Hemolytic activity of mycobacterial TlyA (Rv1694) is independent of its rRNA methylation activity

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In the present study, we show that the mycobacterial TlyA (Rv1694) is a 'moonlighting' protein, while the intracellular form methlylates the 2′-O-ribose of ribosomal RNA (conferring susceptibility to the second line of the antibiotic capreomycin); the extracellular form is suspected to be a non-conventional membrane damaging entity. The purified Rv1694 can self-assemble into large oligomers upon contact with phagosomal membranes or red blood cells. Rv1694 has the well-conserved K^{69}–D^{154}–K^{182}–E^{238} residues that are essential for site-specific methyltransferase activity. Using the *Escherichia coli* model system, we show that mutation of any of these residues can result in resistance to capreomycin, while the mutated TlyA can reach the cell wall of the bacterium. The *E. coli*/TlyA system described here would serve as a simple and reliable model for studying the mechanism of export of TlyA and its homologues, their topology on the cell wall and the nature of contacts it must establish with target phagosomal membranes for their susceptibility.

Keywords: Capreomycin, *Escherichia coli*, hemolytic activity, methylation, mycobacterium.

MYCOBACTERIUM tuberculosis (*Mtb*) is a pathogenic bacillus which causes tuberculosis in humans. It primarily infects the lungs through inhalation of aerosolized bacateria, where it is phagocytosed into alveolar macrophages of the lung. Upon entry into the macrophages, it prevents the maturation of phagolysomes to localize itself within the immature phagosome for its survival and growth. There is enough experimental evidence in the literature regarding the compromise of integrity of mycobacterial phagosomes[1].

Initial studies have suggested that the mycobacterial phagosomal lumen contents can be accessed by the cytoplasmic antigen processing and presentation machinery of major histocompatibility complex (MHC-I) pathway. These observations have indirectly suggested that 'membrane permeability' could play a role during this process. This notion led to the postulation of 'existence of pores or loss of membrane integrity' on the mycobacterial phagosome[2–6]. However, it is interesting to note that inhibition of phagosomal maturation does not appear to compromise the antigen presentation by MHC class I molecule[7]. The phagosomal membranes of BCG-infected cells were found to be permeable to molecules up to 70 kDa (ref. 3). All these observations suggest an obvious possibility, which is an interaction of molecule(s) present on the cell surface (or secreted forms) of the mycobacterium and the phagosomal membrane. Hence, we postulate that this permeability might be due to a cell surface protein of the mycobacterium that may play an immediate role soon after its entry. Among the priority of events the mycobacterium must organize after entry, the need for membrane destabilization could certainly be one of them and there exists a role for a 'pore-forming protein'. Surprisingly, the TlyA of mycobacterium, Rv1694, aligns well with both rRNA methyltransferases and hemolysins. Hence, the controversy: whether TlyA is a hemolysin or rRNA methyltransferase, or it contains both activities.

Multiple sequence alignment of RrmJ class of methyltransferases has revealed that only four residues, corresponding to the K^{38}–D^{124}–K^{164}–E^{199} tetrad in *Escherichia coli* RrmJ, are invariant and common to several site-specific methyltransferases that modify 2′-hydroxyl groups of ribose moieties of ribosomal RNA. Further studies showed that residues K38, D124 and K164 play critical roles in the methyl-transfer reaction of RrmJ. However, E199 of RrmJ showed a reduced specific activity *in vitro*. Current bioinformatic classification has annotated the Rv1694 as a rRNA methyltransferase, since it has the well-conserved K^{69}–D^{154}–K^{182}–E^{238} tetrad like the RrmJ of *E. coli*. We have recently shown that Rv1694 might be a dually active protein, i.e. it is a ribosomal RNA methylase and can also function as a non-conventional membrane-damaging entity[8].

In view of the suspected dual functionality of TlyA, we studied whether or not the amino acid residues K^{69}–D^{154}–K^{182}–E^{238}, important for its catalytic activity, are required for cell-wall localization upon expression in *E. coli*. It should be noted that *E. coli* does not have a natural homologue of TlyA and hence can serve as an easy experimental model. Moreover, the *E. coli* model can also help us understand the host factors responsible for the observed contact-dependent hemolysis since the cell wall of *E. coli* differs from that of mycobacterium. It is also important to understand whether or not the mutated forms can exhibit the contact-dependent hemolysis as well as contribute to drug resistance in the *E. coli* model. Our studies reveal that mutation of D^{154}–K^{182}–E^{238} amino acids results in resistance to capreomycin, but cannot prevent their cell-wall localization and contact-dependent hemolytic activity in the *E. coli* model.

TlyA-pET28a+ construct or its mock vector (pET28a+) transformed *E. coli* strain BL21(DE3) CodonPlus-RIPL were grown in Luria-Bertani (LB) medium or solid LB-agar medium with 30 μg/ml kanamycin(Kan) and chloramphenicol (34 μg/ml), grown at 28°C and 37°C respectively.

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Polyclonal antibody against purified TlyA was raised in NZW rabbit with the approval of Institutional Animal Ethical Committee of National Centre for Cell Science. *E. coli* expressing TlyA or its mutants were grown in LB medium with appropriate antibiotics, harvested at mid log phage, washed thrice and resuspended in PBS (pH 7.4). The bacteria were further incubated with 1 : 100 dilution of TlyA immune rabbit serum for 30 min at room temperature, washed thrice with PBS and labelled with 1 : 100 dilution of Cy3 conjugated goat-anti-rabbit IgG antibody for 45 min followed by staining with DAPI for 2–3 min. The mixture was washed thrice with PBS and a thin smear was made on the glass slides in the presence of antifade for microscopic visualization using 100× objective.

The wild-type (WT) TlyA, cloned in pET28, was used as template for mutagenesis[8]. We have mutated the Asp^{154}, Lys^{182} and Glu^{238} amino acids to alanine using the Quik Change™ site-directed mutagenesis kit supplied by Stratagene to obtain the D154A, K182A and E238A mutants of TlyA. All these mutants have a 6-histidine amino acid tag at their C-terminal which was contributed by the pET28a+ expression vector. These mutants were transformed in BL21(DE3) CodonPlus-RIPL strain and are referred to here as D154A, K182A and E238A.

Contact-dependent lysis of rabbit erythrocytes was carried out by employing a slight modification of earlier published procedures[8,9]. Briefly, bacterial culture grown at 28°C was harvested 3 h after induction with 1 mM IPTG. The bacterial pellet was resuspended and washed thrice in 25 mM sodium phosphate buffer, pH 7.4 and 150 mM NaCl (buffer B). *E. coli* ($\sim 10^7$) cells and rabbit erythrocytes ($\sim 10^5$) were mixed in 1 ml of the same buffer and pulse centrifuged to ensure close contact between bacteria and red blood cells for incubation at 37°C for 30 h. At the end of the incubation period, haemoglobin present in the supernatant was measured at 540 nm.

A 10 ml start-up culture of D154A, K182A, E238A, TlyA (also referred to as WT) and pET control vector was grown till the A_{600} reached about 0.6–0.8. This culture was inoculated with equal number of cells into 25 ml LB medium containing kanamycin (30 µg/ml) with or without 100 µg/ml capreomycin (capreomycin sulphate, Sigma Aldrich, USA). The culture was incubated at 28°C with vigorous shaking and A_{600} was measured at regular intervals of time.

Coupled *in vitro* transcription–translation was carried out with the S30 extracts of *E. coli* expressing TlyA and D154A. The S30 extract was prepared by growing recombinant *E. coli* in LB medium in the presence of appropriate antibiotics at 28°C, induced with 40 µM IPTG at A_{600} of ~0.35 and harvested after 3 h. Bacteria were subjected to ultrasonic disruption (40% amplitude, ten times, 10 sec) in buffer C (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol), and centrifuged for 30 min at 30,000 *g* to obtain the supernatant. *In vitro* translation activity was measured in the absence and presence of 80 ng/ml capreomycin. We have used α-hemolysin from *Staphylococcus aureus* as a reporter gene for the quantitative translation. Lysis of rabbit RBC with *in vitro* generated α-hemolysin is the direct measure of the translation efficiency, which was monitored at A_{595} nm.

