Extracellular DNA of slow growers of mycobacteria and its contribution to biofilm formation and drug tolerance

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Abstract

DNA is basically an intracellular molecule that stores genetic information and carries instructions for growth and reproduction in all cellular organisms. However, in some bacteria, DNA has additional roles on the outside as an extracellular DNA (eDNA), an essential component for the formation of antibiotic tolerance bacteria in biofilm. Mycobacteria include life-threating human pathogens, most of which are slow growers. However, little is known about the nature of pathogenic mycobacteria's eDNA. Here we found that eDNA is present in slow growers of mycobacterial pathogens, such as Mycobacterium tuberculosis, M. intracellulare, and M. avium at exponential growth phase. In contrast, eDNA is little in all tested rapid growers of mycobacteria. The physiological impact of disrupted eDNA on slow growers of mycobacteria include reduced formation of pellicle, a floating biofilm, and enhanced susceptibility to isoniazid and amikacin. Isolation and sequencing of eDNA revealed that it is identical to the genomic DNA in M. tuberculosis and M. intracellulare. In contrast, accumulation of phage DNA in eDNA of M. avium, suggests that the DNA released differs among mycobacterial species. Our data show important functions of eDNA for biofilm formation and drug tolerance in slow growers of mycobacteria.

Introduction

DNA is an essential molecule for all cellular organisms which stores genetic information required for development, growth, reproduction, and evolution. It has been also shown that DNA has an important role outside of the cell and it is termed as extracellular DNA (eDNA)[1-4]. Initially eDNA was detected in the supernatant of liquid cultures of biofilm forming bacterial species, *Pseudomonasaeruginosa*[3-6]. The role of eDNA is enormous in some bacterial life: eDNA is a major component necessary for genome repair, nutrition and horizontal gene transfer, transformation[2,5,7], required component for biofilm formation[3,4]. eDNA has long been known as one of the most abundant molecules in mucous biofilm, formed by different microorganisms such as halophiles[8]. Biofilm formation give the bacteria a growth advantage in that it helps to become more tolerant to harsh conditions, resistant to antibiotics, persist in chronic disease and can even reduce efficacy of vaccines[9-15]. Biofilm formation thus causes health-threating impact on pathogenic bacterial infection.

Mycobacteria include life-threating human pathogens. Currently *Mycobacterium tuberculosis* var. *tuberculosis* (*Mtb*) kills more people than any other single infectious agents and 1.5 million people died from tuberculosis in 2018[16]. Non-nontuberculous mycobacterial (NTM) diseases are increasing especially in developed countries[16]. It is known that more than six months chemotherapy is required to treat tuberculosis. Incompletion of such long-term chemotherapy frequently occurs without adequate control of patients, which in turn causes development of drug resistant tuberculosis. As for NTN diseases, more than one-year chemotherapy is required and most of NTM diseases are resistant to treatment, even when *in vitro* drug-sensitivity test show effectiveness. These suggest that biofilm formation of

mycobacterial pathogens to induce phenotypic drug tolerance that requires long-term chemotherapy for curing.

Mycobacteria, such as, *Mtb, Mycobacterium avium, Mycobacterium marinum, Mycobacterium ulcerans* and *Mycolicibacterium smegmatis* are known to form many types of biofilms[17−24]. Pellicle is a kind of biofilm at the air-liquid interface and one of the standard models of analysis of biofilm formation of mycobacteria. Generally, biofilm formation of bacteria is induced by oligotrophy that reflects harsh conditions. In contrast, formation of mycobacterial pellicle is enhanced by high carbon dioxide and low oxygen tensions, and eutrophy rather than oligotrophy[19,20,25−28]. These characteristics might be reflected with biofilm formation of *Mtb* in lungs with high density of carbon dioxide and inside oxygen-depleted tuberculosis granuloma. Biofilm formation by mycobacteria can be related to long-term habitat of NTM in bathrooms which is a major infectious source in human environment[29]. Components of mycobacterial biofilms are also unique, in part because in addition to exopolysaccharides (EPS) matrix contain of free mycolic acids, glycopeptidolipids, and other lipid-containing molecules[27]. Despite the presence of such unique components in mycobacterial biofilms, eDNA is found in mycobacteria biofilm as seen in other bacteria.

eDNA can be released by different mechanism. The most common mechanism is bacterial lysis, but lytic-independent mechanisms (eDNA releasing independently of cell lysis) also exist[4,6,7,30]. It was first found in *P. aeruginosa* and later seen in the biofilm matrix of other many bacterial genera, including *Enterococcus faecalis, Bacillus cereus, M. avium* subsp. *Hominissuis*[2,3,6,24,30]. pH-dependent export of eDNA was observed in *M. avium* and FtsK/SpoIIIE were identified its responsible molecules. Condition of appearance of eDNA depends on environments: attachment surface, nutrients, mechanical challenges, and stress conditions etc.[31].

Understanding factors mediating biofilm formation of pathogens is an important step for disease control but the nature of eDNA is largely unknown in mycobacteria. In this study we evaluated the physiological functions of mycobacterial eDNA.

Results

Different level of eDNA between slow and rapid growers of mycobacteria.

We first assessed the level of eDNA among mycobacterial species. We assessed slow-growing *Mycobacterium*, such as, *Mycobacterium tuberculosis* var. BCG Tokyo 172 (BCG), *Mycobacterium intracellulare* 13950, *Mycobacterium tuberculosis* H37Rv (*Mtb*), and *Mycobacterium avium* subsp. *hominissuis* 104 and fast-growing mycobacterial species, such as, *Mycolicibacterium fortuitum* subsp. *fortuitum* ATCC 6841, *Mycolicibacterium phlei* 5865[T] (ATCC 19249), *Mycolicibacterium smegmatis* mc^2 155, and *Mycobacteroides abscessus* subsp. *abscessus* ATCC 14472. Bacteria were cultured in 7H9-ADC media until exponential growth phase with optical density value 600 nm (OD_{600}) of 0.2. We then stained the bacteria with both SYTOX Green (SG) and Calcein violet with an acetoxy-methyl ester group (CV-AM).

SG stains eDNA only, because it cannot penetrate an intact cell membrane and cell wall[32]. CV-AM is an indicator of the presence of esterase, which is a marker of bacterial viability[33].

The percentage of SG and CV-AM double positive BCG, *M. intracellulare*, *Mtb* and *M. avium* was 29.42%, 50.06%, 15.03%, and 32.92% respectively (Fig. 1 a, d, g, j and Fig. 3). Heat killing the bacteria resulted in the decrease of double-positive bacteria to under 0.5% and increased SG-single positive indicating that CV-AM does not stain dead bacteria (Fig. 1 c, f, i, l and Fig. 3).

Given the unexpected higher percentage of double stained bacteria compared to a previous study[34], we treated bacteria with Benzonase[@]Nuclease I (DNase I) and saw a change in the percentage of double positive bacteria. We found a substantial decrease in double positive bacteria in all tested slow growers. These data suggest the presence of DNase I sensitive eDNA on every tested strain of slow-growing mycobacteria, although more than half of the portions were resistant to DNase I treatment (Fig. 1 b, e, h, k, and Fig. 3).

By contrast, the amount of double stained fast-growing mycobacteria (*M. fortuitum, M. phlei, M. smegmatis*, and *M. abscessus*) was low and did not exceed 7%. These small portions of double-stained population were constantly low when bacteria were treated with DNase I (Fig. 2 and Fig. 3), showing little eDNA on rapid growing mycobacteria.

We repeated experiments and showed the average of eDNA amounts with or without DNase I treatment in Fig. 3. The data demonstrate that more than 27% of living slow growers are eDNA-positive, while most of rapid-growers are eDNA negative. Taken together, these data indicate that the presence of eDNA is remarkable on slow-growing mycobacteria but not on fast-growing ones when bacteria are cultured in standard eutrophic media for mycobacteria (7H9 supplemented with ADC).

The impact of eDNA on pellicle formation.

It has been shown that eDNA promotes a biofilm formation in *P. aeruginosa, Enterococcusspp.* and others. Mycobacterial species including pathogenic and non-pathogenic organisms form biofilms in the various environmental reservoirs; we thought that eDNA would contribute to biofilm formation in slow growers of mycobacteria.

In order to address this, we cultured BCG, *M. intracellulare*, and *M. avium* on Sauton media, a standard media for mycobacterial growth with pellicle formation, with or without addition of DNase I every week. Two to three weeks later, these bacteria formed thin pellicle on the surface of media and piled up, leading to the formation of a thick floating biofilm. After five weeks we filtered the bacterial suspension with a 0.45 µm filter and measured the weight of the pellicle formed. We observed a reduction in biomass of all tested mycobacterial species pellicles by addition of DNase I (Fig. 4). BCG, *M. intracellulare,M. avium* had above 2-fold difference between treated and untreated DNase I samples. These data show that disruption of eDNA led to a decrease in pellicle formation, thus eDNA has a structural role and promotes formation of biofilms of slow growers of mycobacteria.

eDNA aids in tolerance of mycobacteria to certain drugs.

Biofilm formation has been shown to be advantageous in bacterial growth especially when it comes to tolerance to antibiotics. However, due to solid clumped mycobacterial biofilm, measuring the exact number of drug-tolerant mycobacteria in biofilm are difficult. We thus examined whether eDNA alone contributes to mycobacterial tolerance to drugs using planktonic bacteria.

BCG, *M. intracellulare* and *M. avium* were pre-cultured in 7H9-ADC media to an OD_{600} of 0.1, diluted to OD_{600} of 0.001, and further cultured in the presence or absence of DNase I for 72 hours. The cultures were then incubated with isoniazid (INH), rifampicin (RMP), amikacin (AMK), or clarithromycin (CLA) for 6 or 24 hours and followed by a subsequent assessment of viability by counting the colony forming units (CFUs). Viability of bacteria was then assessed by CFUs. Data are represented as a percent of viable cells relatively to untreated of DNase I cells (Fig. 5).

Similar to pellicle formations on Sauton media, we found that even DNase I treatment leads to decrease in BCG viability (data not shown). In this condition, DNase I treatment increased the efficacy of AMK and INH but not RMP. Significant reduction of CFUs was observed when BCG was treated with AMK for both 6 and 24 hours, and with INH for 24 hours (Fig. 5).

As for NTM, similar to BCG, treatment *M. intracellulare* and *M. avium* by DNase I reduced CFU in drug-free condition. There was a greater reduction of CFU in DNase I - treated NTM exposed to AMK and CLA than in untreated controls. However, DNase I treatment did not boost sensitivity in most cases. We only observed significantly increased drug sensitivity of *M. avium* when treated with CLA for 6 hours (Supplementary Fig. S1).

Nature of eDNA.

In order to know nature of eDNA of mycobacteria, we purified both eDNA and genomic DNA (gDNA) from BCG, *M. intracellulare*, *Mtb*, and *M. avium* as shown in Fig. 6. We sequenced both eDNA and gDNA using the MiSeq Illumina next-generation sequencer. Comparison of eDNA and gDNA sequences showed that eDNA of most of tested strains corresponds to genome DNA. eDNA of BCG, *M. intracellulare,* and *Mtb* are identical with their gDNA, suggesting that the eDNA resulted from mycobacterial lysis or export of entire gDNA (Fig. 7). Expected to have a comparison, *M. avium* on the other hand had DNA sequences that were specific to the eDNA (Fig. 7). These sequences encoded MAV_0779 to 0842 that are genes of Prophage phiMAV_1[35] (Fig. 8). This suggests that phage DNA release is an additional way of eDNA construction in *M. avium* 104.

Discussion

Biofilm formation is involved in resistance against chemotherapy in mycobacterial diseases, most of which are caused by slow-growers[17,20,23,27]. eDNA is a component of the bacterial biofilm[20,24,26]. Unexpectedly, this study found that slow growers of mycobacteria release a higher amount of eDNA in

contrast to rapid growers of mycobacteria. Slow growing pathogens, such as *M. tuberculosis* complex and MAC release detectable amounts of eDNA which afforded pellicle formation that resist drug treatment. eDNA also contributed to drug tolerance even in planktonic exponential phase (Fig. 4-5). These data suggest a more prominent function of eDNA in slow growers of mycobacteria thus conferring a growth advantage and drug tolerance.

Rapid growers of mycobacteria have little eDNA at the exponential growth phase. This may suggest the importance of other molecules for biofilm development in rapid growers. Recht et al. showed that glycopeptidolipids and mycolic acid are essential for initial surface attachment during biofilm formation for *M. smegmatis*[28]. Thus, fast-growing bacteria probably may start to release eDNA at the later phase of biofilm formation for biofilm development[4,25].

Treatment with antituberculosis drugs INH, RMP, and aminoglycosides AMK, CLA at two conditions (with and without DNase I at 6 and 24 hours of treatment), showed heterogeneous results but revealed contribution of eDNA to tolerance of mycobacteria to certain kinds of drugs, such as INH, AMK, and CLA. By contrast, DNase I treatment did not show any influence on the effect of RMP. It may be because RMP is highly lipophilic. Mycobacterial cell walls consist up to 60 % of lipids. Mycobacterial lipids has many biological functions, including resistance of majority of mycobacteria species to most broad-spectrum antibiotics by low permeability rate. Lipophilic nature of RMP may allow to drug act independently from eDNA.

Treatment with AMK also showed significant decrease of viability in contrast to control, and sensitivity to AMK was increased by addition of DNase I (Fig. 5). Thus, the presence of eDNA somehow influences BCG's viability. AMK is an aminoglycoside that irreversibly binds to the 30S subunit and inhibits protein synthesis in ribosomes. Possible reason of heterogeneous data of aminoglycosides about viability of mycobacteria with/without DNase I treatment would be a dissimilarity of local environment[27].

To investigate if eDNA found in mycobacteria is genomic in origin or has an eDNA-specific sequence, extraction of eDNA was done, sequenced, and was directly compared to gDNA sequence. eDNA sequence identity with gDNA in BCG, *M. intracellulare*, and *Mtb* means that eDNA was likely occurred by bacterial lysis. Since eDNA was seen in slow growers of mycobacteria but was little in rapid growers (Fig. 1), it can be considered that a certain population of slow-growing mycobacteria die even at exponential growth phase. This can be explained by the bacterial death caused by entering the lysis cycle of intrinsic lysogenic bacteriophages. We actually observed accumulated phage DNA in the eDNA of *M. avium* 104, suggesting that some population of *M. avium* was lysed by phage lysis.

However, we did not identify phage DNA accumulation in the eDNA of other examined slow growers of mycobacteria. Another possibility of eDNA construction is frequent failure of replication, resulting to the death of mycobacteria. It is known that even in active disease state, a certain *Mtb* population shifts to dormant phase, which necessitates the long treatment regimen for tuberculosis[32]. It is also known that a constant population of bacteria, past the stationary phase, lose viability and eventually die. Bacterial

death releases gDNA to become eDNA which may support the survival of other live bacteria. Recent studies suggest that bacterial apoptosis and its mechanisms is important in biofilm development[10,12]. In case of nutrient limitation or antibiotics exposure, part of the bacterial population is sacrificed to provide a source of nutrients or eDNA that can be picked up viable cells and used for DNA repair, transformation, quorum sensing etc.[9,15,17]. Such proactive biological mechanism might be involved in the construction of eDNA in slow growers of mycobacteria during planktonic exponential phase.

Using a high biofilm forming strain of *M. avium* subsp. *hominissuis*, Rose et. al observed a significant effect eDNA on the development of biofilm and drug tolerance of *M. avium*[29]. Cloning of certain parts of the eDNA by polymerase chain reaction-based method and their sequencing showed that gDNA is the source of eDNA. Interestingly, comparison of the quantity of eDNA in the biofilm matrix and living number of bacteria by CFU in the same study didn't show significant decrease in CFU despite the increase in the amount of eDNA. This statement is an introduction to the other factor that is the source of eDNA in addition to bacterial death. There can be a mechanism that transfer large-size of DNA resemble to bacterial conjugation[37].

Similar to our study, other groups also showed that eDNA induces tolerance of bacterial biofilms. DNA release occurs during drug-mediated killing of bacteria. Thus, powerful new drug, such as RMP (Fig. 5) that sterilizes mycobacteria covered by eDNA is required for controlling mycobacterial diseases. This study provides basic knowledge for development of control strategies against intractable mycobacterial diseases.

Methods

Bacterial strains, growth, and reagents.

All mycobacteria strains were grown on Middlebrook 7H9 broth (BD, Franklin Lakes, NJ) supplemented with 0.2% (v/v) glycerol, 0.05% (v/v) Tween 80 (MP Biomedicals, Santa Ana, CA), and 10% ADC enrichment (5% bovine serum albumin [Wako Pure Chemical Industries, Osaka, Japan], 0.81% NaCl, and 2% D-glucose) (7H9-ADC broth) or on Mycobacteria 7H11 agar (BD) supplemented with 0.5% (v/v) glycerol and 10% OADC enrichment (ADC enrichment supplemented with 0.06% [v/v] oleic acid) (7H11-OADC agar).

Hygromycin B (HYG), kanamycin sulfate (KAN), RMP, AMK, and CLA were purchased from Wako Pure Chemical Industries [Osaka, Japan]; INH purchased from SIGMA-ALDRICH [St. Louis, USA]. AMK stock solutions were prepared in water. Stock solutions of all other compounds were prepared in 100% dimethyl sulfoxide (DMSO) and then filter sterilized (pore size 0.45 μm). These components were frozen in aliquots at -20 °C.

CV-AM and SG were purchased from Invitrogen (Life-Technologies Corporation, California, USA). Each aliquot of CV-AM purchased was dissolved in 250 microliters (μl) of 100% dimethyl sulfoxide (DMSO). SG

was diluted from the manufacture's 5 mM stock solution to 50 μM working solution by DMSO. They were frozen at -30 °C until use.

DNaseItreatment and double staining.

Bacterial culture was adjusted to an OD_{600} of 0.01. Each sample was divided into two tubes of 1 ml each and 1 μl (2 units) DNase I (SIGMA-ALDRICH) added to one of them. Samples were then incubated overnight at 37 °C with rotation 4 rpm. One hundred μl was transferred to a fresh tube. Five μl CV-AM staining solution was added to tubes and incubated for 60 minutes at 37 °C. Then, 1 μl of 0.5 uM SG staining solution was added and incubated for 15 minutes at RT. To remove excess amount of stain the sample was washed with 7H9-ADC once (for *Mtb* samples fixation was performed using 4% paraformaldehyde/PBS in BSL 3). All samples were diluted in 150 μM NaCl containing 0.05% Tween 80 and analysed by NovoCyte Flow Cytometer [ACEA Biosciences Inc., San Diego, CA, USA] using excitation of 488 (SG) nm and 405 (CV-AM) nm. Control, live, HK, untreated and treated cells by DNase I were also prepared[31].

Biofilm formation.

BCG, *M. intracellulare*, *M. avium* strains were grown on Sauton media containing KAN and HYG without (-) or with Takara DNase I [Kusatsu, Shiga, Japan (total 50 units)].

All samples were incubated for 5 weeks at 37 °C with weekly addition of DNase I. Then, samples were centrifuged at 2,500 g for 20 minutes to pellet the biomass. The biofilm supernatant was pellet collected and filtered through a 0.45 μm filter and BCG, *M. intracellulare* and *M. avium* wet biomass weight.

Bacterial viability after antibiotics treatment.

BCG, *M. intracellulare* and *M. avium* were grown until an OD_{600} of 0.1 in 7H9/ADC broth. The cultures were then diluted in fresh medium to an OD_{600} of 0.001 with/without DNase I and cultured for a further 72 hours at 37 °C on the rotator. After that bacteria were diluted in fresh medium to an OD_{600} of 0.001 and treated with INH (0.1 mg/ml), RMP (0.5 mg/ml), AMK (2.5 mg/ml), and CLA (5 mg/ml) for 6 and 24 hours; we also compared control samples without antibiotics. After antibiotics treatment, we prepared ten-fold serial dilutions of each sample from 10^{-1} to 10^{-6} and then plated in triplicate on 7H11-OADC agar. The plates were cultured at 37 °C for 3 weeks. The number of colonies were then counted and CFUs (CFU/ml) were calculated.

DNA extraction and electrophoresis.

Bacteria strains were grown to an OD_{600} of 1.5 in 7H9/ADC broth. Followed by collection of the BCG, *M. intracellulare,Mtb,* and *M. avium* pellet by centrifuging the samples at 3500 rpm at room temperature for 20 minutes. Bacterial pellets were suspended in 200 μl of TE lysis buffer [1 M Tris-HCL (pH 8.0) and EDTA (ethylenediaminetetraacetic acid) in Treff tube and frozen at -80 °C. Then, samples were washed with

chloroform/methanol, dried, resuspended by 200 ul TE with 20 ul of 1M Tris-HCl pH 9.0 buffers. Next 2.2 ul of 10 mg/mL lysozyme and 5 µl of 10 mg/ml proteinase K (Invitrogen) in the presence of 10% SDS were added. Samples were incubated overnight at 37 °C. All samples were incubated at 50°C for more than 3 hours to lyse the cell wall and membrane. The DNA was then extracted from each sample by addition of an equal volume of 3 M pH 5.2 sodium acetate and phenol: chloroform: isoamylalcohol solution (25:24:1 vol/vol/vol) and mixed gently by inverting the tubes for a few minutes. Next, all the samples were centrifuged for 10 minutes, 15,000 g at RT, and the supernatant containing DNA was transferred to a sterilized Eppendorf tube. Samples were then washed with isopropanol and were centrifuged for 10 minutes above 15,000 g at RT again. After decanting the supernatant, 400 µl of 70% ethanol was added, and the pellet was dissolved. The mixture was centrifuged for 10 minutes at 15,000 g at RT, and the supernatant was decanted gently. After that, RNase A (23 µl of 10 mg/ml; Macherey-Nagel, Germany) was added to DNA samples, and incubated at 37°C for more than 30 minutes. Equal volume of 3 M pH 5.2 Sodium Acetate and phenol: chloroform: isoamylalcohol solution were added and centrifuged, again for 10 minutes with 15,000 g at RT. The supernatant was transferred to a sterilized eppendorf tube, and washed with of chilled isopropanol and 70% alcohol again. The pellet was air-dried and incubated with TE buffer at 50 °C overnight for precipitation and frozen at −20 °C for storage.

The same protocol was used for eDNA extraction, but without the bacterial membrane destruction step. The quality and quantity of the DNA and eDNA samples was assessed using the Agilent 2200 TapeStation [Agilent Technologies, California, USA] was done using gDNA and eDNA[36].

Next-generation sequencing of DNA.

eDNA and gDNA samples from bacteria such as BCG, *M. intracellulare,Mtb* and *M. avium* were sequenced by MiSeq Illumina sequencer.

Sequencing libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) according to the manufacture's procedure and purified by AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA). The DNA concentration of each purified library with adapters was measured by Qubit[TM] Fluorometer with Qubit dsDNA HS assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the library size was checked by Agilent 2100 Bioanalyzer with High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA). Based on the measured DNA concentration and the size, the molarity of each DNA library was calculated and normalized to 4 nM. Each 4 nM DNA library was pooled and sequenced by the MiSeq system with MiSeq Reagent Kit v3 (Illumina) by following the manufacture's instructions.

Comparison of gDNA and eDNA.

The genomic sequences and the annotation data of BCG (BCG Tokyo), *M. intracellulare* (ATCC 13950), *Mtb* (H37Rv), and *M. avium* (104) were obtained from the release 40 of Ensemble Bacteria[37]. The paired-end sequences of gDNA and eDNA samples were filtered using Sam tools[39] with maxi parameter of 0.5. The filtered sequences were mapped to each *Mycobacterium* species genome using BWA[38]. The number

of reads mapped for each gene was calculated using feature counts[40]. The counts were then normalized by the total read number in a sample, yielding the relative abundance of a gene in a sample. The abundance ratio of eDNA to gDNA was calculated by dividing the relative abundance of eDNA by that of gDNA.

Statistics.

UNIANOVA statistics was performed using IBM SPSS 22.0 software (SPSS, Chicago, IL, USA). Data was analyzed using Games-Howell post-hoc test, Wilcoxon/Kruskal-Wallis non-parametric test, and Student t-test. Differences were considered significant when the P-value was <0.05.

Declarations

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Author contributions

Conceptualization: S.M.

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Competing interests

The authors declare that no competing interest exists.

References

1. Catlin, B. W. Extracellular deoxyribonucleic acid of bacteria and a deoxyribonuclease inhibitor. *Science (80-.).* **124**, 441–442 (1956).

2. Antonova, E. S. & Hammer, B. K. Genetics of Natural Competence in Vibrio cholerae and other Vibrios. *Microbiol. Spectr.* **3**, (2015).

3. Vilain, S., Pretorius, J. M., Theron, J. & Brözel, V. S. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. *Appl. Environ. Microbiol.* **75**, 2861–2868 (2009).

4. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. Extracellular DNA required for bacterial biofilm formation. *Science (80-.).* **295**, 1487 (2002).

5. Wesley Catlin, B. Interspecific transformation of neisseria by culture slime containing deoxyribonucleate. *Science (80-.).* **131**, 608–610 (1960).

6. Allesen-Holm, M. *et al.* A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* **59**, 1114–1128 (2006).

7. Hamilton, H. L., Domínguez, N. M., Schwartz, K. J., Hackett, K. T. & Dillard, J. P. Neisseria gonorrhoeae secretes chromosomal DNA via a novel type IV secretion system. *Mol. Microbiol.* **55**, 1704–1721 (2005).

8. Toyofuku, M. *et al.* Environmental factors that shape biofilm formation. *Biosci. Biotechnol. Biochem.* **80**, 7–12 (2016).

9. Flores-Valdez, M. A. Vaccines directed against microorganisms or their products present during biofilm lifestyle: Can we make a translation as a broad biological model to tuberculosis? *Front. Microbiol.* **7**, (2016).

10. Bayles, K. W. The biological role of death and lysis in biofilm development. *Nat. Rev. Microbiol.* **5**, 721–726 (2007).

11. Hall, C. W. & Mah, T. F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews* vol. 41 276–301 (2017).

12. Allocati, N., Masulli, M., Di Ilio, C. & De Laurenzi, V. Die for the community: An overview of programmed cell death in bacteria. *Cell Death and Disease* vol. 6 e1609–e1609 (2015).

13. Baskin, H., Bayrakal, V. & Bahar, I. H. Effects of gentamicin, amikacin and netilmicin on the pathogenic factors and two extracellular quorum sensing systems of *Pseudomonas aeruginosa* strains. *Turkiye Klin. J. Med. Sci.* **32**, 1319–1326 (2012).

14. Singh, S., Singh, S. K., Chowdhury, I. & Singh, R. Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents. *Open Microbiol. J.* **11**, 53–62 (2017).

15. ADELBERG, E. A. & PITTARD, J. CHROMOSOME TRANSFER IN BACTERIAL CONJUGATION. *Bacteriological reviews* vol. 29 161–172 (1965).

16. Tuberculosis. https://www.who.int/news-room/fact-sheets/detail/tuberculosis.

17. Xiang, X., Deng, W., Liu, M. & Xie, J. Mycobacterium biofilms: Factors involved in development, dispersal, and therapeutic strategies against biofilm-relevant pathogens. *Crit. Rev. Eukaryot. Gene Expr.***24**, 269–279 (2014).

18. Esteban, J. & García-Coca, M. Mycobacterium biofilms. *Frontiers in Microbiology* vol. 8 2651 (2018).

19. Aung, T. T. *et al.* Biofilms of pathogenic nontuberculous mycobacteria targeted by new therapeutic approaches. *Antimicrob. Agents Chemother.***60**, 24–35 (2016).

20. Basaraba, R. J. & Ojha, A. K. Mycobacterial Biofilms: Revisiting Tuberculosis Bacilli in Extracellular Necrotizing Lesions. in *Tuberculosis and the Tubercle Bacillus* 533–539 (ASM Press, 2017). doi:10.1128/9781555819569.ch24.

21. Falkinham, J. O. Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine* vol. 23 529–551 (2002).

22. Schulze-Röbbecke, R., Janning, B. & Fischeder, R. Occurrence of mycobacteria in biofilm samples. *Tuber. Lung Dis.***73**, 141–144 (1992).

23. Yamazaki, Y., Danelishvili, L., Wu, M., MacNab, M. & Bermudez, L. E. *Mycobacterium avium* genes associated with the ability to form a biofilm. *Appl. Environ. Microbiol.***72**, 819–825 (2006).

24. Rose, S. J., Babrak, L. M. & Bermudez, L. E. *Mycobacterium avium* possesses extracellular DNA that contributes to biofilm formation, structural integrity, and tolerance to antibiotics. *PLoS One***10**, (2015).

25. Totani, T. *et al.* Effects of nutritional and ambient oxygen condition on biofilm formation in *Mycobacterium avium* subsp. *Hominissuis* via altered glycolipid expression. *Sci. Rep.***7**, 1–12 (2017).

26. Zambrano, M. M. & Kolter, R. Mycobacterial biofilms: A greasy way to hold it together. *Cell* vol. 123 762–764 (2005).

27. Ojha, A. K. *et al.* Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol.***69**, 164–174 (2008).

28. Recht, J. & Kolter, R. Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. *J. Bacteriol.***183**, 5718–5724 (2001).

29. Nishiuchi, Y. *et al.* The recovery of *Mycobacterium avium-intracellulare* complex (MAC) from the residential bathrooms of patients with pulmonary MAC. *Clin. Infect. Dis.***45**, 347–351 (2007).

30. Barnes, A. M. T., Ballering, K. S., Leibman, R. S., Wells, C. L. & Dunnya, G. M. *Enterococcus faecalis* produces abundant extracellular structures containing DNA in the absence of cell lysis during early biofilm formation. *MBio***3**, (2012).

31. Rose, S. J. & Bermudez, L. E. Identification of bicarbonate as a trigger and genes involved with extracellular DNA export in mycobacterial biofilms. *MBio***7**, (2016).

