

1 **Clay-induced DNA double-strand breaks underlay genetic diversity, antibiotic resistance and**  
2 **molecular basis of asbestosis**

3

4 Alexandro Rodríguez-Rojas<sup>1,2\*</sup>, Renate Radek<sup>1</sup>, Jerónimo Rodríguez-Beltran<sup>2</sup>, Jesús Blázquez<sup>2</sup> and  
5 Jens Rolff<sup>1</sup>

6

7 <sup>1</sup>Institut für Biologie, Freie Universität Berlin. Königin-Luise-Str. 1-3 14195 Berlin, Germany.

8 <sup>2</sup>Departamento de Microbiología Bacteriana, Centro Nacional de Biotecnología (CNB-CSIC),  
9 28049 Madrid, Spain.

10

11

12 \*Author to whom correspondence should be addressed: a.rojas@fu-berlin.de

13

14

15 **Abstract**

16

17 Some natural clays and synthetic nanofibres present in the environment have a severe impact on  
18 human health. After several decades of research, the molecular mechanism of how asbestos induce  
19 cancers is not well understood. Different fibres, including asbestos, can penetrate and transform  
20 both, bacterial and eukaryotic cells. We found that sepiolite and asbestos cause double-strand breaks  
21 in bacteria when the incubation occurs under friction forces. Since antibiotics and clays are used  
22 together in animal husbandry, we propose that this mutagenic effect constitutes a pathway to  
23 antibiotic resistance due to the friction provided by peristalsis of the gut from farm animals. We also  
24 discuss the possibility that the same mechanism could generate bacteria diversity in natural  
25 scenarios with a role in the evolution of species. Finally, we provide a new model on how asbestos  
26 promotes mutagenesis and cancer based on the genotoxicity that we observe in bacteria.

27

28

29 **Keywords:** sepiolite, mutagenesis, antibiotic resistance, double-strand break, microbiota evolution,  
30 genetic diversity, asbestos, asbestosis, carcinogenesis

31

32

33

34

1

## 35 **Introduction**

36 A significant concern arises from fibrous clays or industrial nanofibres which are responsible for  
37 severe human diseases such as asbestosis [1]. These materials are considered genotoxic and  
38 carcinogenic, likely due to their ability to damage DNA [2]. They have assayed in several  
39 experimental models including bacteria and cell in cultures, but they display a poor correlation with  
40 mutagenicity or carcinogenesis found *in vivo* [3,4]. Short or long periods of exposure to fibres have  
41 been failing to identify a molecular basis of DNA damage in different several genotoxicity tests [1].  
42 Thus, nowadays the mechanisms underlying the genotoxicity and carcinogenicity of asbestos and  
43 other fibres remain obscure.

44  
45 The transformation of bacteria by foreign DNA can be achieved if clay fibres are spread applying  
46 some friction or vibrations. This phenomenon is known as Yoshida effect [5]. This transformation  
47 relies on the ability of mineral nanofibres or nanoneedles to adsorb DNA and penetrate bacterial  
48 cells under sliding friction forces [6]. By its mechanical nature, the Yoshida effect can be used to  
49 transform diverse bacterial species [8–10]. The action of sepiolite and other clays fibres is not only  
50 capable of delivering DNA into the receptor bacteria but also able to promote the releasing of DNA  
51 by disrupting the cell envelope of the portion of the population by the abrasive action of clays [11].  
52 Before Yoshida began his experiments with bacteria, the ability of asbestos to transform eukaryotic  
53 cells has been reported at the end of the eighties [12].

54  
55 Clays have the potential to enhance antibiotic resistance in farming activities [13]. In natural  
56 sceneries, sediments and stones (gastroliths) are frequently swallowed by animals resulting  
57 unavoidably in the exposure of their microbiota to pebbles, sand, and clays. Soils and waters are a  
58 primary source of antimicrobials, either by natural microbial production or environmental antibiotic  
59 pollution, a major selective pressure that favours resistant strains [14,15]. Even, gut microbes can  
60 produce antibiotic compounds [16].

61  
62 Here, we experimentally show how fibrous clays such as sepiolite and asbestos are not only able to  
63 transform bacteria, but also induce mutagenic DNA double-strand breaks (DSBs) when they are  
64 exposed to friction forces. Additionally, we propose a molecular mechanism of action for asbestos,  
65 that were strong inducers of DSBs in *Escherichia coli* if friction is present. The observed  
66 mutagenesis can contribute to antibiotic resistance via mutations in farming settings. Besides, it is a  
67 potential source of bacterial diversity in the natural context we hypothesise that this novel mutation  
68 supply has important contribution for the speciation processes of metazoa.

## 69 **Results and Discussion**

70

71 Different type of clays are able to transform bacteria penetrating the cell envelope. We reasoned that  
72 this penetration could also interact with the DNA and promote mutations. We first investigated  
73 whether the treatment with sepiolite under friction forces (as in transformation) has an impact on  
74 the mutant frequency of *Escherichia coli* measured by plating in the antibiotic fosfomycin (figure  
75 1). Simultaneously, we checked the effect of the treatment on cell viability (figure 2). We did not  
76 detect any significant differences in mutant frequencies when the cells were merely exposed to  
77 sepiolite without applying any friction on agar plates surface. In contrast, we found values around  
78 six-fold increase in mutant frequency when friction is present for two or three minutes ( $P=0.006$ )  
79 and a modest value when the treatment time lasted for one minute but not significant. Interestingly,  
80 we noted that only cells in the stationary phase displayed an increase in mutant frequency, while no  
81 significant mutagenesis was observed when bacteria came from exponential cultures (figure S1).  
82 We initially attributed this difference to a higher sensitivity to the treatment (figure 2).

83

84 Under friction forces on an hydro-gel, sepiolite and other nanosized acicular materials can penetrate  
85 bacterial cells [5]. Many minerals containing metals such as iron, aluminium or copper are toxic for  
86 bacteria and their toxicity is caused by the generation of reactive oxygen species (ROS) [17]. We  
87 reasoned that the release of metal ions inside the cell could elicit the Fenton reaction and promote  
88 mutagenesis. Despite the addition of 2-2' bipyridyl shortly before treatment mutagenesis was still  
89 observed (figure S2). This result supports the idea that the mutagenic effect in this situation does not  
90 depend on the metals present in the fibres.

91

92 A second likely explanation is the physical interaction of individual clay fibres in motion during  
93 penetration directly damage the DNA, causing strand breaks (DSBs). Physical or mechanical stress  
94 on the DNA duplex is a relevant cause of DSBs [18]. To evaluate this possibility, we designed a  
95 simple experiment where *E. coli* DH5-alpha strain (*recA* deficient) carrying a plasmid (pET-19b)  
96 were subjected to treatment with sepiolite and sliding friction while the addition of sepiolite without  
97 friction and bacterial cells alone were used as controls. The extracted plasmid from the sepiolite  
98 group (under friction) presented a significantly high level of linearised molecules when compared to  
99 the control groups ( $P=0.00016$ ). Thus, we inferred that the joint action of sepiolite and friction are  
100 responsible for induction of double-strand breaks in the DNA. Interestingly, no increase in nicked  
101 DNA (single-strand break) was observed, indicating that if this type of lesion occurs, it happens at a  
102 non-detectable rate by this technique. Typically, during plasmid DNA extraction, three molecular

103 conformations are found: the supercoiled (which migrates very fast), nicked DNA (which is also  
104 closed circular but relaxed due to single strand breaks and it has an intermediate migration rate) and  
105 linear molecules (with a lower migration speed) [19]. These latter DNA molecules were especially  
106 abundant in the friction-sepiolite treated group at the time that they are present in a low level in  
107 control groups (figure 3).

108

109 The view of mutagenic DSBs by mechanical shearing is very consistent with the absence of  
110 mutagenic effect in exponentially growing bacteria. If the organism is diploid (even if the diploidy  
111 is only transient, as in replicating bacteria or replicating haploid yeast), then homology-directed  
112 repair can be used [18]. There is a pathway to deal with lethal double-strand breaks by non-  
113 homologous DNA end joining at the cost of introducing mutations [18]. Because *E. coli* lacks a  
114 pathway to join non-homologous ends, homologous recombination is the only means to salvage  
115 broken chromosomes [20]. But how can *E. coli* repair DSBs in stationary phase by homologous  
116 recombination? Stationary-phase cultures contained cells with several chromosome copies [21]. In  
117 exponentially growing *E. coli* DSB repair is non-mutagenic [22,23]. However, breaks repair  
118 becomes mutagenic during the stationary phase and requires the Sigma S factor (RpoS), the SOS  
119 response, and the error-prone DNA polymerase PolIV. The change from one situation to the other  
120 has been described as a switch from high-fidelity repair in the exponential phase to error-prone  
121 DNA double-strand breaks during the stationary phase [22,23]. Because DSBs are lethal unless  
122 repaired, and repair action requires RecA protein [22,23], we designed an experiment to confirm  
123 this notion. We repeated the experiment of sepiolite mutagenesis with a RecA deficient strain that is  
124 impaired in the SOS response triggering. We found that *recA* gene inactivation completely  
125 abolished sepiolite mutagenesis (figure 3). Thus, the lower level of mutant frequency in the *recA*  
126 deficient strain could be explained by the death of cells that suffered DSBs and were unable to  
127 repair them. Mutations introduced by DSB repair are considered a mechanism of diversity via  
128 mutagenic repair in bacteria [24,25].

129

130 Potentially, the mutagenicity of clay treatment is also enhanced in stationary phase cells due to  
131 DNA being more tightly compacted than in the exponential phase [26]. Indeed, in *Escherichia coli*,  
132 DNA goes to a co-crystallization state with the stress-induced protein Dps offering protection to  
133 several types of stress, normally chemical damage [27]. However, crystallization is often associated  
134 with less flexibility or added fragility to direct physical contact. In contrast, less compacted DNA of  
135 proliferating *E. coli* is elastic and soft [28], which may limit the number of DSBs. It is then possible  
136 that mineral fibres under friction can break DNA strands more easily in the stationary than in the

137 exponential phase.

138

139 To reunite more evidence that penetration and interaction of fibres with DNA cause DSBs inside the  
140 cell, we performed a direct observation of sepiolite-treated bacteria by scanning electromicroscopy.  
141 We noticed that fibres look compatible in dimensions able to penetrate bacteria without completely  
142 destroying the envelope. Additionally, we observed bacteria directly penetrated by fibres while  
143 those that were exposed to mineral without friction were not (figure 5). This observation joined to  
144 the failure of 2-2' bipyridyl in suppressing the mutagenic effect of sepiolite point to the mechanical  
145 action as causing agent of the damage. The notion of mechanical breaks is in good agreement with  
146 the results in cell-free systems. In these experiments, breakage of plasmid DNA was not directly  
147 associated with the amount of iron released by asbestos fibres when they are incubated together [1].

148

149 Clays (e.g. sepiolite) are jointly used with antibiotics in farming as growth promoters. This practice  
150 improves growth and animal product quality, and these additives are common in feed for broiler  
151 chickens and pigs [29,30]. Sepiolite is considered to be safe, stable and chemical inert hence; it is  
152 also used in tablet formulation for human medicine [31]. Recently, we proposed that clays used as  
153 animal feed additive can increase the risk of horizontal gene transfer (HGT) among microbes,  
154 resulting possibly in a rise of antibiotic resistance [11,32].

155

156 Sepiolite also contains very short fibres (figure S3). In the case of asbestos, there is a certainty that  
157 long fibres are much more dangerous by their carcinogenic potential. We decided to investigate if,  
158 in bacteria, the length of sepiolite fibre is relevant for mutagenesis. We prepared a suspension of  
159 short fibres (less than 1  $\mu\text{m}$ ). The exposure of stationary phase bacteria to this preparation did not  
160 cause any significant DNA damage if compared with the control and in contrast with the long-fibre  
161 original mineral suspension (figure 6).

162

163 Bacterial genotoxicity experiments are considered an important step in the assessment of mutagenic  
164 properties of chemicals, drugs or materials in general [33]. Because asbestos fibres resemble  
165 sepiolite ones, we decided to test if asbestos fibres provoke an increase in mutagenesis. We  
166 employed for this analysis the crocidolite asbestos (figure S4), the most the most hazardous one. In  
167 our assay, the addition of asbestos to bacteria in the plates without friction did not increase the  
168 mutant frequency. In contracts, the application of friction when the fibres were present increases the  
169 mutant frequency even more than sepiolite (figure 7), probably by the same mode of action. Yoshida  
170 *et al.* have suggested that asbestos and other clays can be potentially mutagenic base on integrity

171 analysis of genomic DNA from treated bacteria [34].

172

173 Based on our results and by compiling the current knowledge about asbestos-induced carcinomas,  
174 we propose the following model. People exposed to asbestos fibres inhale them during prolonged  
175 periods and fibres accumulate in the respiratory tract. The movement of the fibres into the pleural  
176 cavity is well documented [35]. The cyclical mechanical movement between the parietal pleura  
177 (covering membrane of the inner surface of the thoracic cavity) and the visceral pleura (covering  
178 membrane of the lung surface) provokes the movement of asbestos, transpassing occasionally the  
179 epithelial cell membranes and fiscally interacting with the DNA. In eukaryotic cells, double strand  
180 breaks generate chromosome aberrations or fragmentations. If this physical interaction happens in  
181 the right time, with adequate intensity, could induce DSBs. With years of continued exposure and  
182 related with other symptoms due to direct toxicity, the higher frequency of double-strand breaks  
183 increases the probability of malignancy by finding the randomly the right mutations. The proposed  
184 model for eukaryote cells would need *in vitro* testing with appropriate cell lines but this is beyond  
185 the scope of the current study and left for future research directions.

186

187 What other implications has our finding that nanofibres induce DSB in bacteria? It has been  
188 suggested that gut microbes play a crucial role in keeping species apart or enhance the speciation  
189 [36]. It is tempting to speculate that animals that use gastroliths or sediment ingestion expose their  
190 microbiota to the abrasive action of stone derivative fibres. Therefore, the shaping of their own  
191 microbes is expected to contribute to their own speciation trajectories. Among animals that use or  
192 used gastroliths in their evolutionary trajectories, we find several branches of fishes, amphibians,  
193 reptiles (including dinosaurs) and birds. Gastroliths also regularly occur in several groups of  
194 invertebrates [37]. Wings (2007) recommends making a distinction between lithophagy and  
195 geophagy. Lithophagy (stones larger than 0.063 mm in diameter) is defined as the deliberate  
196 consumption of stones that turn into gastroliths after their ingestion. Geophagy is the consumption  
197 of soil and it is known for reptiles, birds, and mammals. These soils, rich in clays, salts or fat, serve  
198 mainly as a food supplement for the supply of specific minerals or for medical purposes [37]. Both  
199 concepts can contribute to getting together all the components that this mechanism needs to operate:  
200 gut microbiota, gut mucin mucoïd layer (hydro-gel) and friction forces provided by the peristaltic  
201 pressure of digestive tract in animals, especially the gizzard and the stomach.

202

203 One interesting question is why sepiolite from limestone gastroliths does not damage the animal  
204 gut. A convincing explanation is that the mucoïd layer in the gut protects it from the action of these

205 sharp fibres at the time that serve as a protective layer for gut epithelium. It is known that in  
206 mammals, this mucoid layer is around 200  $\mu\text{m}$  thick and is under continuous renovation [38].  
207 Sepiolite is a natural clay mineral characterised by a nanofibre structure with average dimensions  
208 less than or equal to 0.2 micrometers in diameter, and from 2 to 5 micrometers in length, although  
209 longer fibres can be present.

210

211 An implication of our study is that is important to take other factors in consideration during  
212 assessing of genotoxicity by certain materials. In our case, was essential to introduce the variable of  
213 friction. Until now, many studies associate clay-induced damage mostly with oxygen reactive  
214 species [1]. DNA damage can be produced by oxidoreduction processes generated by metal  
215 containing-fibres. Asbestos fibres are carcinogenic for both, humans and experimental animals [39].  
216 Carbon nanotubes, a novel industrial material with many applications, are another example of a  
217 potentially dangerous material. The genetic alterations provoked by these nanotubes in rat  
218 malignant mesothelioma were similar to those induced by asbestos. The nanoscale size and needle-  
219 like rigid structure of CNTs appear to be associated with their pathogenicity in mammalian cells,  
220 where carbon atoms are major components in the backbone of many biomolecules [39].  
221 Coincidentally, carbon nanotubes can be used to transform bacteria with plasmids [40] in a similar  
222 fashion that asbestos [12,41] and sepiolite do [5,42]. It would not be surprising that all these fibrous  
223 nanomaterials share their ability to mechanically induce DSBs.

224

225 It is worthy to note that in all studies related to the assessment of carcinogenesis by fibres (mainly  
226 in asbestos), there was a key element missing: the friction forces. It is known that asbestos produces  
227 DNA breaks leading to the formation of micronucleus (a type of chromosome aberration). This kind  
228 of damage seems to be caused more by mechanical action rather than ROS generation, which can  
229 worsen the situation but not necessarily has to be determinant. A good example is that in the lung  
230 cancer caused by asbestos, the most affected part of the organ is the pleural tissue. The pleura is an  
231 area in a continuous movement that creates a strong pressure and thus seems to be a good candidate  
232 to provide the friction force that mechanically can enhance the fibre penetration of the epithelial  
233 cells, being the size of the fibre is a determinant factor.

234

235 Based on our results and by compiling the current knowledge about asbestos-induced carcinomas,  
236 we propose the following model. People exposed to asbestos fibres inhale them during prolonged  
237 periods and fibres accumulate in the respiratory tract. It is frequent to find asbestos fibres into the  
238 pleural cavity, and maybe they increase the friction coefficient in the pleural space, a parameter that

239 in physiological conditions has a very small value [35]. The cyclical mechanical movement between  
240 the parietal pleura (covering membrane of the inner surface of the thoracic cavity) and the visceral  
241 pleura (covering membrane of the lung surface) provoke the movement of asbestos, transpassing the  
242 epithelial cells and promoting DSBs. In eucaryotic cells, double strand breaks generate chromosome  
243 aberrations or fragmentations. With years of continued exposure and related with other symptoms  
244 due to direct toxicity, the higher frequency of double-strand breaks increases the probability of lung  
245 cancer. The proposed model would need *in vitro* validation with epithelial cells but this is beyond  
246 the scope of the current study and left for future research directions. This model does not exclude  
247 other toxic and genotoxic mechanism of asbestosis such as reactive species arising from metal  
248 action or inflammatory response. It is also possible that irruption of fibres can break some  
249 microtubules and disrupt the spindle in inside mitotic cells, something that may cause chromosome  
250 aberrations and led to carcinogenesis [43]. Its is possible that the whole pathological process is  
251 limited to a threshold of fibres in the target tissues, the low probability of introducing the right  
252 mutations and some additional factors that are in agreement with the association to chronicle  
253 exposure. Hence, it can take from 10 to 40 years for the development of symptoms or tumours after  
254 asbestos exposure. This model does not exclude other toxic and genotoxic mechanism of asbestosis  
255 such as reactive species arising from metal action contained in the fibres or inflammatory response.  
256 Indeed, if ROS is taking part, the introduction of fibres directly into to cytoplasmic compartment  
257 would be a good approach to study these interactions.

258

259 If our hypothesis is certain, the poor correlation between DNA damage *in vivo* and *in vitro* [3] may  
260 be explained by the limited or lack of penetration of asbestos in experimental designs. Thus, the  
261 introduction of some friction can be a cornerstone in determining a molecular mechanism of  
262 carcinogenic fibres. The mechanism(s) underlying asbestos toxicity associated with the  
263 pathogenesis of mesothelioma has been a challenge to unravel for more than seven decades [44].  
264 Based on bacterial DNA damage induced by sepiolite and asbestos fibres that we observe in this  
265 study, we propose that the coelomic movement (more prominent in pleural space), with the  
266 participation of clay fibres, may account to generate sliding friction forces to allow penetration by  
267 fibres. Thus, this factor deserves to be investigated as an important parameter in fibre  
268 carcinogenesis studies *in vitro*, including the tests of genotoxicity with bacteria and human cells in  
269 culture. Although Yoshida described the ability of nanoclays to penetrate bacteria, there was a clear  
270 antecedent indicating that chrysotile (a type of asbestos) were able to transform monkey cells in  
271 culture by exogenous plasmid DNA [12]. Unfortunately, the authors did not describe in details how  
272 the incubation steps were carried out. It is logic to think that this transformation requires penetration

273 of the plasmatic membrane. One could believe that the penetration can account for pore generation  
274 and make the cells to burst, but not necessarily. A good example is the microinjection technique,  
275 implying necessarily membrane transpassing by microneedles or micropipettes [45], often of bigger  
276 diameter than asbestos fibres and the cells resist it.

277

278 The most important limitation of our study is the lack of an animal model to test if our finding of  
279 mutagenicity in bacteria by clays occurs *in vivo*. We theoretically anticipated that clays present in  
280 livestock feed could promote antibiotic resistance and virulence in pathogenic bacteria by their  
281 transformation ability, we extend now this possibility to the antibiotic resistance via mutations.  
282 However, testing conditions are hindered by the fact that experiments would require at least S1  
283 security level, and this is difficult to achieve with livestock animals [32]. Another important issue is  
284 the need for a co-action of several parameters responsible for a successful introduction of  
285 mutations. Because of labour-intensity of this type of experiments, we fix only one concentration  
286 for sepiolite and asbestos as proof of principle to match the one that is described in the literature as  
287 the optimal for transformation value. Nevertheless, the transformation of plasmid DNA requires  
288 penetration and sepiolite and other clays have shown this capacity in a wide range of concentrations  
289 although it diminishes at high concentration due to the killing of bacteria [10,46–48]. We discussed  
290 in a previous article that the values of pressure in the gut of many animal species, meeting the  
291 criteria very well [32]. The presence of an hydrogel does not seem to be a problem since both mucin  
292 layer of the gut or mucoid secretion in the respiratory tract can play that role, particularly if fibres  
293 have the capacity to change viscosity locally or gradients of viscosity exist across these body  
294 compartments.

295 There is the recent debate about a possible link between talcum powder and ovarian cancer risk  
296 associated with asbestos contamination in talc. Although the risk is small, some studies are  
297 indicating a low or moderate but significant chance of cancer, while other consider that there is not  
298 [49–51]. This debate points that is necessary to advance the understanding of molecular base of  
299 DNA damage by asbestos and other industrial fibres. If other studies confirm our proposal of  
300 mechanical/physical DNA breaks, it is possible to suggest that some genotoxicity assays intended to  
301 unveil mutagenic properties of materials, such as the test of Ames, should be modified accordingly  
302 to include a standardised procedure of friction or promoting some sort of shaking during incubation  
303 steps. Similarly, several *in vitro* test, with both bacteria and eukaryotic cells, were modified by  
304 researchers and regulatory agencies where introduced the metabolic activation by fraction S9 of  
305 liver homogenate [52].

306 Overall, one of the most significant contributions of this article is that provide for the first time a  
307 bacterial model to test genotoxicity of nanofibers and uncover a new mechanism of action for  
308 asbestos that correlates better with *in vivo* observations. Asbestosis is a world health and  
309 environmental problem, which molecular basis has been a challenge during several decades [44].  
310 One more observation in favour of our mechanism is that although asbestos fibres are widely  
311 distributed in the anatomy of patients [44,53], the most common cancers caused by asbestos  
312 originate in lungs (mostly mesothelioma). If the most explored mechanism of action is based on  
313 reactive radicals (chemical damage), why is not there big differences in the frequencies of other  
314 types of carcinoma such us leukaemia, lymphoma or digestive track system among exposed  
315 populations? In the last place, and not less important, is the tighter contact of slippery membranes (a  
316 monolayer of flattened epithelial-like cells ) of the mesothelium. This area, the pleural space, is in  
317 continuous movement and constitute preferential target of asbestos-induced carcinogenesis. Of  
318 particular interest are free-floating mesothelial cells of the cavity, that even proliferate under  
319 damaging conditions [54]. The free-floating cells are the ideal candidates to be penetrated by  
320 asbestos in the pleural space. They may be more sensitive to suffer direct (physical) or indirect  
321 (chemical) DNA damage and become into a mesothelioma. If the mechanical penetration due to  
322 coelemic movement is going to be tested *in vitro*; a cell tissue culture system should be designed in  
323 order to reflect closely the situation of pleural space including floating cells. We suggest an  
324 experimental design as starting point for this type of experiment (supplementary figure S5). It is  
325 clear that the current systems to assess carcinogenicity risk with nonofibres (asbestos, carbon nan-  
326 tubes, silicates, glass fibres etc) has to be modified because the current set-ups fail most of the time  
327 in detection of damage [4].

328 Finally, it did not escape to our attention that sepiolite transformation technique gained some  
329 popularity in the last years because there is no need to prepare competence cells [7,10,42,55],  
330 diverse bacteria can be transformed [32] in both stationary and exponentially growing bacteria are  
331 equally transformable. However, to prevent undesired mutations in both, plasmid and genomic  
332 DNA, we recommend using exponential phase bacteria, where mutagenesis is not significant.

333

334

335

336

337

338

339 **Methods**

340

341 **Bacteria and growth conditions.** The *E. coli* MG1655 wild-type strain and its derivative mutants  
342 were cultured in Lysogenic Broth (LB). All experiments were performed at 37°C, with shaking in  
343 liquid culture. All solid cultures were grown in LB agar 1.5% for standard procedures and 2% for  
344 the sepiolite treatment. All cultures were supplemented with antibiotic when appropriate.

345

346 **Mutant frequency estimation of sepiolite treated cells.** Approximately  $2 \times 10^9$  bacterial cells per  
347 ml of *E. coli* MG1655 and its derivative mutants from overnight or mid-exponential growing  
348 cultures were centrifuged and resuspended in 100 µl of sterilized transformation mixture, consisting  
349 of sepiolite (Kremer Pigmente, Germany) suspended in aqueous solution at a final concentration of  
350 0.1 mg/ml. Resuspended cells were spread on plates containing fresh Müller-Hinton-Agar (Sigma-  
351 Aldrich, Germany) medium solidified with 2% agar, and Petri dishes were pre-dried in a biological  
352 safety flow cabinet for 20 minutes before use. Friction force was provided by streaking bacterial  
353 cultures plus sepiolite with sterile glass stir sticks gently pressed onto the medium surface for 1, 2  
354 and 3 minutes, applying as much pressure as possible without breaking the agar gel. Petri dishes  
355 were incubated at 37°C for 2 hours to allow DNA repair if any damage occurred. The plates were  
356 washed gently four times with 5 ml of 0.9% sodium chloride solution using a 5 ml pipette and the  
357 bacterial suspensions were transferred to 10 ml tubes to recover the cells by centrifugation at 3000 g  
358 for 10 minutes. The resulting pellets were resuspended in a final volume of 1 ml of fresh LB an  
359 incubate during 1 hour at 37°C to allow the cells to recover. Appropriate dilutions were plated onto  
360 LB plates to estimate bacterial viability and in LB plus fosfomycin (50 µg/ml) to estimate the  
361 number of resistant mutants. Plates were incubated overnight at 37°C. Each experiment consisted of  
362 5 replicates and was repeated at twice. Mutant frequencies were calculated by using the on-line  
363 web-tool Falcor [56].

364

365 **Mutant frequency estimation of asbestos treated cells.** The procedure was carried out identically  
366 that the one described for sepiolite in this section. The time was set to 2 minutes and the same  
367 concentration that was used, 0.1 mg/ml. We used the crocidolite asbestos analytical standard (SPI  
368 Supplies, USA). The asbestos fibres in distilled water were autoclaved and sonicated in bath during  
369 10 minutes before use to render an homogeneous suspension.

370

371 **Long fibre-depleted sepiolite.** To assess the role of long fibre of sepiolite in mutagenesis, we  
372 obtained a sepiolite preparation depleted of fibres longer than 1 µm. A 100 ml sepiolite suspension

373 (1 mg/ml) in distilled water was passed through Pall® Acrodisc® glass fibre syringe filters (Sigma,  
374 USA) several times. The resulting suspension was desiccated by evaporation at 70°C overnight. A  
375 non-filtered solution was used as a control. From the obtained powder, two suspensions were  
376 prepared to a final proportion of 0.1 mg/ml. These two solutions were used for a mutagenesis  
377 experiment plating in fosfomycin as indicated previously, using a friction time of two minutes.

378

379 **Influence of 2-2' bipyridyl on sepiolite mutagenesis.** The effect of 2-2' bipyridyl, a metal  
380 chelating agent [57], on sepiolite mutagenesis was determined by measuring its influence on the  
381 mutant frequency for a selected concentration of sepiolite, where mutagenesis was observed. The  
382 experiment consisted of adding a titrating concentration of 2-2' bipyridyl (200 µM) to chelate  
383 metals 5 minutes before the treatment. Cultures treated with sepiolite and friction without the  
384 addition of 2-2' bipyridyl and bacteria alone without sepiolite were used as a control. The mutant  
385 frequencies for these groups were determined as described elsewhere in this section.

386

387 **Assessing double-strand breaks with a plasmid system.** To evaluate if sepiolite under friction  
388 treatment induces double-strand breaks in plasmid DNA, the strain *Escherichia coli* DH5 alpha  
389 (fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17)  
390 carrying the plasmid pET-19b (Novagen, Germany) was treated with sepiolite and sliding friction  
391 forces during one minute. Several samples were recovered from the plates and pooled to  
392 compensate viability losses due to friction. The recovery was done by washing the surface with 5  
393 ml 0.9 % NaCl saline solution four times as described for mutagenesis experiments. The recovered  
394 pellets were washed with 1 ml of TE buffer and the OD<sub>600</sub> adjusted to 1 for each type of sample.  
395 Plasmid DNA samples were extracted using a Qiagen mini plasmid extraction kit (Qiagen,  
396 Germany). Added sepiolite with or without friction and no sepiolite groups were used as a control  
397 group. Each experiment consisted of five replicates. The same amount of plasmid DNA per  
398 replicate was applied per well to an agarose gel that was stained with SYBR® Gold Nucleic Acid  
399 Gel Stain kit (Molecular Probes, USA). A NdeI (Promega, USA) digested aliquot of pET-19b was  
400 used as control of linear migration rate. The proportion of linear molecules of the plasmid were  
401 compared among groups using a densitometry analysis by ImageJ [58].

402

403 **RecA deficient strain construction.** The recA null mutant was constructed following the protocol  
404 previously described [59]. Briefly, the following primers were used to amplify the chloramphenicol  
405 cassette: 5'-CAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAATGGT-  
406 GTAGGCTGGAGCTGCTTC-3' and 5'-ATGCGACCCTTGTGTATCAAACAAGACGATTAA-

407 AAATCTTCGTTAGTTTCATGGGAATTAGCCATGGTCC-3' (forward and reverse respectively)  
408 from pKD3 donor plasmid. Underlined sequences represent the pairing sequence to the plasmid,  
409 while the remaining parts belong to the homologous upstream and downstream regions of the *recA*  
410 gene. The mutant was checked by PCR amplification using the primers c1 5'-  
411 TTATACGCAAGGCGACAAGG-3' and c2 5'-GATCTTCGGTCACAGGTAGG-3' in combination  
412 with specific primers for upstream and downstream regions of *recA* gene: 5'-  
413 ATTGCAGACCTTGTGGCAAC-3' and 5'-CGATCCAACAGGCGAGCATAT-3' respectively.  
414 Additionally, we tested phenotypically the increased susceptibility to UV light and mitomycin C in  
415 comparison to the parental strain. The antibiotic resistance gene was eliminated using the pCP20  
416 plasmid as described previously [59].

417

418 **Scanning electron microscopy of *E. coli* treated with sepiolite.** Approximately  $2 \times 10^9$  cfu of  
419 stationary phase *E. coli* MG1655 were treated with sepiolite and friction force was applied for one  
420 minute as described for the mutagenesis experiment. Circular agar blocks were taken from agar  
421 plates with a sterile cork borer (1 cm of diameter). Then, a thin surface layer was cut off, placed on  
422 a circular glass coverslip (1.5 cm of diameter) and incubated for 45 minutes at room temperature in  
423 a laminar flow cabinet to allow air drying of the samples. The cover glasses with dehydrated agar  
424 sections were mounted on aluminium stubs using double-sided adhesive tape and coated with gold  
425 in a sputter coater (SCD-040; Balzers, Union, Liechtenstein). The specimens were examined with a  
426 FEI Quanta 200 scanning electron microscope (FEI Co., Hillsboro, OR) operating at an accelerating  
427 voltage of 15 kV under high vacuum mode at different magnifications. At least 5 sections from  
428 independent plates were observed to check physical penetration by the mineral. Some samples of  
429 sepiolite or asbestos (crocidolites) alone were processed and observed in the same way.

430

431 **Statistical analysis.** To compare experimental groups, Kuskal-Wallis test or One-way ANOVA test.  
432 In case of case of significance were used followed by Bonferroni-corrected one-tailed Mann-  
433 Whitney U test or Tukey HSD Test respectively. P values less than or equal to 0.05, after correction  
434 if needed, were considered statistically significant. All tests were performed with the statistic  
435 software R [60].

436

437

438

439

## 440 **Acknowledgements**

441

442 This work was supported by The Collaborative Research Centre (CRC) via project 973 “Priming  
443 and Memory of Organismic Responses to Stress”. J.B. was supported by Plan Nacional de I+D+i  
444 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación  
445 Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in  
446 Infectious Diseases (REIPI RD15/0012)-co-financed by European Development Regional Fund "A  
447 way to achieve Europe" ERDF and Fondo de Investigación Sanitaria Grant PI13/00063. We are  
448 grateful to Alejandro Couce, Enrique González-Tortuero and Olga Makarova for critical comments  
449 about the manuscript.

450

## 451 **References**

452

- 453 1. Jaurand MC (1997) Mechanisms of fiber-induced genotoxicity. *Environ Health Perspect* 105  
454 Suppl: 1073–1084. Available: [http://www.pubmedcentral.nih.gov/articlerender.fcgi?](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1470173&tool=pmcentrez&rendertype=abstract)  
455 [artid=1470173&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1470173&tool=pmcentrez&rendertype=abstract). Accessed 15 April 2016.
- 456 2. Huang SXL, Jaurand M-C, Kamp DW, Whysner J, Hei TK (2011) Role of mutagenicity in  
457 asbestos fiber-induced carcinogenicity and other diseases. *J Toxicol Environ Health B Crit*  
458 *Rev* 14: 179–245. Available: [http://www.pubmedcentral.nih.gov/articlerender.fcgi?](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3118525&tool=pmcentrez&rendertype=abstract)  
459 [artid=3118525&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3118525&tool=pmcentrez&rendertype=abstract). Accessed 6 April 2016.
- 460 3. Daniel FB (1983) In vitro assessment of asbestos genotoxicity. *Environ Health Perspect* 53:  
461 163–167. Available: <http://www.ncbi.nlm.nih.gov/pubmed/6363052>. Accessed 2 November  
462 2016.
- 463 4. Fowler P, Homan A, Atkins D, Whitwell J, Lloyd M, et al. (2016) The utility of the in vitro  
464 micronucleus test for evaluating the genotoxicity of natural and manmade nano-scale fibres.  
465 *Mutat Res Toxicol Environ Mutagen* 809: 33–42. Available:  
466 <http://www.ncbi.nlm.nih.gov/pubmed/27692297>. Accessed 6 January 2017.
- 467 5. Yoshida N, Sato M (2009) Plasmid uptake by bacteria: a comparison of methods and  
468 efficiencies. *Appl Microbiol Biotechnol* 83: 791–798. Available:  
469 [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19471921)  
470 [cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=19471921](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19471921).
- 471 6. Dong L, Johnson DT (2005) Adsorption of acicular particles at liquid-fluid interfaces and the  
472 influence of the line tension. *Langmuir* 21: 3838–3849. Available:  
473 <http://www.ncbi.nlm.nih.gov/pubmed/15835945>. Accessed 18 June 2013.
- 474 7. Yoshida N, Ide K (2008) Plasmid DNA is released from nanosized acicular material surface  
475 by low molecular weight oligonucleotides: exogenous plasmid acquisition mechanism for

- 476 penetration intermediates based on the Yoshida effect. *Appl Microbiol Biotechnol* 80: 813–  
477 821. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18704395>. Accessed 18 June 2013.
- 478 8. Rodríguez-Beltrán J, Rodríguez-Rojas A, Yubero E, Blázquez J (2013) The Animal Food  
479 Supplement Sepiolite Promotes a Direct Horizontal Transfer of Antibiotic Resistance  
480 Plasmids between Bacterial Species. *Antimicrob Agents Chemother* 57: 2651–2653.  
481 Available: <http://www.ncbi.nlm.nih.gov/pubmed/23529735>. Accessed 18 June 2013.
- 482 9. Djordjevic SP, Stokes HW, Roy Chowdhury P (2013) Mobile elements, zoonotic pathogens  
483 and commensal bacteria: conduits for the delivery of resistance genes into humans,  
484 production animals and soil microbiota. *Front Microbiol* 4: 86. Available:  
485 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
486 [artid=3639385&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3639385&tool=pmcentrez&rendertype=abstract). Accessed 10 June 2013.
- 487 10. Wilharm G, Lepka D, Faber F, Hofmann J, Kerrinnes T, et al. (2010) A simple and rapid  
488 method of bacterial transformation. *J Microbiol Methods* 80: 215–216. Available:  
489 <http://dx.doi.org/10.1016/j.mimet.2009.12.002>. Accessed 18 June 2013.
- 490 11. Rodríguez-Beltrán J, Rodríguez-Rojas A, Yubero E, Blázquez J (2013) The animal food  
491 supplement sepiolite promotes a direct horizontal transfer of antibiotic resistance plasmids  
492 between bacterial species. *Antimicrob Agents Chemother* 57: 2651–2653. Available:  
493 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
494 [artid=3716135&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3716135&tool=pmcentrez&rendertype=abstract). Accessed 7 April 2014.
- 495 12. Appel JD, Fasy TM, Kohtz DS, Kohtz JD, Johnson EM (1988) Asbestos fibers mediate  
496 transformation of monkey cells by exogenous plasmid DNA. *Proc Natl Acad Sci U S A* 85:  
497 7670–7674. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
498 [artid=282254&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=282254&tool=pmcentrez&rendertype=abstract). Accessed 1 July 2013.
- 499 13. Rodríguez-Rojas A, Rodríguez-Beltrán J, Valverde J, Blázquez J (2015) Can Clays in  
500 Livestock Feed Promote Antibiotic Resistance and Virulence in Pathogenic Bacteria?  
501 *Antibiotics* 4: 299–308. Available: <http://www.mdpi.com/2079-6382/4/3/299>. Accessed 16  
502 July 2015.
- 503 14. Blázquez J, Couce A, Rodríguez-Beltrán J, Rodríguez-Rojas A, Blázquez J, et al. (2012)  
504 Antimicrobials as promoters of genetic variation. *Curr Opin Microbiol* 15: 561–569.  
505 Available: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?>  
506 [cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=22890188](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22890188). Accessed 24 May 2013.
- 507 15. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, et al. (2015) Tackling  
508 antibiotic resistance: the environmental framework. *Nat Rev Microbiol* 13: 310–317.  
509 Available: <http://dx.doi.org/10.1038/nrmicro3439>. Accessed 22 November 2015.
- 510 16. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, et al. (2016) Human commensals  
511 producing a novel antibiotic impair pathogen colonization. *Nature* 535: 511–516. Available:  
512 <http://www.nature.com/doi/10.1038/nature18634>. Accessed 6 January 2017.
- 513 17. Williams LB, Metge DW, Eberl DD, Harvey RW, Turner AG, et al. (2011) What makes a

- 514 natural clay antibacterial? *Environ Sci Technol* 45: 3768–3773. Available:  
515 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
516 [artid=3126108&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3126108&tool=pmcentrez&rendertype=abstract). Accessed 26 June 2015.
- 517 18. Lieber MR (2010) The mechanism of double-strand DNA break repair by the  
518 nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 79: 181–211. Available:  
519 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
520 [artid=3079308&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3079308&tool=pmcentrez&rendertype=abstract). Accessed 11 July 2014.
- 521 19. Sambrook J, Russell DW (2001) *Molecular cloning : a laboratory manual*. 3rd ed. Cold  
522 Spring Harbor [New York]: Laboratory Press.
- 523 20. Persky NS, Lovett ST (2008) Mechanisms of Recombination: Lessons from *E. coli*. *Crit Rev*  
524 *Biochem Mol Biol* 43: 347–370. doi:10.1080/10409230802485358.
- 525 21. Akerlund T, Nordström K, Bernander R (1995) Analysis of cell size and DNA content in  
526 exponentially growing and stationary-phase batch cultures of *Escherichia coli*. *J Bacteriol*  
527 177: 6791–6797. Available: <http://www.ncbi.nlm.nih.gov/pubmed/7592469>. Accessed 6  
528 January 2017.
- 529 22. Ponder RG, Fonville NC, Rosenberg SM (2005) A switch from high-fidelity to error-prone  
530 DNA double-strand break repair underlies stress-induced mutation. *Mol Cell* 19: 791–804.  
531 Available: <http://www.ncbi.nlm.nih.gov/pubmed/16168374>. Accessed 3 April 2016.
- 532 23. Shee C, Gibson JL, Darrow MC, Gonzalez C, Rosenberg SM (2011) Impact of a stress-  
533 inducible switch to mutagenic repair of DNA breaks on mutation in *Escherichia coli*. *Proc*  
534 *Natl Acad Sci U S A* 108: 13659–13664. Available:  
535 <http://www.ncbi.nlm.nih.gov/pubmed/21808005>. Accessed 14 November 2016.
- 536 24. Boles BR, Thoendel M, Singh PK (2004) Self-generated diversity produces “insurance  
537 effects” in biofilm communities. *Proc Natl Acad Sci U S A* 101: 16630–16635. Available:  
538 <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?>  
539 [cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=15546998](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15546998).
- 540 25. Rosenberg SM, Shee C, Frisch RL, Hastings PJ (2012) Stress-induced mutation via DNA  
541 breaks in *Escherichia coli*: a molecular mechanism with implications for evolution and  
542 medicine. *Bioessays* 34: 885–892. Available:  
543 <http://www.ncbi.nlm.nih.gov/pubmed/22911060>. Accessed 6 January 2017.
- 544 26. Dorman CJ (2013) Genome architecture and global gene regulation in bacteria: making  
545 progress towards a unified model? *Nat Rev Microbiol* 11: 349–355. Available:  
546 <http://dx.doi.org/10.1038/nrmicro3007>. Accessed 6 April 2016.
- 547 27. Wolf SG, Frenkiel D, Arad T, Finkel SE, Kolter R, et al. (1999) DNA protection by stress-  
548 induced biocrystallization. *Nature* 400: 83–85. Available:  
549 <http://www.ncbi.nlm.nih.gov/pubmed/10403254>. Accessed 12 April 2016.
- 550 28. Pelletier J, Halvorsen K, Ha B-Y, Paparcone R, Sandler SJ, et al. (2012) Physical

- 551 manipulation of the Escherichia coli chromosome reveals its soft nature. Proc Natl Acad Sci  
552 U S A 109: E2649-56. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22984156>. Accessed  
553 5 January 2017.
- 554 29. Ouhida I, Pérez JF, Piedrafita J, Gasa J (2000) The effects of sepiolite in broiler chicken diets  
555 of high, medium and low viscosity. Productive performance and nutritive value. Anim Feed  
556 Sci Technol 85: 183–194.
- 557 30. Parisini P, Martelli G, Sardi L, Escribano F (1999) Protein and energy retention in pigs fed  
558 diets containing sepiolite. Anim Feed Sci Technol 79: 155–162. Available:  
559 [http://www.animalfeedscience.com/article/S0377-8401\(99\)00008-5/abstract](http://www.animalfeedscience.com/article/S0377-8401(99)00008-5/abstract). Accessed 18  
560 June 2013.
- 561 31. Viseras C, Lopez-Galindo A (1999) Pharmaceutical applications of some spanish clays  
562 (sepiolite, palygorskite, bentonite): some preformulation studies. Appl Clay Sci 14: 69–82.  
563 Available: [http://dx.doi.org/10.1016/S0169-1317\(98\)00050-7](http://dx.doi.org/10.1016/S0169-1317(98)00050-7). Accessed 18 June 2013.
- 564 32. Rodríguez-Rojas A, Rodríguez-Beltrán J, Valverde JR, Blázquez J (2015) Can Clays in  
565 Livestock Feed Promote Antibiotic Resistance and Virulence in Pathogenic Bacteria?  
566 Antibiotics 4: 299–308. Available:  
567 <https://doaj.org/article/d9391353bbb7436d98103496d945305b>. Accessed 3 April 2016.
- 568 33. Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, et al. (2009) Mutagenicity  
569 testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme.  
570 Mutagenesis 24: 341–349. Available: <http://www.ncbi.nlm.nih.gov/pubmed/19535363>.  
571 Accessed 28 October 2016.
- 572 34. . NY, . TN, . KO (2004) Mutagenesis of Bacteria by Fibrous or Clay Minerals. J Biol Sci 4:  
573 532–536. Available: <http://www.scialert.net/abstract/?doi=jbs.2004.532.536>. Accessed 15  
574 November 2016.
- 575 35. Misericocchi G, Sancini G, Mantegazza F, Chiappino G (2008) Translocation pathways for  
576 inhaled asbestos fibers. Environ Health 7: 4. Available:  
577 <http://www.ncbi.nlm.nih.gov/pubmed/18218073>. Accessed 2 November 2016.
- 578 36. Yong E (2013) Gut microbes keep species apart. Nature. Available:  
579 [http://www.nature.com/news/gut-microbes-keep-species-apart-1.13408?WT.ec\\_id=NEWS-](http://www.nature.com/news/gut-microbes-keep-species-apart-1.13408?WT.ec_id=NEWS-20130723)  
580 20130723. Accessed 5 April 2016.
- 581 37. Wings O (2007) A review of gastrolith function with implications for fossil vertebrates and a  
582 revised classification - Acta Palaeontologica Polonica. Acta Palaeontol Pol 52: 1–16.  
583 Available: <https://www.app.pan.pl/article/item/app52-001.html>. Accessed 5 April 2016.
- 584 38. Hansson GC (2012) Role of mucus layers in gut infection and inflammation. Curr Opin  
585 Microbiol 15: 57–62. Available: </pmc/articles/PMC3716454/?report=abstract>. Accessed 29  
586 October 2015.
- 587 39. Toyokuni S (2013) Genotoxicity and carcinogenicity risk of carbon nanotubes. Adv Drug

- 588 Deliv Rev 65: 2098–2110. Available: <http://www.ncbi.nlm.nih.gov/pubmed/23751780>.  
589 Accessed 15 April 2016.
- 590 40. Rojas-Chapana J, Troszczynska J, Firkowska I, Morsczeck C, Giersig M (2005) