done as follows: pPR27_6935 and pVV16_6935 were amplified using primers that are listed in Table S2, and the amplified products were then ligated into pPR27 and pVV16 that had been restricted with XbaI. All constructs were verified by DNA sequencing.

2.4. Expression and purification of CwlM

For expression in *E. coli*, the *Rv3915* and *MSMEG_6935* genes were amplified with two primers (Table S2) using H37Rv and Mc^2155 chromosomal DNA and then cloned into pCold I in-frame with His-tags using a ligation-independent cloning strategy. The protein was expressed in the BL21 (DE3) strain as follows. An overnight preculture that was grown at 37 °C was used to inoculate 2 l of LB broth. The cells were grown at 37 °C until the OD_{600nm} reached 0.6, at which point expression was induced by the addition of 0.1 mM IPTG and the cells were incubated overnight at 16 °C. The cells were spun down and resuspended in 20 ml of lysis buffer that contained 50 mM Tris, pH 8, 0.4 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol and 1 mM benzamidine. After sonication, cell debris was removed by centrifugation. The supernatant was incubated with binding buffer. The protein was purified using a His-Trap HP column (affinity chromatography), an anion exchange column (ion exchange chromatography) and a gel filtration column (Superdex-200) that was connected with an AKTA Purifier 100 (GE) system.

2.5. Western blot analysis

The total protein was collected by centrifugation and was quantified using the BCA reagent (Beyotim, P0012). The images were obtained by a CanoScan LiDE 100 scanner (Canon). Protein blots were measured using Image-J software.

2.6. qPCR analysis

Total mRNA was extracted following instructions from Takara RNAiso Plus (Code No.: 9108). *M. smegmatis* biofilms were sampled at 0, 3, 6, 9, 24 and 96 h. *M. tuberculosis* biofilms were sampled at 0, 3, 6, 9, 12, 14, 20 and 28 d. The total RNA was reverse transcribed into cDNA at different time points according to the Tiangen reverse transcription kit instructions. *M. tuberculosis* H37Rv cDNA was used as the template, the *Rv3915* gene was the target gene, and the *M. tuberculosis sigA* gene served as the internal reference. For *M. smegmatis*, Mc^2155 cDNA was used as the template, the *MSMEG_6935* gene was the target gene, and the *M. smegmatis sigA* served as the internal reference (Table S2). Fluorescent quantitative PCR buffers that were prepared using FastStart Universal SYBR Green Master (ROX) (Roche) were mixed with the template and primers and tested on an ABI Step One Plus Fluorescence Quantitative PCR system. The 25 µl reaction was repeated 3 times; the *M. smegmatis* PCR amplification reaction program was performed as follows: predenaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 30 s, 60.7 °C for 30 s and 72 °C for 60 s. For *M. tuberculosis* samples, the PCR amplification reaction program used the following parameters: predenaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s. The gene expression levels at different time points were calculated by the $2^{-\Delta\Delta CT}$ method. Data were analyzed by using GraphPad Prism 5 software for two-way ANOVA analysis, and the difference was determined to be statistically significant based on the *t*-test Compared with the control group, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.7. Construction of M. smegmatis deletion mutants and recovery strains

Plasmid pPR27_6935 was transformed into prepared Mc^2155 competent cells by electroporation, and the cells were cultured at 32 °C overnight in a medium (7H9B-glycerol) that contained gentamicin

(100 μg/ml) and kanamycin (50 μg/ml). The bacterial strains that were grown in 7H9B-glycerol medium were coated on a plate that contained carnosine and 5% sucrose 7H11 solid medium, which was then cultured at 42 °C. The colonies that grew on the 7H11 plates were cultured in a 7H9B-glycerol medium at 42 °C, and their identities were verified by PCR or Xba I endonuclease digestion. Plasmid pVV16_6935 was transformed into prepared Mc^2155 competent cells by electroporation, and the cells were cultured at 37 °C on a medium (LB agar plates) that contained hygromycin (250 μg/ml). The colonies were grown in an LBT medium, and their identities were verified by PCR or Xba I endonuclease digestion.

2.8. Determination of autolysis of M. smegmatis that was induced by Triton X-100

M. smegmatis was cultured to OD_{600nm} = 0.3, and the culture was divided into two groups (10 ml each). The two groups were centrifuged at 4500 rpm for 10 min, and the precipitated bacteria were collected. The experimental group was supplemented with medium that contained CwlM protein (200 μg/ml) and was cultured at 37 °C until the late stage of logarithmic growth. The cells were collected and washed three times with cold Tris−HCl buffer (0.05 M Tris, pH 7.2) that contained 0.1% (v/v) Triton X-100 and finally suspended in 200 μg/ml CwlM and 0.1% Triton X-100 in Tris−HCl buffer to reach OD_{580nm} = 1.0. This cell suspension was incubated at 30 °C, and the value of OD_{580nm} in the Mc^2155 and Mc^2155 + CwlM groups was determined every 30 min (The maximum time is 6.5 h.).

2.9. Determination of M. tuberculosis autolysis ability

M. tuberculosis that grew to the logarithmic growth phase were collected and washed three times with modified Sauton medium. The control group and the experimental group were treated with Sauton medium and Sauton medium that contained 200 μg/ml $CwlM_{TB}$, respectively. The concentration of the solution was OD_{600nm} = 0.2, and the shaking culture was carried out at 37 °C. The activity of isocitrate dehydrogenase (ICD) was determined every 3 days. The assay samples were prepared according to the bacterial ICD activity colorimetric assay kit instructions.

2.10. Crystalline violet quantitation of biofilms

The biofilm medium was gently removed with a 1 ml syringe, the planktonic bacteria were removed three times with PBS, and the biofilm was suspended with 1 ml of PBS and shredded into 1.5 ml tubes, which were then centrifuged at 12,000*g* for 5 min. In total, 500 μl 1% crystal violet solution was for 5 min, and the sample was centrifuged at 12,000*g* for 2 min. The stained biofilm was washed with PBS, and the biofilm was dried at room temperature. Next, the sample was incubated with 500 μl of 80% ethanol solution for 5 min, and 200 μl of each sample was placed in a 96-well plate. A microplate reader measured the OD_{550nm} of each sample. Two-way ANOVA data were analyzed by using GraphPad prism analysis software; compared with the control group, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.11. Correlation analysis between autolysis levels and biofilm products

The autolysis levels and biofilm products of *M. tuberculosis*, *M. smegmatis*, deletion strain MSΔ6935 and recovery strain MSΔ6935-R were analyzed by using GraphPad prism analysis software, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.12. Extracellular matrix observation by scanning electron microscopy

Sterile coverslips that were treated with paraformaldehyde were placed on the bottom of a 24-well plate, and bacterial solution that was diluted with 2 ml of Sauton medium (OD_{600nm} = 0.2) was inoculated into the 24-well plates and incubated overnight at 37 °C. Images of these samples were observed and collected using a scanning electron microscope.

2.13. The detection of eDNA and eRNA

The H37Rv was cultured in accordance with the above methods. The experimental group was added with the final concentrations of DNase and RnaseA were 200 ug/ml, and the control group was added with inactivated enzyme respectively. The suspended solids in the medium in 0, 3, 6, 9, 12 15, 18, 21, 24, and 28 day were stained with SYTOX orange stain and RNA select respectively and washed with PBS for three times. The fluorescence value of eDNA and eRNA results were obtained with a spectrofluorophotometer (Ravaioli et al., 2011).

The Mc^2155 was centrifuged at 4500*g* for 5 min at logarithmic growth stage and the sediment was washed with Sauton medium three times. Sauton medium was used to suspend the bacterial liquid to OD_{600nm} = 0.2, and 2 ml of suspended bacterial liquid was placed in 24-well cell culture plate. The final concentrations of DNase and RnaseA were 200 ug/ml in each hole of the experimental group and 200 ug/ml inactivating enzymes in the control group respectively. The biofilm was incubated at 37℃ to the desired time to record the growth of biofilm. The biofilms that were cultured in 24-well cell culture plate were placed on sterile glass slides and cultured for 12 h. The medium was removed, and the biofilms were washed three times with PBS. The biofilms were stained with SYTOX orange stain and RNA select. The results were examined using a fluorescence microscope (Olympus BX53). Image analyses and export were performed using a Fluoview ver. 1.7.3.0 (Olympus, Japan).

2.14. Statistical analysis

The group means were compared using one-way ANOVA, two-way ANOVA, and Student's *t*-test was used to determine the significance of differences. For *p* values, * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ compared with the control were considered statistically significant. The data are representative of triplicate experiments and presented as the mean value ± the SD.

3. Results

3.1. The establishment of biofilm models of M. tuberculosis and M. smegmatis in vitro

To explore the mechanism of the formation of *M. tuberculosis* and *M. smegmatis* biofilms, we established *in vitro* models of *M. tuberculosis* and *M. smegmatis* biofilms. Moreover, the morphology of the biofilm at different time points during the biofilm formation process was observed and recorded. The results of *M. tuberculosis* biofilm formation showed that no formation was obvious in the first 12 days; the biofilm appeared at the bottle wall at 14 days, formed at the gas-liquid interface at 20 days and continued to grow on the bottle wall. The biofilm rapidly aggregated and grew at the gas-liquid interface at 28 days (Fig. 1A). Compared with *M. tuberculosis* biofilms, the time taken for the formation of *M. smegmatis* biofilms is shorter. The results showed that *M. smegmatis* biofilms did not appear during the period from 0 h to 6 h; the bacterial fragments gathered at the bottle wall at 9 h; the milky *M. smegmatis* biofilm formed clearly on the bottle wall and gas-liquid interface at 24 h, which is the formation stage of the biofilm. In addition, the biofilm formed clear yellow folds at 72 h, which is the mature period of *M. smegmatis* biofilms (Fig. 1B). Correlations between the autolysis levels and the ability of biofilm forming of *M. tuberculosis*, *M. smegmatis*, deletion strain MSΔ6935 and recovery strain MSΔ6935-R were examined, and the results showed that the ability of biofilm forming was positively correlated with the autolysis levels (Fig. 1C).

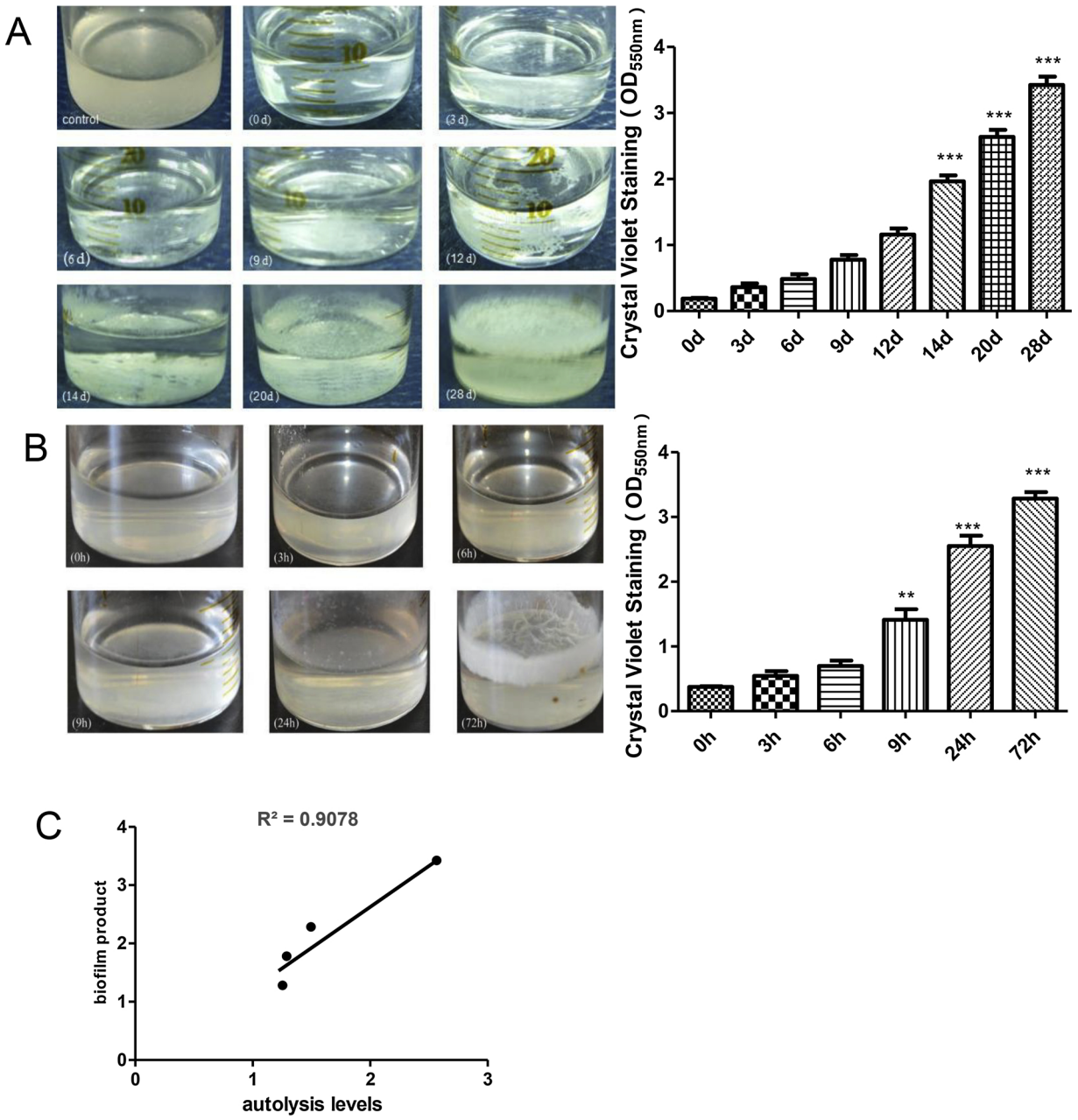

Fig. 1. The establishment of biofilm models of *M. tuberculosis and M. smegmatis in vitro*. *In vitro* models of *M. tuberculosis and M. smegmatis* biofilm were established by *M. tuberculosis* H37Rv and *M. smegmatis* Mc^2155 in 28 and 3 days, respectively. *M. tuberculosis* biofilms were observed at 0, 3, 6, 9, 12, 14, 20, 28 days, and their OD_{550nm} were analyzed (A) by Crystal Violet Staining. *M. smegmatis* biofilms were observed at 0, 3, 6, 9, 24, 72 h, and their OD_{550nm} were analyzed (B) by Crystal Violet Staining. The "biofilm product" was detected by Crystal Violet Staining and the "autolysis level" was detected by the absorbance at 580 nm. The analysis of the correlations between autolysis levels and biofilm products of *M. tuberculosis*, *M. smegmatis*, deletion strain MSΔ6935 and recovery strain MSΔ6935-R was performed using GraphPad Prism (C), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

3.2. eDNA and eRNA were the main constituents of the M. tuberculosis and M. smegmatis biofilms

While examining the major components of the mycobacterial biofilms, the existence of eDNA and eRNA in *M. tuberculosis* and *M. smegmatis* biofilm was detected. DNA inhibitor DNase I and RNA inhibitor RNaseA were added to the culture medium at different times during the formation of the *M. tuberculosis* and *M. smegmatis* biofilms. In *M. tuberculosis* biofilms, the results showed that no biofilms were present in the groups to which DNase I was added for 15–28 days, but a small amount of biofilm was formed in the group that received DNase I during days 3–9 (Fig. 2A). In addition, the RNaseA experiment showed that no biofilms were formed in the RNaseA-added groups (Fig. 2B). The *M. tuberculosis* biofilm was stained and then examined using the fluorescence microplate reader; the results showed that the fluorescence value of eDNA in the DNase I-added 15–28 day group was significantly lower than that of the control (Fig. 2C). Moreover, the RNaseA-added results showed that the fluorescence value of eRNA in the 9–28 days group was also significantly lower than that of the control (Fig. 2D). In *M. smegmatis* biofilms, the results showed that there were no biofilms in the groups in which DNase I was added for 2 and 3days, but a small amount of biofilm was formed in the group that received DNase I for only one day (Fig. 2E). In addition, the RNaseA results showed that no biofilms were formed in the RNaseA-added groups (Fig. 2F). These results proved that eDNA and eRNA were the main constituents of the *M. smegmatis* biofilm. To further verify this

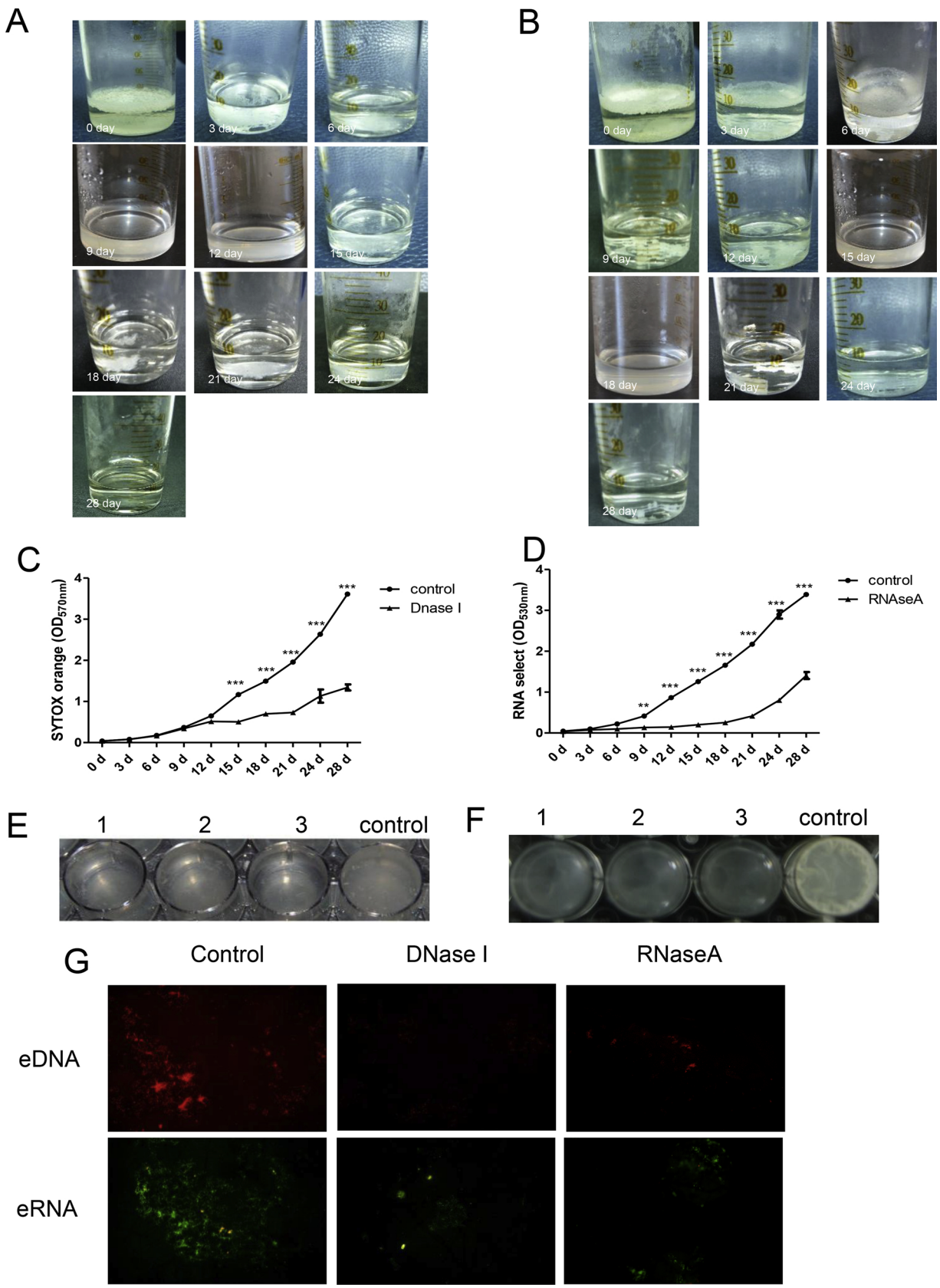

Fig. 2. eDNA and eRNA were the main constituents of *M. tuberculosis and M. smegmatis* biofilms. The biofilms of *M. tuberculosis* were treated with DNase I (A) or RNaseA (B) every 3 days and observed at days 0, 3, 6, 9, 12 15, 18, 21, 24, and 28. The *M. tuberculosis* biofilms that were treated with DNase I (C) or RNaseA (D) were stained by SYTOX orange (OD570 nm) stain or RNA select (OD530 nm), respectively, and observed using a fluorescence microplate reader. The biofilms of *M. smegmatis* were treated with DNase I (E) or RNaseA (F) and observed at days 1, 2, and 3. The *M. smegmatis* biofilms that were treated with DNase I or RNaseA were stained by SYTOX orange stain or RNA select, respectively, and the images were collected by fluorescence microscope (G).

conclusion, the mature *M. smegmatis* biofilm was stained with SYTOX orange acid stain and RNA select. The results showed that there were some red and green filaments in the control. Moreover, there were no red and green filaments in the DNase I- or RNaseA-added groups, but some red and green spots appeared (Fig. 2G). Together, the results confirmed that eDNA and eRNA were the main constituents of the *M. tuberculosis* and *M. smegmatis* biofilms.