We have designed and characterized the individual point mutants of TlyA, viz. D154A, K182A and E238A in order to understand its diverse functions, i.e. rRNA methylation versus haemolytic phenotype, since they constitute its catalytic site. It is relevant to note that *in silco* analysis by Feder *et al.*[10] categorized Rv1694 as an rRNA methyltransferase, since it has the well-conserved K^{69}–D^{154}–K^{182}–E^{238} tetrad and possesses the specific methylation 2′-hydroxyl groups of ribose in the ribosomal RNA. All the mutants of TlyA described here were confirmed by DNA sequencing and expressed in BL21(DE3)-RIPL strain. All mutants have exhibited good expression in *E. coli* as shown in Figure 1 *a* and can be purified to homogeneity with the help of the carboxy terminal 6-histidine tag using Ni-nitrilotriacetic acid matrix.

The contact-dependent haemolytic activity of WT and the catalytically inactive mutants were assessed as described earlier[8,9]. All these mutants of TlyA, upon close contact with rabbit red blood cells, have shown significant hemolytic activity without and with preimmune rabbit serum after ~30 h of incubation at 37°C (Figure 1 *b*). The purpose of the immune serum is to block the contacts needed for hemolysis and as expected, the specific immune serum has been able to neutralize the hemolytic

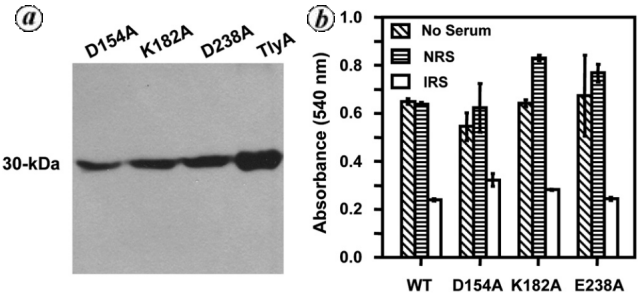

Figure 1. ***a***, Normal expression of TlyA and its mutants in *Escherichia coli*. The wild-type TlyA and its mutants, viz. D154A, K182A and E238A are expressed *E. coli* BL21(DE3)-RIPL strain as described in the text. An aliquot of recombinant *E. coli* were lysed and boiled in 1X SDS-PAGE sample buffer, electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose and detected with HRP conjugated anti-6xHis antibody. ***b***, TlyA muants exhibit normal contact-dependent hemolysis. Contact-dependent hemolytic activity of *E. coli* expressing the wild-type TlyA (WT) and D154A, K182A and E238A mutants was measured by incubating the respective bacteria with rabbit RBC at 37°C for 24 h in the presence of normal rabbit serum (NRS) or immune rabbit serum (IRS) or no serum (NS). RBC lysis was monitored by measuring the absorbance at 540 nm of a cell-free supernatant. Error bars represent the standard deviation of three independent measurements.

activity. There was no significant difference in hemolysis between wild type and mutants studied here. Interestingly, all the mutants have been able to reach the cell wall of *E. coli*, which can be visualized by staining with Cy3 conjugated secondary antibody (Figure 2). It should be noted that neither the immune rabbit serum or normal rabbit serum or the Cy3 conjugated secondary antibody alone gives any staining of the *E. coli* cells. Hence, the cell-wall staining seen is specific for TlyA expressing *E. coli* only.

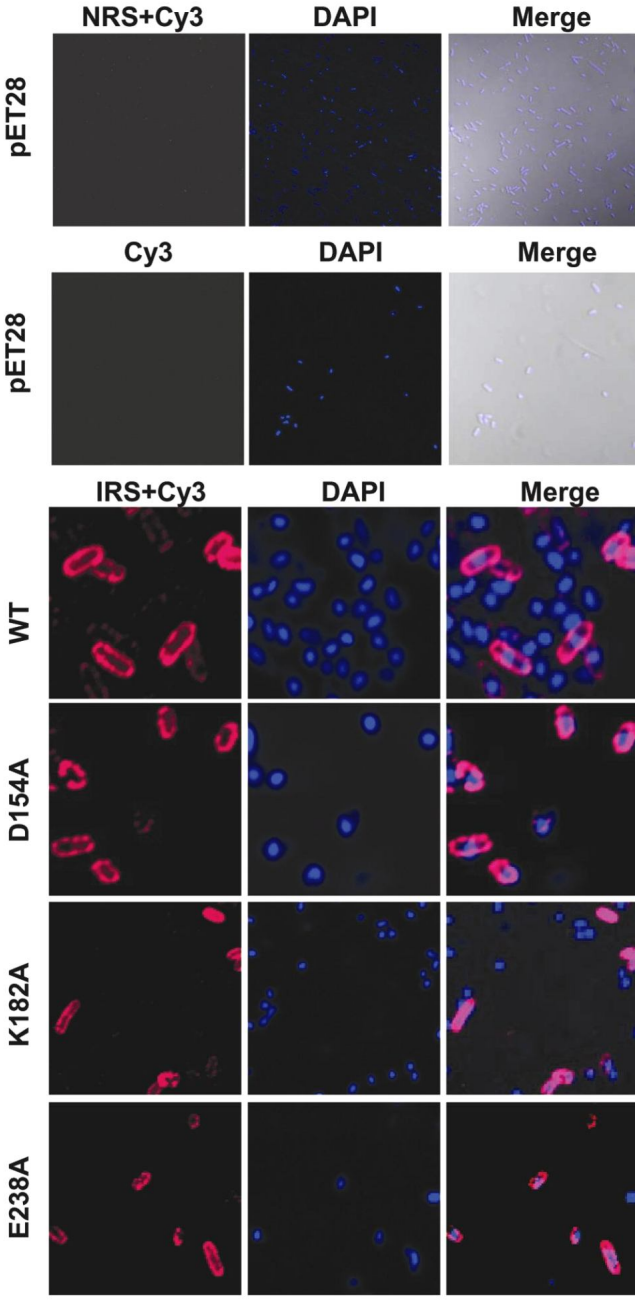

Figure 2. TlyA is localized to cell surface in *E. coli*: Live *E. coli* expressing the indicated mutant or WT was incubated with immune rabbit serum followed by goat-anti-rabbit-Cy3 staining (red fluorescent) and DAPI staining (blue fluorescence). The bacteria were neither fixed nor permeablized during this procedure to avoid intracellular staining, if any. The uppermost panel represents the control antibody staining (normal rabbit serum). These labelled bacteria were observed under confocal microscope with 100× objective. Result shown is one representative image of three independent experiments.

As the TlyA aligns well with the RrmJ/FtsJ, a 2-O-ribose 23S rRNA methyltransferase (MTases), we sought to study the susceptibility of *E. coli* expressing the mutants of TlyA through *in vitro* culture. We have examined the methylation activity of TlyA mutants. Capreomycin binds to methylated ribosomes and inhibits their translational ability. However, the drug is not effective when the ribosomes are not methylated. For example, normal *E. coli* is not sensitive because it has no natural homologue of *Mtb* TlyA. If a functional form of TlyA is expressed in the bacteria, it is expected to methylate the 16S and 23S rRNA which results in susceptibility to capreomycin. In other words, if TlyA is expressed in *E. coli*, it acquires a new property, i.e. susceptibility to capreomycin[8,11].

Figure 3 *a* shows that the WT expressing *E. coli* has exhibited a retarded or no growth in the presence of 100 µg/ml capreomycin in comparison with *E. coli*/pET28a. The growth rates of *E. coli*/D154A and *E. coli*/pET28a were the same in the absence of capreomycin, as shown in Figure 3 *a* (growth rate of *E. coli*/K182 and *E. coli*/E238 were also the same; data not shown here). Further, in the presence of capreomycin, the growth rate of *E. coli* expressing D154, K182 and E238 mutants was the same as that of the vector control (although the growth rate was slower in the presence of capreomycin compared to its absence), whereas *E. coli*/TlyA exhibited retarded growth in comparison to all the mutants and vector control. All these observations indicate that point mutations at D154, K182 or E238 of TlyA abolish their ribosomal RNA methylation activity conferring resistance to capreomycin.

In view of the above observation, we next examined the translational ability of the ribosomes of *E. coli* expressing the D154A mutant as an example. Capreomycin inhibits translation activity by binding to 16S and 23S ribosomal RNA only when methylated at nucleotide positions C1409 and C1920 respectively. Hence, ribosomal RNA isolated from the bacteria expressing the WT or mutated TlyA should accordingly exhibit subtle differences in their ability to translate a given target gene in the presence of capreomycin. We have made S30 extract of WT and D154A expressing *E. coli* and carried out *in vitro* transcription and translation of α-hemolysin of *Staphylococcus aureus* as a reporter gene, as shown in Figure 3 *b*. This method is simple, reliable and can be judged even without a spectrophotometer[12]. In addition, the kinetics of RBC hemolysis of α-hemolysin is well studied and fast in comparison to TlyA. *In vitro* translation reaction was done in the presence and absence of 80 ng/ml capreomycin. Total hemolysis is proportional to the total amount of α-hemolysin translated during the reaction. The lytic

activity of the S30 extracts of WT and D154A was same in the absence of capreomycin, whereas 80 ng/ml capreomycin had abolished the translational activity of the WT S30 extract, while the D154A has exhibited nearly the same activity.

Recently, we have provided several evidences that the *Mtb tlyA* gene product, Rv1694, being a ribosomal RNA methylase, is also a non-conventional hemolysin-like molecule. It has the ability to assemble into ordered oligomers, capable of permeabilizing the membrane that comes into contact with it based on the evidence that it can form oligomers on isolated phagosomes of RAW264.7 macrophages[8].

The Rv1694 has been shown to specifically methylate the C1409 and C1920 of 16S and 23S ribosomal RNA respectively by an earlier study. This methylation results in sensitivity towards the capreomycin[11]. Hence, mutation of amino acids important for RNA methylation activity will result in resistance to capreomycin. All the point mutants of TlyA, viz. D154A, K182A and E238A have exhibited normal growth in the presence of capreomycin compared to the wild-type *E. coli*/TlyA, which did not grow in the presence of capreomycin, suggesting the catalytic role for D154, K182 and E238 (Figure 3). Similarly, the S30 extracts of bacteria expressing the mutated constructs have exhibited normal translational efficiency in the presence of capreomycin (Figure 3 *b*). Interestingly, all the mutants have also reached the cell wall of *E. coli* and also exhibited contact-dependent hemolysis as shown in Figure 2. These observations prove that the point mutants, D154A, K182A and E238A have near-normal membrane damaging activity, similar to the wild-type protein. Hence, it is of interest to know whether *Mtb* can mutate this protein, export it to the cell wall to impair the phagosomal maturation process, while acquiring capreomycin resistance.

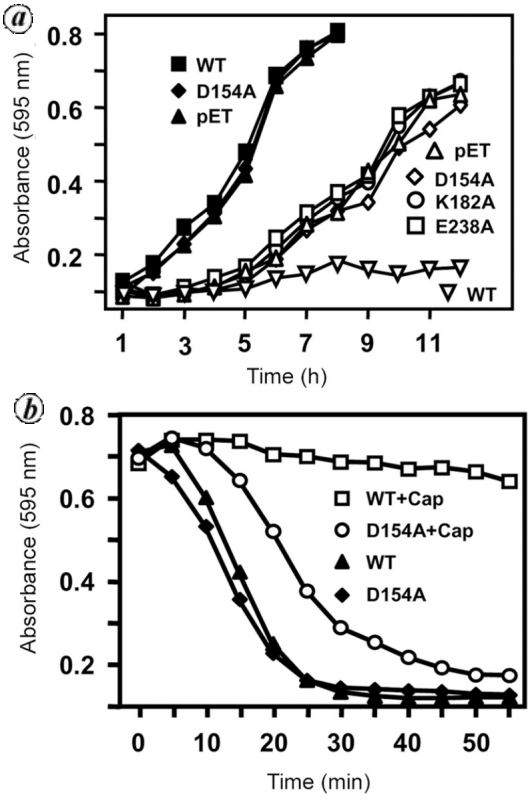

Figure 3. ***a***, D–K–E mutants of TlyA are resistant to capreomycin. Growth curves of *E. coli* expressing mock transformed or transformed with wild type, D154A, K182A and E238A constructs were examined for growth in the presence of capreomycin (100 µg/ml) as described in the text. Open and filled symbols respectively, represent growth of bacteria in the presence and absence of capreomycin in the growth medium. Only D154A mutant was selected for the effect of capreomycin and other mutants show similar growth pattern. Data shown here are representative of one of the two independent experiments. ***b***, S30 extract of D154A expressing *E. coli* has poor translational activity in the presence of capreomycin. S30 extracts of wild-type TlyA (WT) and D154A were supplied with a supercoiled plasmid of staphylococcal α-hemolysin, and *in vitro* transcription and translation was carried out in the absence or presence of 80 ng/ml capreomycin. Various lines represent the translational activities of the ribosomal mix in the absence of capreomycin for WT (◆) and D154A (▲) and in the presence of 80 ng/ml capreomycin for WT (□) and D154A (○). Data shown here are representative of one of the two independent experiments.

Based on the accumulated knowledge in the literature, it is pertinent to ask the question: at which stage can TlyA play a role for the bacterium? Numerous microarray experiments as well as gene essentiality studies have not found any instance of 'upregulation' of the *tlyA* gene[13]. However, this is not surprising because both activities of TlyA (rRNA methylation and membrane damaging) do not favour upregulation or higher degree of expression for two reasons. The first being, if the TlyA expression is upregulated as a rRNA methyltransferase, the bacterium will be susceptible to second-line therapy for the treatment of XDR version of the disease (beneficial to the patient). There are three options that can aid the XDR phenotype: (a) base-change mutations in the rRNA at C1409 and C1920 which cannot be methylated and hence second-line antibiotics cannot bind; (b) mutations in the TlyA protein that abolish the rRNA methylation activity and (c) both options 'a' and 'b' together. However, among 400 clinical isolates studied so far, majority of isolates have mutations in the rRNA, except three instances where mutations of TlyA have been observed[14]. At present, it is debatable whether this reflects a non-prominent role for TlyA as a rRNA methylase for the bacterium.

The second reason being, if the TlyA is to function as a hemolysin, higher degree of expression results in higher degree of the membrane damaging phenotype, which could be detrimental to the survival of *Mtb* either before phagocytosis (cell adhesion and rapid plasma membrane damage due to higher expression of TlyA) or after phagocytosis (damage of the phagosomal compartment that allows the passage of the bacterium into cytosol, which may expose it to the MHC-1 pathway). Both these reasons together suggest that controlling the expression of TlyA is of prime importance to the bacterium. This logic

is also valid as most pathogenic bacteria control the expression of their virulence factors rather tightly.

The data presented here reveal a novel aspect. The TlyA that is devoid of rRNA methylation activity (contributed to evolution of second-line antibiotic resistance) can still reach the cell wall of the bacterium and aid the survival strategies. This indirectly suggests that the cell-wall association is not dependent on the rRNA methylation activity. Based on these observations, we have a valid speculation that TlyA could be a virulence factor for the bacterium. For example, infection of THP-1 macrophages with of *Mycobacterium avium* subsp. *paratuberculosis* has resulted in 5-fold upregulation of its *tlyA* gene (protein ID: MAP_1401), which has over 80% identity with the *Mtb* protein studied here. We have predicted the possibility of TlyA acting as a virulence factor post-infection. *Mycobacterium avium* subsp. *paratuberculosis* is a gut-associated pathogen of humans and linked to Crohn's disease. This observation also suggests that regulation of gene expression of mycobacterial species may be linked to their environment. Future studies that focus on the cellular location and quantitation of the membrane proteins under a variety of environments might reveal new clues to the establishment of tuberculosis disease. We also believe that the *E. coli* model employed by us may not be perfect, but is reasonable enough to understand the function of TlyA *per se*, and also such proteins of other bacteria in the absence of the host bacterium's milieu.

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Quantification of *Sugarcane yellow leaf virus* in *in vitro* plantlets and asymptomatic plants of sugarcane by RT-qPCR

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Yellow leaf caused by *Sugarcane yellow leaf virus* (SCYLV) is a serious viral disease affecting production and productivity in many ruling sugarcane varieties in India. Usually the characteristic disease symptoms appear during maturity phases of the crop; also many of the infected varieties do not exhibit disease symptoms and disease expression is influenced by virus titre and other factors, including the prevailing climate. The present study was taken up to quantify and

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