nature portfolio

Peer Review File

An anti-virulence drug targeting the evolvability protein Mfd protects against infections with antimicrobial resistant ESKAPE pathogens

Corresponding Author: Dr Nalini RAMA RAO

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have sufficiently addressed most of the reviewers' concerns. They completed in vivo experiments with multiple doses and included complementary data to reinforce NM102's direct interaction with Mfd. Specifically, they used ITC to demonstrate NM102's affinity for Mfd, provide evidence that NM102 specifically decreases the ATPase activity of Mfd (they used additional, functionally related ATPase proteins as controls in the revised manuscript), and mutated four residues in silico to support further that NM102 specific binds in the ATP binding pocket of Mfd. To further strengthen the manuscript, I would suggest that the authors mutate one of these residues use that variant as a control in their ITC experiment.

Reviewer #3

(Remarks to the Author)
The revised manuscript describes the effects of NM102 a compound that was identified by in silico tools and tested for its ATPase inhibition activity. In vivo assays showed that NM102 reduces bacterial load in the lungs of mice inoculated with Klebsiella and Pseudomonas. Also, the decrease in bacterial load does not occur in the mfd mutant, suggesting that the NM102 is specific to Mfd. These experiments could have included the complemented strain. The complemented strain was not included in these experiments because of previous experiments in B. cereus and gene synteny. The paper could include the genetic arrangement of the mfd gene in those pathogens.
NM102 was shown to on inhibit the ATPase activity of MfdC, and hydrolyzing ATP affects all the enzymatic steps of Mfd. So, the authors did not characterize DNA loading, translocase, and repair activities of Mfd. Instead, they focused on the in vivo effects of Mfd in the context of host-microbe interactions and the acquisition of antibiotic resistant mutations. The mice experiments measured colonization of the lungs after 24 hours and showed a significant decrease in bacterial load. Expanding these experiments to assess survival or disease development would have strengthened the potential of NM102 as a therapeutic compound.
NM84 showed a similar level of inhibition as NM102. However, the authors do not describe why NM84 was not pursued.
NM102 was tested against eukaryotic ATPases involved in DNA repair. However, these experiments did not include bacterial RecG, a conserved ATPase involved in DNA repair and recombination, particularly when cells are under stress. Could NM102 inhibition of Mfd, a factor proposed to modulate gene expression in conditions of stress, affect RecG expression and activity? Answering such question would strengthen the case of the NM102 specificity for Mfd.
The combined use of NM102 and meropenem were tested in the wt. What is the effect of Meropenem on growth or bacterial load on the Mfd mutant compared to the Wt. The synergy of the double treatment suggest that Mfd influences peptidoglycan synthesis, perhaps by affecting gene expression. conducted in the wt. Maybe, Mfd-deficient cells are more sensitive to meropenem than the parent. This observation would support the concept that Mfd is important for antibiotic tolerance – the case in Helicobacter pylori (Lee et al). These experiments would make a more compelling case for the manuscript.
In C. difficile, Mfd deficient cells overproduce toxins (virulence factor). So, inhibition of Mfd may not be effective in the treatment of some pathogens. Perhaps, this should be included in the discussion. Also, some discussion on the likelihood of developing resistance to NM102 needs to be included in the manuscript. Some discussion or speculation comparing the two

(the one presented here and the one form the Merrikh lab) inhibitors of Mfd is granted. Are both targeting ATPase activity?
Other points
Line 74. Martin et al 2020 did not study teixobactin. Please check all references
Line 88 Lee et al showed that H. Pylori Mfd-deficient cells are sensitized (lower MIC than the parent) to antibiotics. The authors indicate that Mfd increases mutagenesis conferring antibiotic resistance.
Line 444 antibioresistance
Line 459 and that NM102 treatment can kill bacteria in contexts where other therapies fail due to
resistant strains. This sentence is not clear.
Line 603 Lea Coursen to Lea Coulson

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have thoroughly addressed the reviewer's concerns and provided well-reasoned responses. Notably, they demonstrate that NM102 strongly inhibits ATP binding in the ATP pocket of Mfd, further strengthening their manuscript.

Other comments:
- Explain why NM102 was the 'best inhibitory molecule' (Line 139). How many other compounds showed an inhibition rate above 80%, and why did you select NM102 over another?
- Line 188: Missing 'were selected/chosen'
- Line 218: Italicize P. aeruginosa
- When discussing in vivo work, report the percent reduction in bacterial load to stay consistent.
- Line 300: mg/kg
- Line 344: Provide a brief explanation of why you selected rifampicin (i.e., high frequency of resistance in vitro)
- Provide a citation for lines 503-504.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns experimentally and editorially.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author)

- The authors have sufficiently addressed most of the reviewers' concerns. They completed in vivo experiments with multiple doses and included complementary data to reinforce NM102's direct interaction with Mfd. Specifically, they used ITC to demonstrate NM102's affinity for Mfd, provide evidence that NM102 specifically decreases the ATPase activity of Mfd (they used additional, functionally related ATPase proteins as controls in the revised manuscript), and mutated four residues in silico to support further that NM102 specific binds in the ATP binding pocket of Mfd. To further strengthen the manuscript, I would suggest that the authors mutate one of these residues use that variant as a control in their ITC experiment.

We warmly thank the reviewers for the comments that allowed to considerably improve the manuscript. We deeply thank for acknowledging that we have sufficiently addressed most of the reviewers' concerns.

In order to further support the specific binding of NM102 in the ATP pocket of Mfd, we performed additional experiments, as suggested by the reviewer. To establish a complete correlation between *in silico* with *in vitro* affinities, we performed competitive assay, using ITC, and calculated the dissociation constant of ATP binding in the presence or absence of NM102. Rather than using a mutant which would lack both ATP and NM102 binding and thus be less quantitative, we performed quantitative assays that allowed to measure the competitive binding of NM102 at the ATP site. We could show that: (i) ATP binds to Mfd with a lower affinity than NM102, consistently with *in silico* analysis, and (ii) ATP binding is strongly inhibited in the presence of NM102, demonstrating the competitive binding of NM102 at the ATPase active site of Mfd. (Fig 1F, page 5). These data clearly demonstrate the direct binding of NM102 specifically in the ATP-pocket of Mfd, thus inhibiting ATP binding. This strongly correlates with the *in silico* analysis showing NM102 binding at the ATP binding site (Fig. 2C, 2D, Supp Table 1).

These new data have been added in the revised version of the manuscript (Revised manuscript, page 5).

"NM102 showed affinity towards Mfd, and the isotherm for the titration of the ligand against the molecule indicated a significant exothermic behavior. The obtained stoichiometry value (n=1.1) indicates a 1:1 binding interaction between the Mfd protein and NM102 with a Kd of 83 +/- 9 µM. To assess the specific and competitive binding of NM102 in the ATP pocket of Mfd, we measured the interaction of ATP with Mfd in the absence and in the presence of NM102 (Fig. 1F). In the absence of NM102, ATP binds to Mfd with a Kd of 145 +/- 9 µM. By contrast, in the presence of NM102, ATP binds to Mfd with a Kd of 430 +/-50 µM. Thus, NM102 binds to Mfd with a better affinity than ATP and successfully inhibits Mfd-ATP interaction which decreased by ca 70% (A = Ka/Kapp = 0.34). These data demonstrate that NM102 binds specifically to the ATP binding site of Mfd and inhibits Mfd-ATP binding in a competitive manner."

Substrate	Inhibitor	K_d (µM)	K_a (M^{-1})
ATP	-	145 ± 9	6896 ± 600
ATP	**NM102**	430 ± 50	2325 ± 300

$$A = \frac{K_{app}}{K_a} = 0.34$$

Fig 1F ITC thermodynamic parameters associated to the interaction of Mfd with ATP in absence or presence of NM102. Kd, dissociation constant Kd, Ka, association constant, A, ratio of Ka in the presence vs absence of inhibitor NM102.