3.3. The expression of the cwlM gene in M. tuberculosis and M. smegmatis during biofilm formation

To explore the expression of the *cwlM* gene in *M. tuberculosis* and *M. smegmatis* during biofilm formation, RNA was extracted at different time points during *M. tuberculosis* and *M. smegmatis* biofilm formation. The expression levels of *Rv3915* (*M. tuberculosis cwlM* gene) and *MSMEG_6935* (*M. smegmatis cwlM* gene) were determined at different time points during biofilm formation using qPCR. The results showed that compared to that of the biofilm at day 0 (control), expression of *Rv3915* did not change significantly ($p > 0.05$) during the early stage (0–12 d) and late stage of *M. tuberculosis* biofilm formation and that *Rv3915* was significantly up-regulated ($p < 0.001$) during the middle and late stages (14–20 d) of *M. tuberculosis* biofilm formation (Fig. 3A). In addition, in *M. smegmatis*, compared to that of the biofilm at hour 0 (control), the expression of *MSMEG_6935* in the early stage (0–6 h) of biofilm formation was not significant ($p > 0.05$), the expression of *MSMEG_6935* was significantly up-regulated ($p < 0.001$) in the middle stage of *M. smegmatis* biofilm formation (9–24 h), and the expression of *MSMEG_6935* was significantly down-regulated ($p < 0.01$) in the late stage of *M. smegmatis* biofilm formation (Fig. 3B).

3.4. Preparation and validation of M. tuberculosis and M. smegmatis CwlM recombinant proteins

To study the role of CwlM in mycobacterial autolysis and biofilm formation, the recombinant expression of *M. tuberculosis* and *M. smegmatis* CwlMs was carried out in *E. coli* (DE3). The recombinant protein expression plasmids pcold-6935-plys and pcold-3915-plys were established and verified by PCR, and the results showed that the *M. tuberculosis* and *M. smegmatis* PCR amplification products were 1221 bp and 1191 bp, respectively, which were consistent with the expected sizes of the target fragments (Fig. 4A). Moreover, the recombinant CwlMs were isolated and purified at room temperature from *E. coli* BL21 (DE3) cell extract by use of an AKTA Purifier 100 (GE) system. The SDS-PAGE results showed that a single protein band appeared between 40 and 50 kD (Fig. 4B) in both *M. tuberculosis* and *M. smegmatis*. In addition, the western blot results showed that the recombinant CwlMs was included in the obtained proteins from *M. tuberculosis* and *M. smegmatis* (Fig. 4C). These results proved that the recombinant *M. tuberculosis* and *M. smegmatis* CwlM proteins were successfully manufactured in *E. coli*.

3.5. The effects of CwlM recombinant proteins on autolysis and biofilm formation in M. tuberculosis and M. smegmatis

To explore the role of CwlM proteins in autolysis and biofilm formation in *M. tuberculosis* and *M. smegmatis*, as mentioned above, the CwlM proteins were expressed in prokaryotes, and their identities were verified by Western blot. The *M. smegmatis* was treated with different concentrations of CwlM protein to determine the best action concentration, and the results showed that the effect of CwlM began to increase significantly from 200 μg/ml (Fig. 5A). *M. tuberculosis* and *M. smegmatis* were each treated with their own CwlM protein to study its effects on autolysis and biofilm formation. Next, the autolysis capacity of *M. tuberculosis* was determined by measuring the activity of isocitrate dehydrogenase (ICD); the results showed that the autolysis capacity of the CwlM-treated group was higher than that of the control group firstly then the CwlM-treated group was lower than that of the control group (Fig. 5B). The results was basically consistent with the previous qPCR results. Moreover, 0.1% Triton X-100 was used to induce *M. smegmatis* and measure its autolytic ability; the results showed that when *M. smegmatis* had been treated with 0.1% Triton X-100, after 2.5 h, the addition of CwlM recombinant protein began to significantly reduce the OD at 580 nm; this reduction is significantly different from the behavior of the control group (Fig. 5C). In addition, the production of *M. tuberculosis* and *M. smegmatis* biofilms was quantified by crystal violet staining. The results showed that *M. tuberculosis* biofilm production in the CwlM-treated group was lower than that of the control group from 9 days onward ($p < 0.05$) (Fig. 5D) and *M. smegmatis* biofilm production in the CwlM-treated group was significantly lower than that of the control group from 48 h onward ($p < 0.001$) (Fig. 5E). Taken together, these results proved that CwlM could enhance the autolytic capacity of *M. tuberculosis* and *M. smegmatis* and reduce their biofilm product.

3.6. Construction and verification of cwlM gene (MSMEG_6935)-deletion strain MSΔ6935 and recovery strain MSΔ6935-R

To further validate the effect of the CwlM protein on mycobacterial autolysis and biofilm formation, we constructed the *cwlM* (*MSMEG_6935*)-deletion strain MSΔ6935 and the *cwlM* recovery strain MSΔ6935-R in *M. smegmatis* Mc^2155, a model bacterium of *M. tuberculosis*. The homologous arms and the kana gene were ligated by fusion PCR to obtain a three fragment-gene that was recombined with the cleavage site (Fig. 6A). Then, the vector pPR27_6935, which included the three fragment-gene, was constructed, and its identity was verified by Xba I endonuclease digestion. The results showed that the digestion products were 2023 bp in size, which was consistent with the expected size of the target fragment (Fig. 6B). The transformed *M. smegmatis* was first screened on a gentamicin resistant plate at 30 °C; second, the bacterium was cultured on a kanamycin and 5% sucrose plate at 42 °C to obtain the *cwlM* (*MSMEG_6935*)-deletion strain

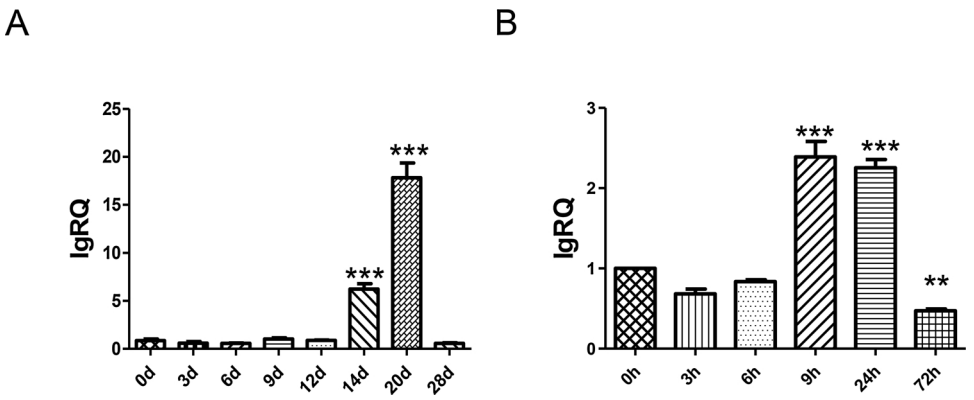

Fig. 3. The expression of *cwlM* in *M. tuberculosis and M. smegmatis* during biofilm formation. The expression levels of *Rv3915* (*M. tuberculosis cwlM* gene) during biofilm formation at 0, 3, 6, 9, 12, 14, 20, and 28 days were determined by qPCR (A). The expression levels of *MSMEG_6935* (*M. smegmatis cwlM* gene) during biofilm formation at 0, 3, 6, 9, 24, and 72 h were determined using qPCR (B), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

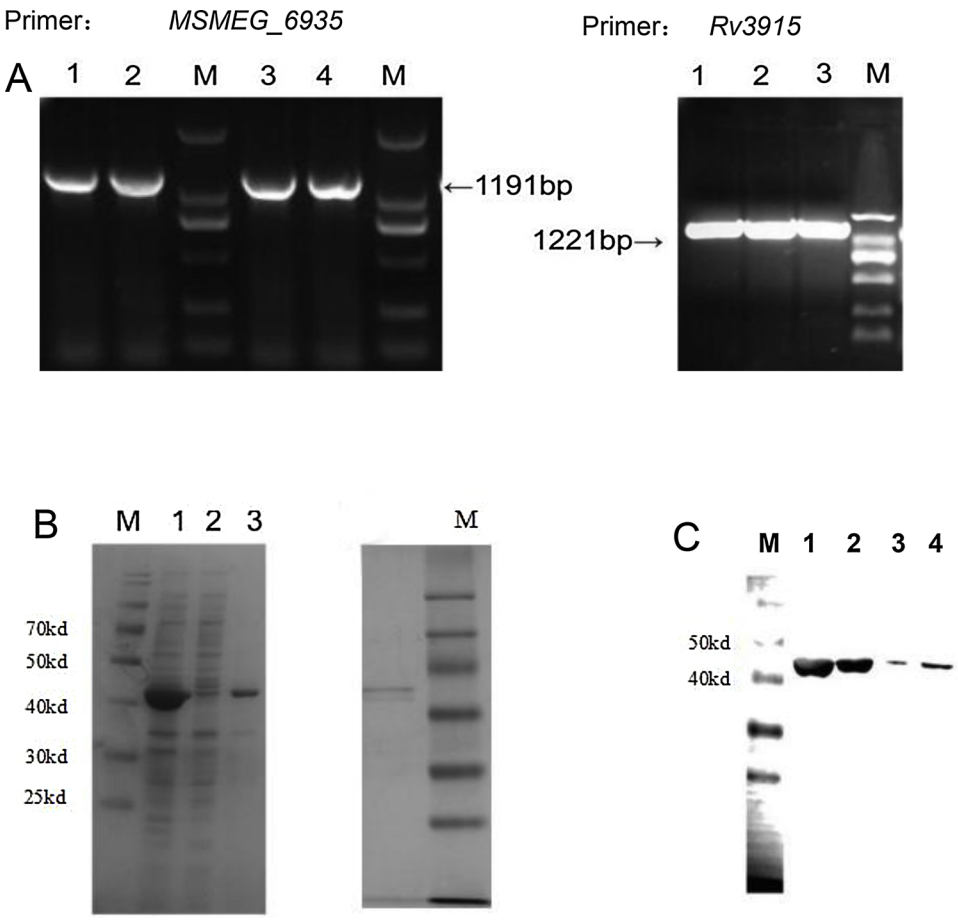

Fig. 4. Preparation and validation of *M. tuberculosis* and *M. smegmatis* CwlM recombinant proteins. The recombinant protein expression plasmids pcold-3915-plys and pcold-6935-plys were verified by PCR, and the PCR amplification products of *M. smegmatis* (left) and *M. tuberculosis* (right) were 1191 bp and 1221 bp (A). M: 2000 bp DNA marker, the other different lanes were repeated samples. The recombinant CwlM proteins were isolated and purified, followed by their verification by SDS-PAGE (B) and Western blot (C). B: left: M: protein marker; 1: inclusion body $CwlM_{MS}$; 2: supernatant $CwlM_{MS}$; 3: purified $CwlM_{MS}$ after purification; right: M: protein marker, and purified $CwlM_{TB}$ after purification. C: M: protein marker; 1: supernatant $CwlM_{MS}$; 2: supernatant $CwlM_{TB}$ 3: purified $CwlM_{MS}$ after purification; 4: purified $CwlM_{TB}$ after purification.

MSΔ6935. In addition, the absence of the *cwlM* gene in the selected *M. smegmatis* colonies was verified by PCR, and the results showed that the wild-strain PCR amplification products were 1191 bp long; in contrast, the PCR amplification products of the MSΔ6935 strain did not have a 1191 bp fragment (Fig. 6C). In addition, the pVV16_6935 vector, which included the *MSMEG_6935* gene, was constructed, and its identity was verified by Xba I endonuclease digestion. The results showed that the digestion products were 1329 bp long, which was consistent with the expected size of the target fragment (Fig. 6D). Then, the transformed *M. smegmatis* was grown on a hygromycin plate at 37 °C, and the presence of *MSMEG_6935* in the selected *M. smegmatis* colonies was verified by PCR. The results showed that the MSΔ6935-R PCR amplification products were 1329 bp in length (Fig. 6E). Together, the results confirmed that the *cwlM* (*MSMEG_6935*)-deletion strain MSΔ6935 and the recovery strain MSΔ6935-R were successfully obtained.

3.7. The effects of *M. smegmatis cwlM* gene deletion on autolysis and biofilm formation

To further verify the role of CwlM proteins in mycobacterial biofilm formation, we established the *M. smegmatis cwlM* (*MSMEG_6935*)-deletion strain MSΔ6935 and the recovery strain MSΔ6935-R. Biofilms of the deletion (MSΔ6935) and recovery strains (MSΔ6935-R) were cultured as described above, and the results showed that no biofilm formed at the gas-liquid interface at 24 and 48 h. A large number of bacteria were found in the bottom, but no biofilm formed at 72 h in the deletion strain. In the recovery strain, biofilm formed at the gas-liquid interface at 48 h and matured at 72 h (Fig. 7A). A 0.1% Triton X-100 solution was used to induce wild, MSΔ6935 and MSΔ6935-R *M. smegmatis* strains, and the results showed that after the *M. smegmatis* wild strain had been treated with 0.1% Triton X-100 for 2.5 h, its OD_{580nm} value began to significantly decrease ($p = 0.0033$); this behavior was significantly different from that of the MSΔ6935 strain (Fig. 7B). In addition, the OD_{580nm} value of the MSΔ6935-R strain began to decrease at 2.5 h ($p = 0.0099$), and the rate of decline was between those of the wild and MSΔ6935 strains (Fig. 7B). SEM images of the biofilm showed that the *M. smegmatis* wild-strain cells were lytic and a layer of gauze-like material was seen around the cells (Fig. 7C). However, this phenomenon was not observed around the cells after the deletion strain (MSΔ6935) was autolyzed (Fig. 7D). Moreover, eDNA and eRNA in the *M. smegmatis* wild strain or MSΔ6935 strain biofilms were detected by immunofluorescence staining. The results showed no red or green filaments, but some red and green spots appeared in the MSΔ6935 strain group; in contrast, some red and green filament was observed in the *M. smegmatis* wild-strain group (Fig. 7E). Together, these results confirmed that the lack of the *cwlM* gene led to a decrease in autolytic capacity and biofilm formation in *M. smegmatis*.

4. Discussion

Tuberculosis is a chronic infectious disease that is caused by *M. tuberculosis* invading the human body and can occur in any part of the body. In addition, the formation of *M. tuberculosis* biofilms has a great impact on resistance, the spread of pathogens, and persistent infections (Trivedi et al., 2016; Zumla et al., 2015). *M. tuberculosis* and *M. smegmatis* have similar cell wall compositions and biofilm formation processes, but *M. smegmatis* is not infectious and pathogenic (Bohsali et al., 2010; Riendeau and Kornfeld, 2003). Moreover, the *M. smegmatis MSMEG_6935* and *M. tuberculosis Rv3915* gene homologies are 77% by BLAST comparison (using NCBI software). These characteristics make *M. smegmatis* an excellent model of *M. tuberculosis*. In this study, biofilm models of *M. tuberculosis* and *M. smegmatis in vitro* were established, and

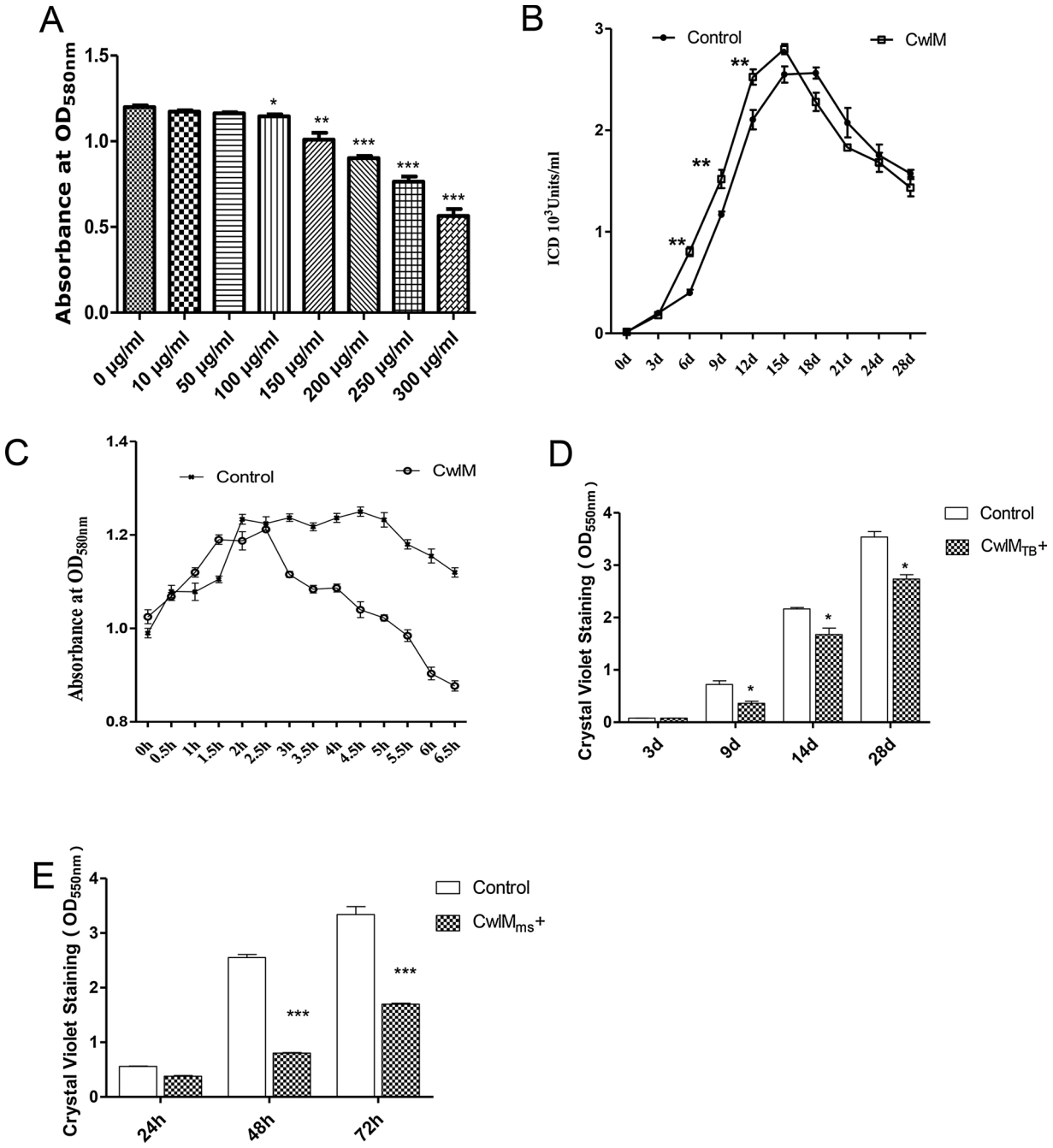

Fig. 5. The effects of CwlM recombinant proteins on the autolysis and biofilm formation of *M. tuberculosis* and *M. smegmatis*. The *M. smegmatis* was treated with different concentrations (0, 10, 50, 100, 150, 200, 250, 300 μg/ml) of CwlM protein for 6.5 h and the autolysis ability was detected by treatment with 0.1% Triton X-100 and measuring OD_{580nm} (A).The autolysis capacity of *M. tuberculosis* was measured by determining the activity of isocitrate dehydrogenase (ICD) (B). The autolytic ability of *M. smegmatis* was detected by treatment with 0.1% Triton X-100 and measuring OD_{580nm} (C). The product of biofilms by *M. tuberculosis* (D) and *M. smegmatis* (E) was quantified by crystal violet staining, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

the results showed that *M. smegmatis* began to form biofilms at 24 h, and they matured at 72 h. In addition, *M. tuberculosis* began to form biofilms at 20 d, and these films matured at 28 d. These results proved that biofilm models of *M. tuberculosis* and *M. smegmatis in vitro* have been successfully established in our study.

Bacterial autolysis is the phenomenon of cell lysis that occurs under the action of a peptidoglycan hydrolase (*i.e.*, autolytic enzyme) that the cell has produced itself (Shockman et al., 1996). Autolysis of a cell occurs because of the degradation of its own cell wall. The peptidoglycan hydrolase that catalyzes autolysis belongs to a cell wall-binding or secreted protein. Therefore, autolysis is closely related to the surface properties of bacteria and is considered a surface property itself. Bacterial cell walls are mainly composed of peptidoglycans, which is fundamental in the survival and development of bacteria. The bacteria can also release peptidoglycan hydrolase to hydrolyze the peptidoglycan of the cell wall. Previous studies have shown that the formation of biofilms is closely related to autolysis (Qin et al., 2007; Rice et al., 2007). The CwlM protein is a peptidoglycan hydrolase (N-acetyl-L-alanine amidase) that can hydrolyze the cell wall and is encoded by *MSMEG_6935* in *M. smegmatis* and *Rv3915* in *M. tuberculosis*. In this study, the *M. tuberculosis* and *M. smegmatis* CwlM proteins were recombinantly expressed, and when *M. tuberculosis* and *M. smegmatis* were

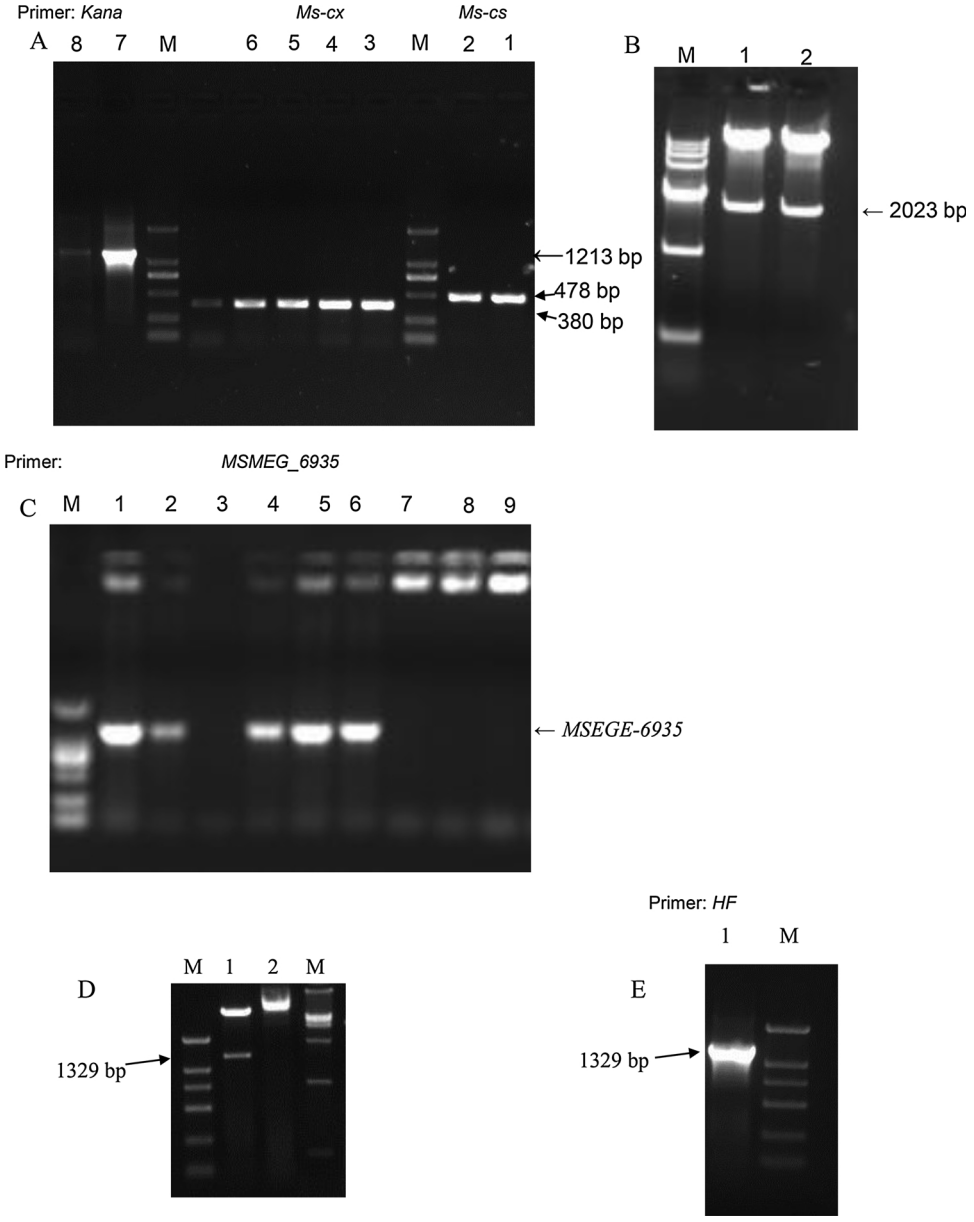

Fig. 6. Construction and verification of the *cwlM* (*MSMEG_6935*)-deletion strain MSΔ6935 and the recovery strain MSΔ6935-R. The homologous arms and the kana gene were ligated by fusion PCR to obtain a three-fragment-gene that was recombined with the cleavage site (A) (M: 2000 bp DNA marker; 1, 2 lanes: upper homologous arm bands; 3, 4, 5, 6 lanes: lower homologous arm bands; 7, 8 lanes: Kana gene). pPR27_6935 included the three fragment-gene, which was verified by Xba I endonuclease digestion (B) (M: 15,000 bp DNA marker; 1, 2 lanes: identified by pPR7 + spd enzyme digestion). The "pPR7 + spd enzyme digestion" means the three-fragment (spd) gene with restriction enzyme recombination was obtained by fusion PCR with the upper and lower homology arms and the Karna gene. The pPR27 shuttle plasmid was successfully constructed with the ligation of spd and pPR27 gene. The gene deletion in the screened *M. smegmatis* colonies was verified by Xba I endonuclease digestion (C) (M: 2000 bp DNA marker; 1, 2, 4, 5 and 6 lanes had *MSMEG_6935* gene; 3, 7, 8 and 9 lanes had no *MSMEG_6935* gene gene). pVV16_6935 included the *MSMEG_6935* gene (D) (M: 2000 bp DNA marker; 1 lanes: Samples (pVV16_6935) containing *MSMEG_6935* gene after Xba I endonuclease digestion, 2 lanes: pVV16_6935 Samples (pVV16_6935) containing *MSMEG_6935* gene); the MSΔ6935-R strain was constructed, and the presence of the additional gene was verified by Xba I endonuclease digestion (E) (M: 2000 bp DNA marker; 1 lane: MSΔ6935-R strain containing *MSMEG_6935* gene).

treated with the recombinant CwlM proteins, their autolyses were enhanced. Moreover, the *M. smegmatis MSMEG_6935*-deletion strain MSΔ6935 was constructed by a gene knockout technique, and the autolytic ability of the deletion strain MSΔ6935 was significantly lower than that of the parental strain, Mc^2155. Together, these results confirmed that the peptidoglycan hydrolase CwlM regulated the autolysis of *M. tuberculosis* and *M. smegmatis* during the formation of their biofilms.

The term bacterial biofilm refers to a large number of microbial communities that are formed by bacteria in the extracellular matrix that is formed by proteins, polysaccharides, lipids and other substances, in addition to the bacteria that are attached to the surface that they contact (Trivedi et al., 2016). In the absence of nutrients, most bacteria do not exist as individuals but rather survive by the formation of a biofilm. Moreover, the biofilm provides a stable protective structure for the bacteria to escape the effects of the host and the antibiotics they contact in a changing environment (Kaur and Singh, 2014). In this study, qPCR showed that the expression of the peptidoglycan hydrolase genes *MSMEG_6935* in *M. smegmatis* and *Rv3915* in *M. tuberculosis* increased significantly in the middle of biofilm formation compared with their expression during early and late stage of biofilm formation. An exogenous peptidoglycan hydrolase was reported to disrupt bacterial metabolic order and be non-toxic and harmless to humans. Therefore, this enzyme has recently been used for clinical treatment, *e.g.*, the combination of autolysin and antibiotics in the treatment of *Staphylococcus aureus* clinical infections (Biziulevicius et al., 2007; Tomasz and Waks, 1975). This study's results showed that there was a positive relationship between autolysis levels and the amount of biofilm that was produced in *M. tuberculosis* and *M. smegmatis*. It is noteworthy that when we treated *M. tuberculosis* and *M. smegmatis* with their own recombinant CwlM proteins to culture their mycobacterial biofilms, the formation of *M. tuberculosis* and *M. smegmatis* biofilms decreased; unexpectedly, the deletion of MSΔ6935 also led to biofilm formation that was lower than that of the parental strain, Mc^2155. To explain the two contrasting results, we speculated that treatment with the recombinant CwlM proteins caused the number of original *M. tuberculosis* and *M. smegmatis* cells to decrease so that biofilm formation also decreased. In the deletion strain MSΔ6935, CwlM protein is never present, leading to less biofilm production because extracellular matrix was not released through the autolysis process. These results reveal that CwlM protein can affect the formation of biofilms by regulating autolysis.

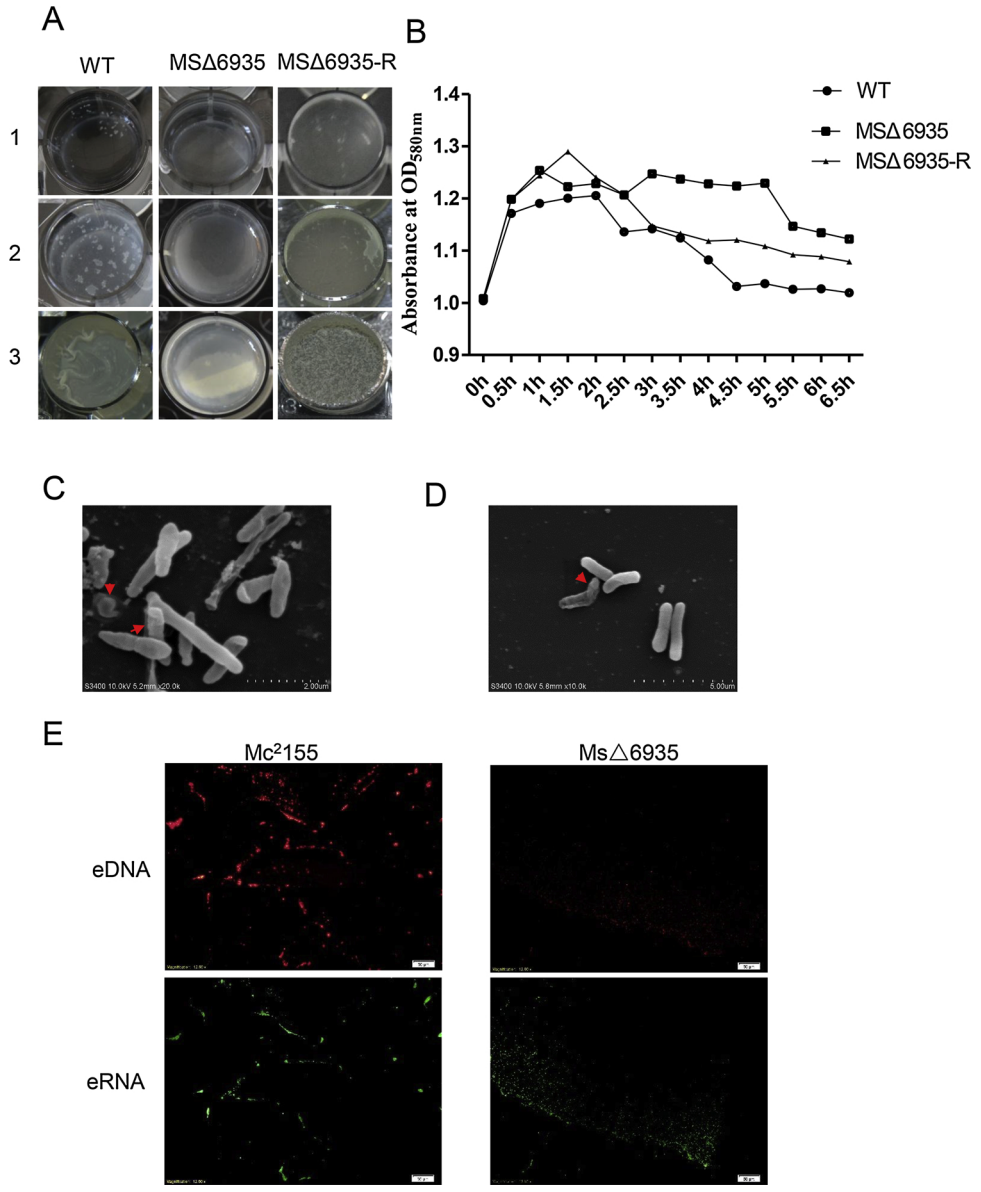

Fig. 7. The effects of *M. smegmatis cwlM* deletion on autolysis and biofilm formation. Biofilms that were produced by the deletion (MSΔ6935) and recovery strains (MSΔ6935-R) were observed at days 1, 2, and 3 (A). The wild, MSΔ6935 and MSΔ6935-R *M. smegmatis* strains were treated with 0.1% Triton X-100, and the autolysis levels were analyzed (B). Biofilms of the wild (C) and the deletion (MSΔ6935) (D) strains of *M. smegmatis* were observed using a scanning electron microscope. eDNA and eRNA in the biofilms that were produced by *M. smegmatis* wild or MSΔ6935 strains were detected by using immunofluorescence staining (E).

eDNA is the main component of bacterial biofilms, has an adhesion function in the biofilm and plays an important role in the aggregation of biofilms, as seen in organisms such as *Listeria monocytogenes* (Harmsen et al., 2010) and *Pseudomonas aeruginosa* (Whitchurch et al., 2002). Thomas et al. showed that inactivation of autolysin can lead to a decrease in eDNA levels and reduced biofilm production (Thomas et al., 2009). DNA is negatively charged and interacts with positively charged polymers to promote the formation of extracellular matrix networks, thereby promoting the formation of biofilm; when it is destroyed, the charge is lost, and biofilm formation is reduced (Okshevsky et al., 2015). Abhishek et al. confirmed that eRNA is present in *M. tuberculosis* biofilms (Trivedi et al., 2016). In the current study, treatment with DNase I and RNaseA could lead to decreased biofilm production. Moreover, there were no eDNA and eRNA filaments in the MSΔ6935 strain. Together, the results showed that eDNA and eRNA were the components of the *M. tuberculosis* and *M. smegmatis* biofilms, and their release into the matrix were related to CwlM.

Overall, this study identified the role CwlM protein expression in the different periods of mycobacterial biofilm formation by establishing *in vitro* biofilm models of *M. tuberculosis* and *M. smegmatis*. Treatment with recombinant CwlM protein and experiments with the *M. smegmatis cwlM* (*MSMEG_6935*)-deletion strain MSΔ6935 demonstrated that the CwlM protein could regulate biofilm formation by autolysis. In addition, eDNA and eRNA were components of the *M. tuberculosis* and *M. smegmatis* biofilms. This study provided a potential target protein and theoretical basis for the development of anti-biofilm drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.12.002.

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