32. invitrogen Molecular Probes. SYTOX® Green Nucleic Acid Stain. 4 (2006).

33. Invitrogen. Calcein Violet, AM. 1–4 (2010).

34. Hendon-Dunn, C. L. *et al.* A flow cytometry method for rapidly assessing *Mycobacterium tuberculosis* responses to antibiotics with different modes of action. *Antimicrob. Agents Chemother.***60**, 3869–3883 (2016).

35. Fan, X., Xie, L., Li, W. & Xie, J. Prophage-like elements present in Mycobacterium genomes. *BMC Genomics* **15**, (2014).

36. Ghatak, S., Muthukumaran, R. B. & Nachimuthu, S. K. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. *J. Biomol. Tech.* **24**, 224–231 (2013).

37. Howe, K. L. *et al.* Ensembl Genomes 2020-enabling non-vertebrate genomic research. *Nucleic Acids Res.* **48**, D689–D695 (2020).

38. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589–595 (2010).

39. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).

40. Liao, Y., Smyth, G. K. & Shi, W. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).

Figures
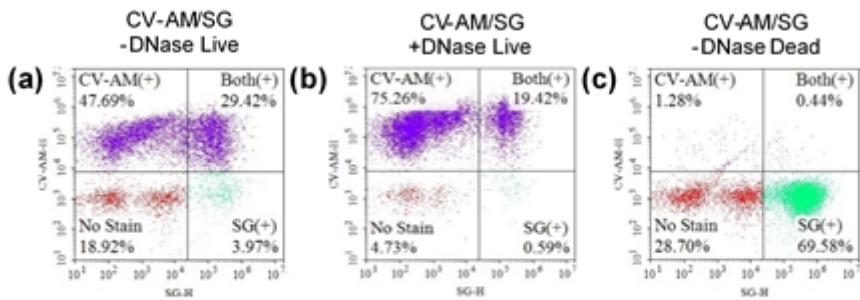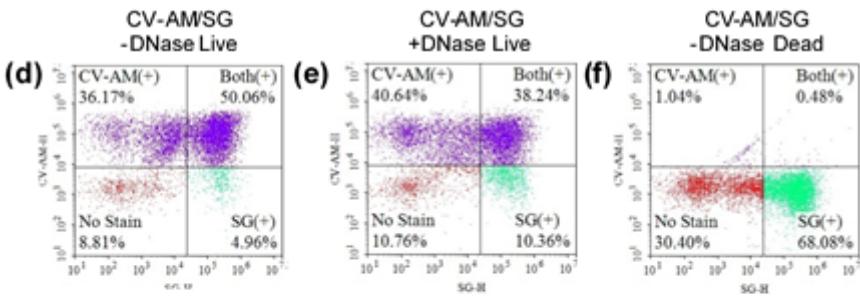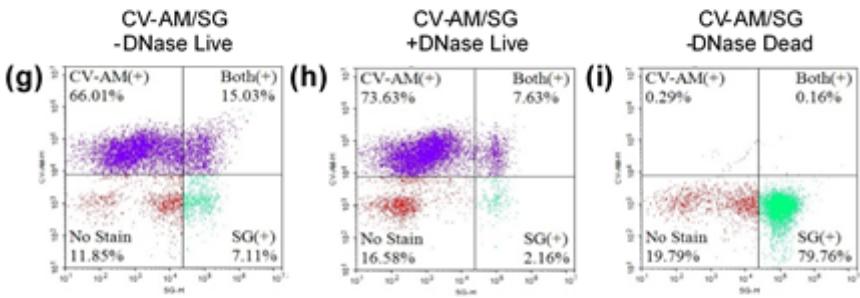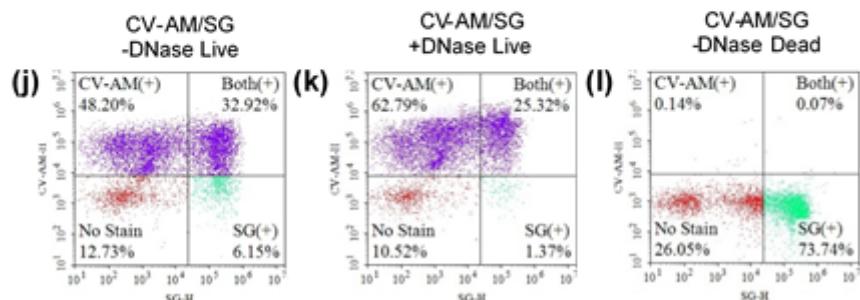
Figure 1

FACS analysis of extracellular DNA (eDNA) on BCG, M. intracellulare, Mtb and M. avium. Representative FACS plots of BCG (a, b, and c), M. intracellulare (d, e, and f), Mtb (g, h and i), and M. avium (j, k and l) stained by CV-AM and SG, respectively. Bacteria were treated with DNase I (b, e, h, and k) or heated at 95°C for 5 min (c, f, i, and l) before staining.
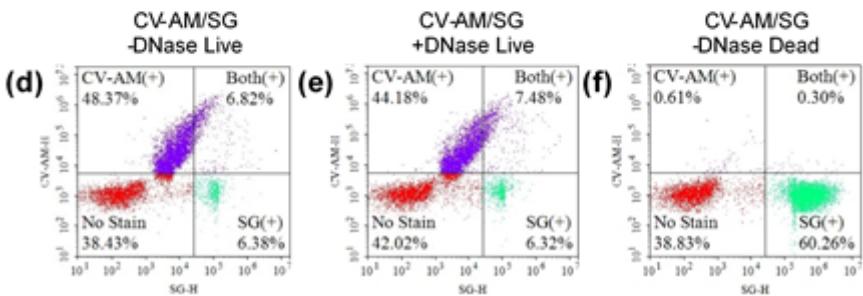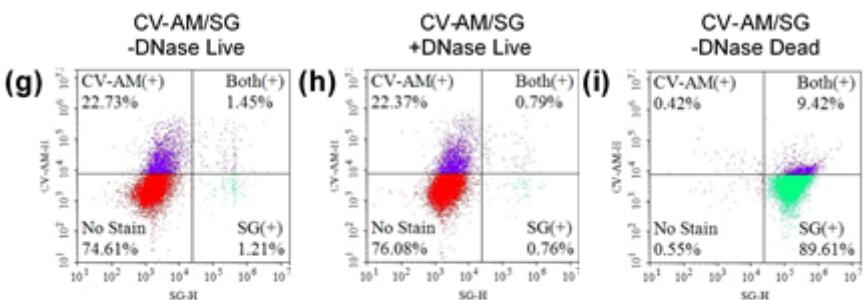
Figure 2

FACS analysis of eDNA on M. fortuitum, M. phlei, M. smegmatis and M. abscessus. Representative FACS plots of M. fortuitum (a, b, and c), M. phlei (d, e, and f), M. smegmatis (g, h and i) and M. abscessus (j, k and l) stained by CV-AM and SG, respectively. Bacteria were treated with DNase I (b, e, h, and k) or heated at 95°C for 5 min (c, f, i, and l) before staining.
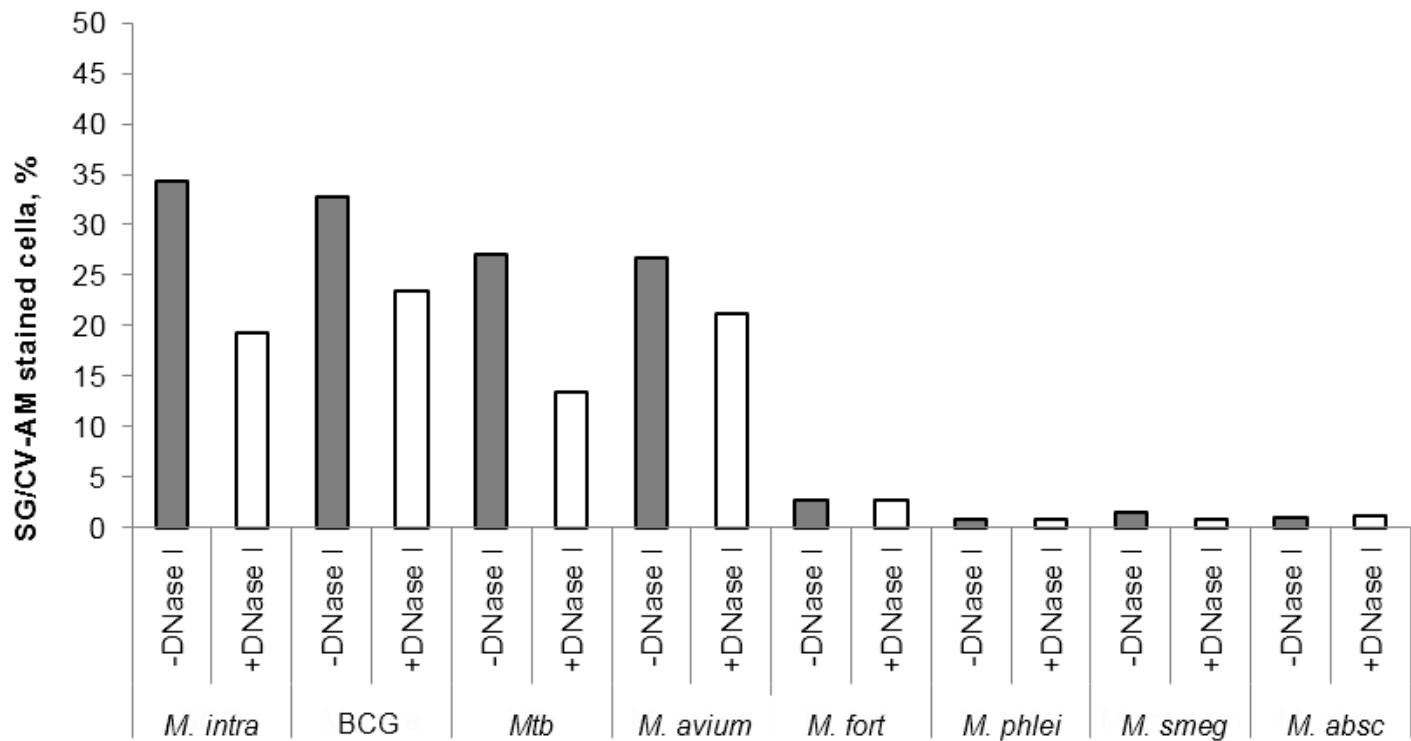
Figure 3

Comparative analysis of eDNA of slow growing and fast-growing mycobacteria. Percentage of double-stained cells DNase I – untreated (-DNase I) or – treated (+DNase I) slow-growing mycobacteria – BCG, M. intracellulare, Mtb, or M. avium and fast-growing mycobacteria – M. fortuitum, M. phlei, M. smegmatis, M. abscessus stained with CV-AM/SG.
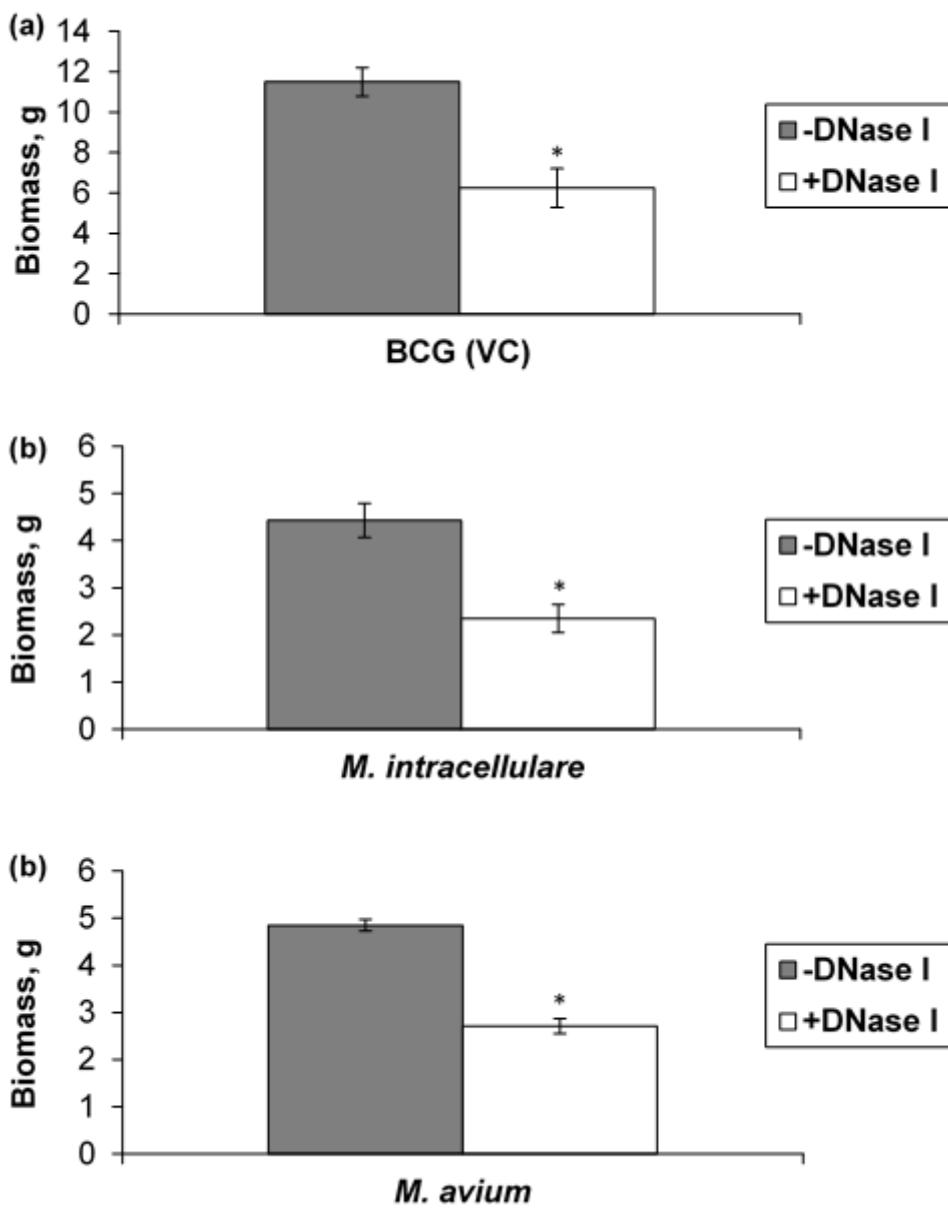
Figure 4

Biomass of mycobacterial pellicles treated with DNase I or not. Five weeks after incubation of BCG (a), M. intracellulare (b), and M. avium (c) on Sauton media with or without addition of DNase I and wet weights of pellicle were measured. Mean ± SD (n = 4). *, $p<0.05$, compared to biomass of DNase I − untreated BCG (-DNase I).
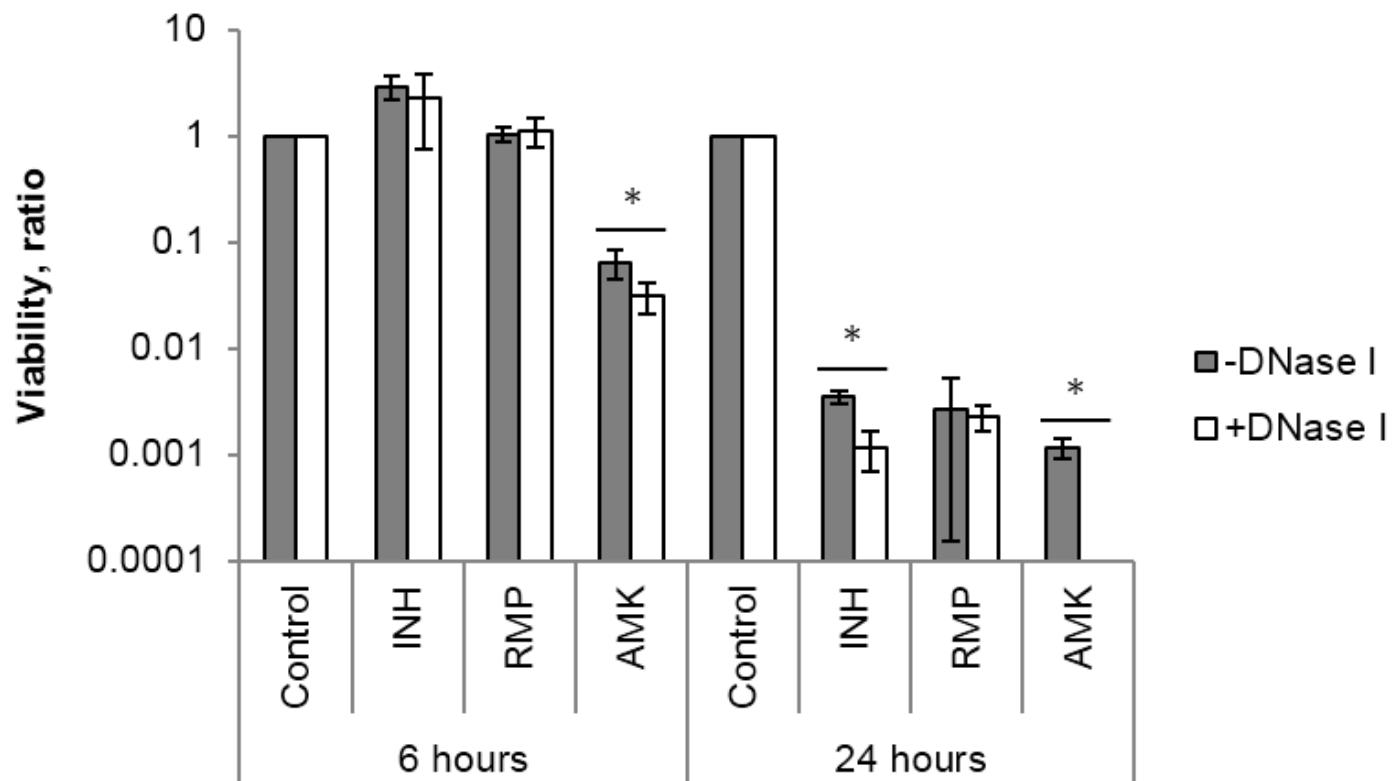
Figure 5

Differential effect of DNase I treatment on drug susceptibility of BCG. BCG was – untreated (-DNase I) or – treated (+DNase I) at 37 °C for 72 hours and further incubated with INH, RMP, AMK or not, for 6 and 24 hours. CFU/ml was determined and normalized with that of control samples. The average viability ratio of each sample (mean ± SD, n = 3) is indicated. *, $p < 0.05$.
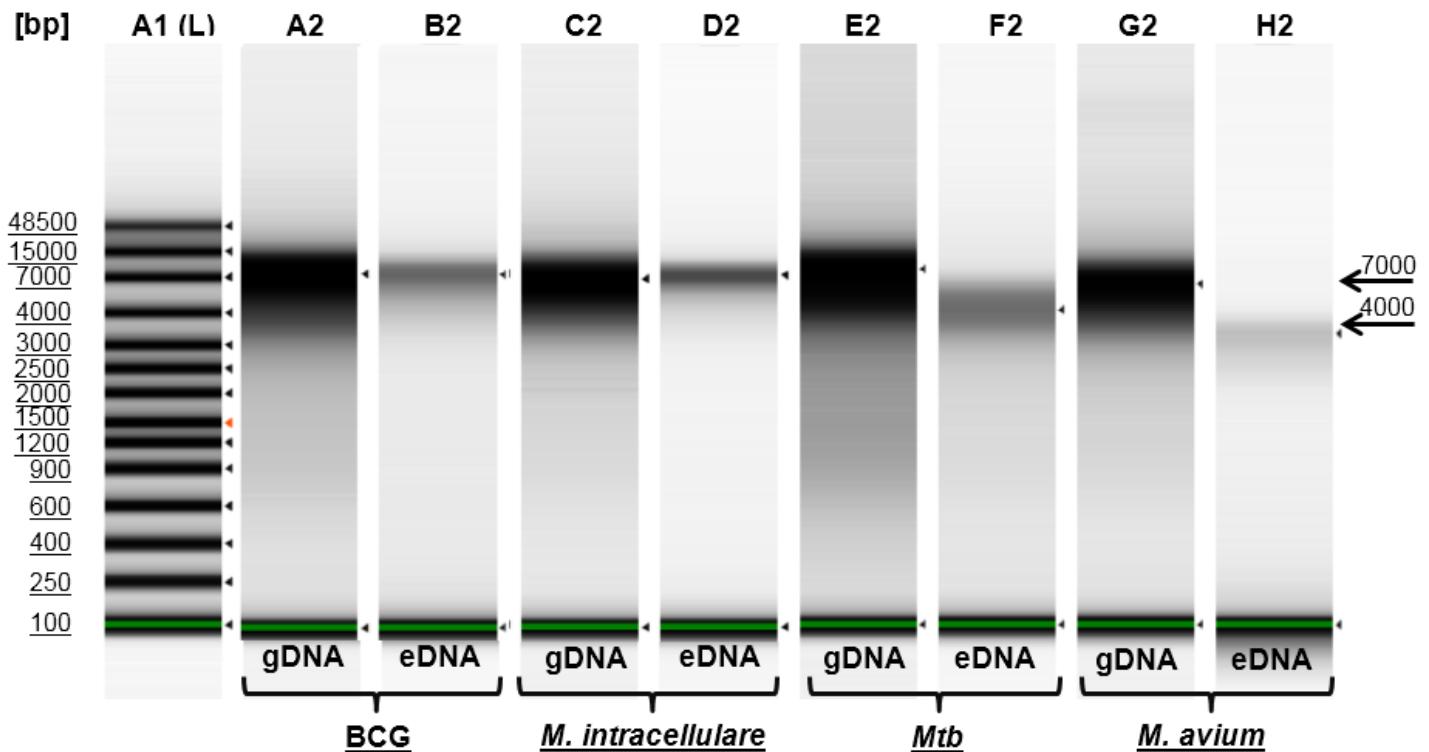
Figure 6

Gel electrophoresis of genomic DNA (gDNA) and eDNA of mycobacteria. gDNA and eDNA extracted from BCG, M. intracellulare, Mtb, and M. avium and fractionated were visualized by Agilent 2200 TapeStation gel electrophoresis. Standards of molecular weight are also loaded. White lines indicate where parts from the same gel (Supplementary Fig. S2) were merged.
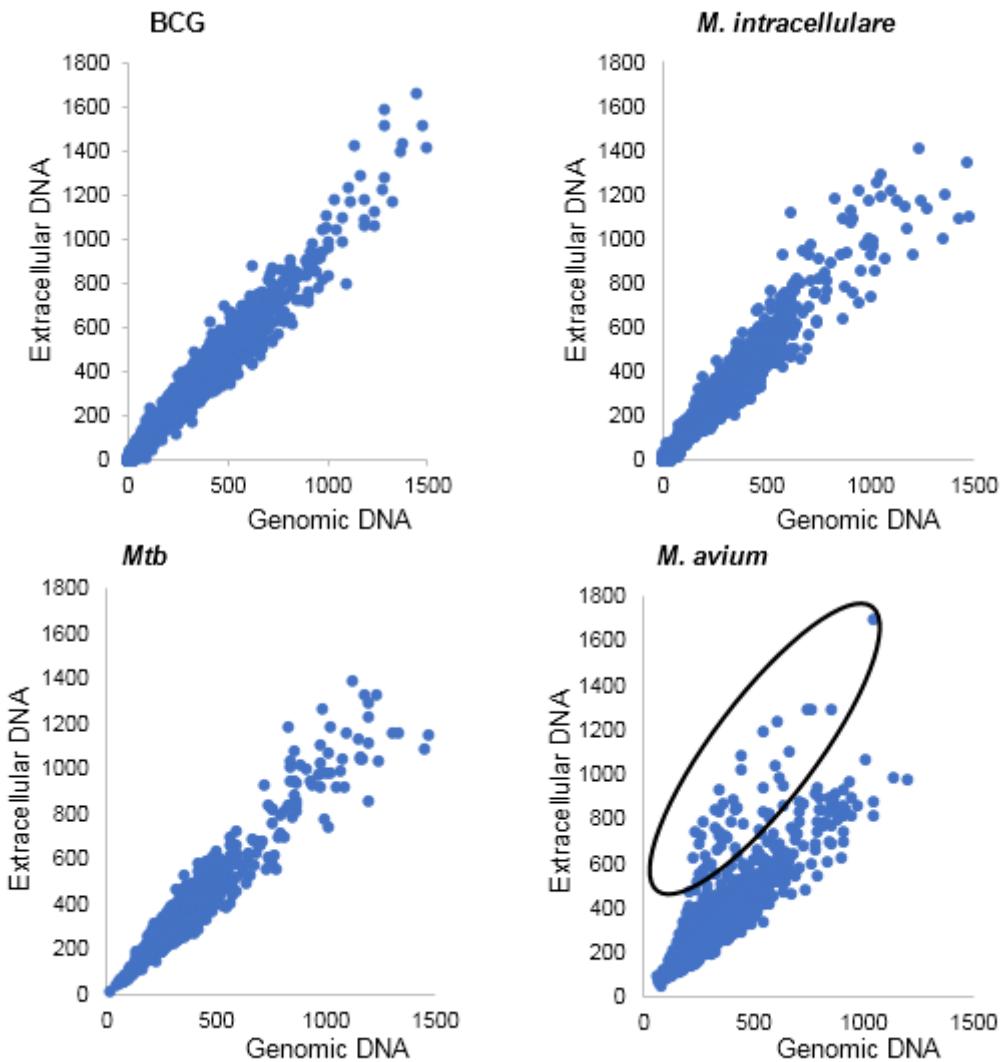
Figure 7

Whole genome sequencing comparison of gDNA and eDNA of BCG, M. intracellulare, Mtb and M. avium. The relative abundance of gDNA and eDNA genes of BCG, M. intracellulare, Mtb and M. avium were calculated. Each gene was plotted where the relative abundance of gDNA and eDNA correspond. The circle indicates accumulated genes in eDNA.
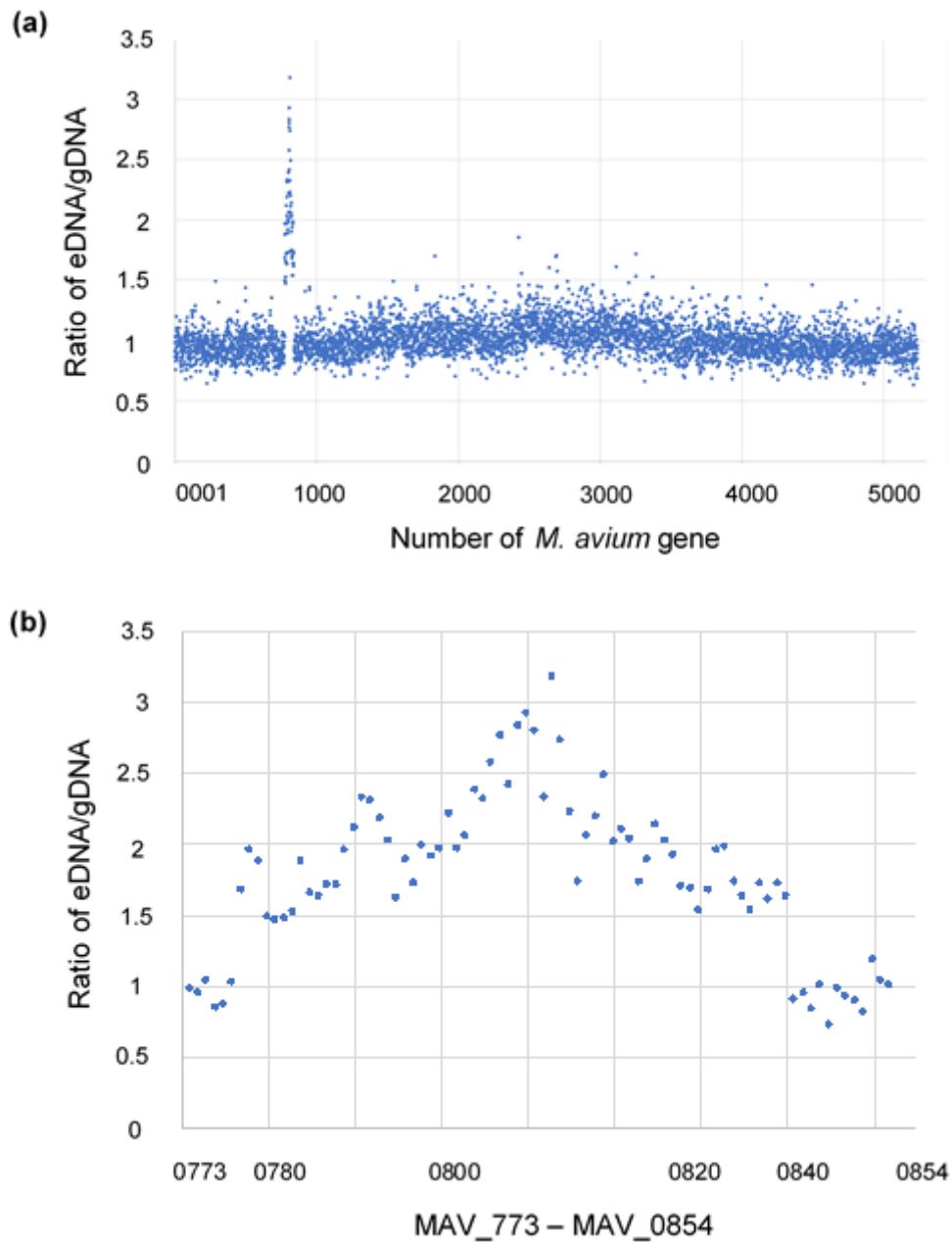
Figure 8

Ratio of gDNA and eDNA of each gene in M. avium 104. (a), The abundance ratio of gDNA to eDNA in M. avium was calculated for each gene. (b), an expansion of (a) from MAV_0773 to MAV_0854.

Supplementary Files

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