As a whole, we provide evidence for Mfd inhibition by NM102, which induces antimicrobial activity and anti-evolvability efficiency. We demonstrate the binding of NM102 in the ATPase pocket and a competitive inhibition of Mfd activity. We also show that NM102 is efficient in reducing the bacterial load of bacteria that shows resistance to currently used antibiotics. As such, NM102 is active on its own to prevent bacterial infections, but more importantly, it is also predicted to synergize the efficacy of existing antibiotics and also to prevent emergence of resistance. These are unique and essential characteristics in the field of drug development and antibiotherapy.

We believe that these findings will be of interest to the broad readership of *Nature Communications* and we hope that the revised version of the manuscript is now suitable for publication.

Reviewer #3
(Remarks to the Author)
- The revised manuscript describes the effects of NM102 a compound that was identified by in silico tools and tested for its ATPase inhibition activity. In vivo assays showed that NM102 reduces bacterial load in the lungs of mice inoculated with Klebsiella and Pseudomonas. Also, the decrease in bacterial load does not occur in the mfd mutant, suggesting that the NM102 is specific to Mfd. These experiments could have included the complemented strain. The complemented strain was not included in these experiments because of previous experiments in B. cereus and gene synteny. The paper could include the genetic arrangement of the mfd gene in those pathogens.

We warmly thank the reviewer for the suggestion. To address it, in the previous revision we had complemented Mfd in *E. coli* and tested the strains during evolvability assays, during which we could show complementation (Fig. 7).

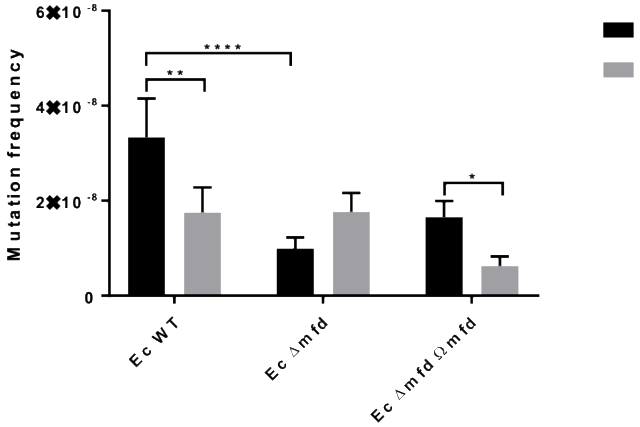
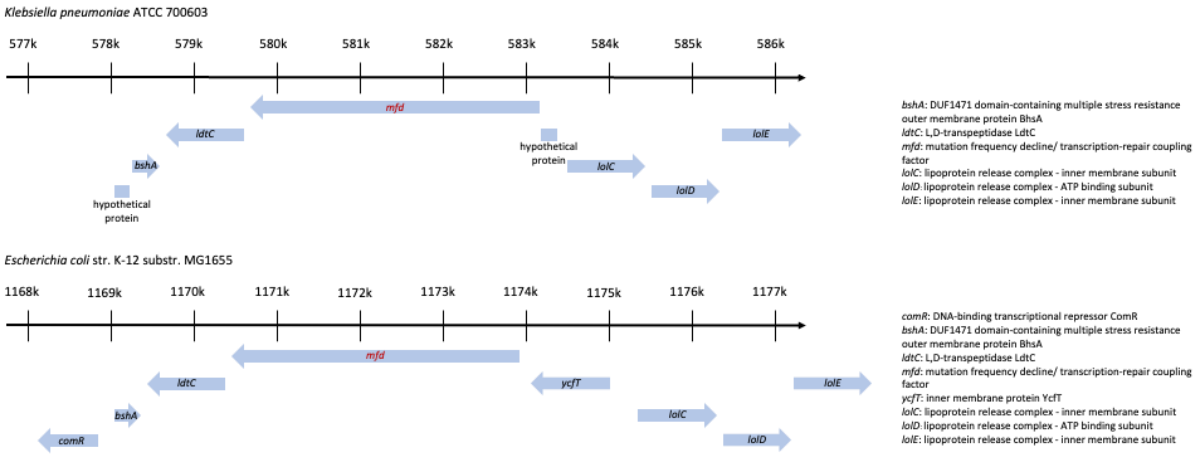


Fig 7. NM102 inhibits Mfd evolvability activity. (A) The mutation rate of *E. coli* wt, Δ*mfd* and Δ*mfd/mfd* strains were measured, following exposure to NM102 (100 µM) or the DMSO control, using the frequency of spontaneous accumulation of resistant mutants to rifampicin as reference measurement.

Herein, to further address the reviewer's comment, we compared the genetic arrangement of the *mfd* genes between *E. coli* and *K. pneumoniae*, and concluded they are highly conserved. This has been added as a supplemental Fig (Supp Fig 9).



Supp Fig.9. Genetic environment of the *mfd* gene in *E. coli* and *K. pneumoniae*. The sequences of the mfd genes and their respective environment were retrieved from ncbi using the following references: *Escherichia coli* str. K-12 substr. MG1655 (GCF_000005845.2) and *Klebsiella quasipneumoniae* strain ATCC 700603 (CP014696.2)

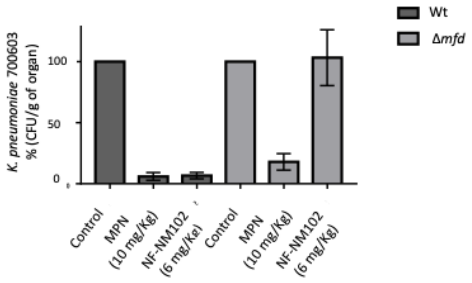
- NM102 was shown to inhibit the ATPase activity of MfdC, and hydrolyzing ATP affects all the enzymatic steps of Mfd. So, the authors did not characterize DNA loading, translocase, and repair activities of Mfd. Instead, they focused on the in vivo effects of Mfd in the context of host-microbe interactions and the acquisition of antibiotic resistant mutations. The mice experiments measured colonization of the lungs, after 24 hours, and showed a significant decrease in bacterial load. Expanding these experiments to assess survival or disease development would have strengthened the potential of NM102 as a therapeutic compound.

We thank the reviewer for this suggestion. However, the survival of the mice was not assessed for several reasons. First of all, we show that NM102 is capable of drastically decreasing the bacterial load in the lung, following *K. pneumoniae* infection. To better document this efficacy, and to carefully address the reviewer's comment, we added a supplementary figure (Supp Fig. 6) indicating the % of recovered cfu after treatment compared to untreated control mice. We show that NM102 decreases by 93% the bacterial load, following *K. pneumoniae* infection. This effect is comparable to the effect of MPN, which decreased by 94% the bacterial load. These data confirmed the antimicrobial activity of NM102 with an efficacy comparable to MPN, used under the same conditions.
We believe that the decrease in bacterial load (log cfu/organ) is one of the standard assessments of efficacy, especially for new drug compound. Indeed, it allows to show the direct effect of the drug on the bacterial survival *in vivo*.

Furthermore, for ethical reasons, survival/disease development assays are now generally reserved for testing compounds at the preclinical level. Indeed, the severity of the protocol to assess survival or disease development is drastically higher and not always granted by ethical committees. It implies a higher dose of bacteria, leading to the death of the host. Although informative, it does not reflect the commonly use of antibiotics, that are usually given at time points of infection to prevent the bacteria to completely invade the host, leading to its death.

We believe that the observed decrease of over 90% of the bacterial load, following treatment with NM102, demonstrates the high potential of NM102 as a promising and innovative therapeutic molecule. These new data have been added in the revised version (Revised manuscript, pages 9, and Supp Fig. 6)

"*NM102 showed similar efficacy to MPN in reducing the K. pneumoniae CFU in the lung of the infected mice at 6 and 10 mg/kg, respectively (Fig 4B), resulting in 94% and 93% reduction of the bacterial load, respectively (Supp Fig. 6)*".



Supp Fig.6. Antimicrobial effect of NM102 *in vivo*. *K. pneumoniae* ATCC 700603 wt and Δ*mfd* were administered intranasally through slow instillation of 20 µL of bacterial suspension (1x 10^7 CFU). 200 µL of NM102 formulation (NF-NM102 6 mg/kg) or empty formulation (control) or MPN (10 mg/kg) were administrated in mice, *via* the i.p. route. Mice were sacrificed by cervical dislocation after 24 h and the CFU in the lung was calculated per gram of organ. For each strain (wt and Δmfd), the cfu values were normalized to the controls.

- NM84 showed a similar level of inhibition as NM102. However, the authors do not describe why NM84 was not pursued.

NM102 was the compound with the higher inhibition of Mfd ATPase activity during our *in vitro* test. We thus focused on NM102 to further assess its efficacy in NO assays and *in vivo*. NM84 has a completely different scaffold and will be tested in another study.

- NM102 was tested against eukaryotic ATPases involved in DNA repair. However, these experiments did not include bacterial RecG, a conserved ATPase involved in DNA repair and recombination, particularly when cells are under stress. Could NM102 inhibition of Mfd, a factor proposed to modulate gene expression in conditions of stress, affect RecG expression and activity? Answering such question would strengthen the case of the NM102 specificity for Mfd.

We thank the reviewer for this excellent suggestion. In agreement with the reviewer's comment, we performed this additional experiment. We challenged the effect of NM102 on the ATPase activity of the RecG protein (Fig2B). A slight activity of NM102 was observed, nevertheless significantly lower than in Mfd. This can be explained by the fact that the initial library screen that allowed to identify NM102 was performed on the data available at the time, that were Mfd sequence of *E. coli* morphed on RecG, with a special attention at the ATP-site to model it in an active conformation (Supp Fig 1). Thus, a residual activity of NM102 towards RecG is not surprising. Overall, we evidence that the activity was leftover, non competitive with ATP and significantly lower than towards Mfd, further emphasizing NM102 specificity for Mfd.

These new data have been added in the revised version. (Revised manuscript, page 6 and Fig. 2B)

"*In addition, the activity of NM102 was also tested towards the bacterial helicase RecG since the library screen that identified NM102 was performed on the Mfd sequence of E. coli morphed with respect to*

the active ADP binding site of RecG (Supp Fig 1). Consistently, a slight activity of NM102 was observed, nevertheless significantly lower than for Mfd, further emphasizing NM102 specificity for Mfd. (Fig. 2B).”

Fig 1B

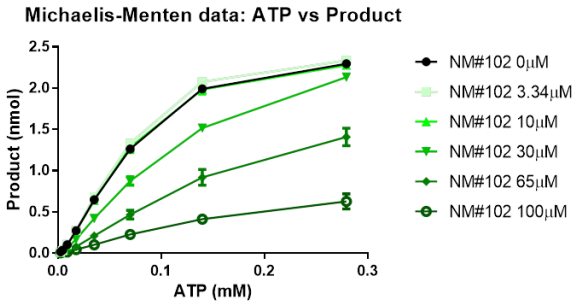
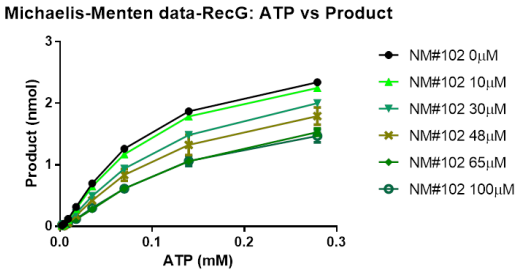


Fig 2B



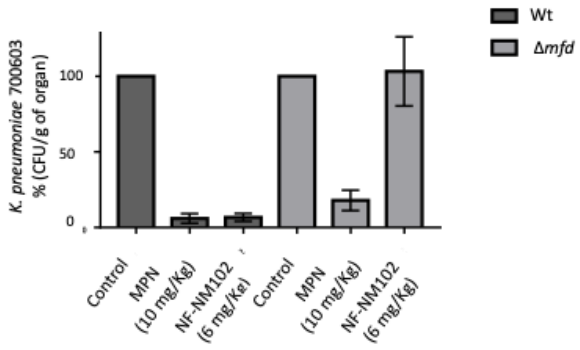
Michaelis Menten plot for inhibition activity of NM102 (0 to 100 µM) on *E. coli* Mfd (Fig 1B) and RecG (Fig 2B) (0.35 µM) ATPase activity with ATP (0 to 0.3 mM). The results are the average of three independent experiments done in duplicate with standard deviation.

- The combined use of NM102 and meropenem were tested in the wt. What is the effect of Meropenem on growth or bacterial load on the Mfd mutant compared to the Wt. The synergy of the double treatment suggests that Mfd influences peptidoglycan synthesis, perhaps by affecting gene expression. conducted in the wt. Maybe, Mfd-deficient cells are more sensitive to meropenem than the parent. This observation would support the concept that Mfd is important for antibiotic tolerance – the case in Helicobacter pylori (Lee et al). These experiments would make a more compelling case for the manuscript.

To answer the reviewer's comment, an additional experiment was performed and the impact of Meropenem (MPN) on the *mfd* mutant strain was assessed *in vivo* (Supp Fig 6). MPN decreased bacterial load of both wt and Δmfd strain with a comparable efficiency (by 90 %). It is striking to note that while NM102 and MPN both decreased by over 90% the bacterial load in the lung for the wt, NM102 did not impact the bacterial load of the *mfd* mutant further highlighting its specificity towards Mfd.

These new data have been added in the revised version (Revised manuscript, page 9, Supp Fig. 6).

“To confirm that NM102 targets Mfd in vivo, mice were also infected with the K. pneumoniae mutant Δmfd. As expected, the Δmfd mutant was less virulent than the wild type strain. MPN inhibited the bacterial burden of the Δmfd mutant (Sup Fig. 6). By sharp contrast, the NM102 nanoformulation showed no effect on the bacterial burden of the Δmfd mutant, further highlighting the inhibitor specificity toward the Mfd target in vivo (Fig 4B, Supp Fig. 6).”



Supp Fig.6. Antimicrobial effect of NM102 *in vivo. K. pneumoniae* ATCC 700603 wt and Δ*mfd* were administered intranasally through slow instillation of 20 µL of bacterial suspension (1x 10^7 CFU). 200 µL of NM102 formulation (NF-NM102 6 mg/kg) or empty formulation (control) or MPN (10 mg/kg) were administrated in mice via the i.p. route. Mice were sacrificed by cervical dislocation after 24 h and the CFU in the lung was calculated per gram of organ. For each strain (wt and Δmfd), the cfu values were normalized to the controls.

- In C. difficile, Mfd deficient cells overproduce toxins (virulence factor). So, inhibition of Mfd may not be effective in the treatment of some pathogens. Perhaps, this should be included in the discussion.

This information has been added in the discussion (Revised manuscript page 14)

"*Besides, in C. difficile, Mfd deficient cells overproduce toxins involved in virulence (Willing et al. 2015). Thus, inhibition of Mfd may not be effective in the treatment of some pathogens*."

- Also, some discussion on the likelihood of developing resistance to NM102 needs to be included in the manuscript.

We thank the reviewer for this suggestion, in agreement with whom, the following paragraph has been added in the discussion (Revised manuscript, page 16).

"*Besides, since Mfd is a non-essential protein, targeting it should decrease the resistance pressure by NM102. NM102 by itself does not kill bacteria and therefore, resistance is not observed in absence of any stress. In vivo, during inflammation, Mfd is essential and NM102 treatment hinders bacterial survival. This is likely to cause bacterial resistance eventually. Nonetheless, we can speculate that this resistance will be reduced in comparison to traditional antibiotics, which apply continuous pressure to both pathogenic and microbiota bacteria over the course of treatment. More research will be required on this topic."*

- Some discussion or speculation comparing the two (the one presented here and the one form the Merrikh lab) inhibitors of Mfd is granted. Are both targeting ATPase activity?

The mechanisms leading to Mfd inhibition is different between NM102 and ARM1. Indeed, in contrast with NM102, ARM1 did not bind to the ATP binding site of Mfd and had no impact on Mfd ATPase activity. To clarify this point, the following paragraph has been added (Revised manuscript, pages 14-15).

"*Interestingly, a small molecule, ARM1, has very recently been identified as an inhibitor of Mfd-dependent mutagenesis, further demonstrating the potential of fighting AMR by targeting this protein involved in the acceleration of evolution leading to increased AMR (Merrikh and Kohli, 2020; Carvajal-Garcia et al, 2024). The mechanisms leading to Mfd inhibition is different between NM102 and ARM1. Indeed, in contrast with NM102, ARM1 did not bind to the ATP binding site of Mfd and had no impact on Mfd ATPase activity (Carvajal-Garcia et al, 2024).*"

- Other points

Line 74. Martin et al 2020 did not study teixobactin. Please check all references

There might be a confusion with two references:
Martin JK et al 2020 developed a new compound named irresistin-16 as stated on line 74, page3
Martin, H.A et al 2019 studied Mfd of B. subtilis as stated on line 441, page 14

- Line 88 Lee et al showed that H. Pylori Mfd-deficient cells are sensitized (lower MIC than the parent) to antibiotics. The authors indicate that Mfd increases mutagenesis conferring antibiotic resistance.

The sentence has been modified to improve clarity (Revised manuscript, pages 3-4).

"*Mfd has been associated with the development of antibiotic resistance in Campylobacter jejuni (Han et al., 2008) and Helicobacter pylori Mfd-deficient cells are sensitized to antibiotics (Lee et al., 2009). Furthermore, Mfd has been suggested to be an evolvability factor that promotes hypermutation in bacteria, thereby accelerating the evolution of antimicrobial resistance (AMR) (Ragheb et al., 2019, Deaconescu, 2021, Browning and Merrikh, 2024, Merrikh, H., and Kohli, R. 2020)*."

- Line 444 antibioresistance

This has been changed to antibiotic resistance

- Line 459 and that NM102 treatment can kill bacteria in contexts where other therapies fail due to resistant strains. This sentence is not clear.

To improve clarity, the sentence has been changed to: "*and that NM102 treatment can kill bacteria that are resistant to current antibiotics*."

- Line 603 Lea Coursen to Lea Coulson

This has been corrected.

We warmly thank the reviewer for all this comments that considerably helped improving the manuscript. We believe that these findings will be of interest to the broad readership of *Nature Communications* and we hope that the revised version of the manuscript is now suitable for publication.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have thoroughly addressed the reviewer's concerns and provided well-reasoned responses. Notably, they demonstrate that NM102 strongly inhibits ATP binding in the ATP pocket of Mfd, further strengthening their manuscript.

Other comments:

- Explain why NM102 was the 'best inhibitory molecule' (Line 139). How many other compounds showed an inhibition rate above 80%, and why did you select NM102 over another?

NM102 was the only compound with an inhibition rate above 80%. It was the molecule with the highest inhibition rate and was selected for this reason.

The sentence line 134-136 was modified accordingly:

"The inhibition rates of Mfd activity ranged from 10% to 85%. The molecule with the highest inhibition rate (85%), named NM102, was selected for further analysis."

- Line 188: Missing 'were selected/chosen'

we modified the sentence and added "selected" (line 183)

- Line 218: Italicize P. aeruginosa

this is done (line 213)

- When discussing in vivo work, report the percent reduction in bacterial load to stay consistent.

This information has been added (lines 275 and 292)

- Line 300: mg/kg

This was corrected

- Line 344: Provide a brief explanation of why you selected rifampicin (i.e., high frequency of resistance in vitro)

This information was added on line 340 "was assessed following exposure to rifampicin that commonly induces high frequency of resistance *in vitro"*

- Provide a citation for lines 503-504.

The reference Ragheb et al 2019, is provided line 473.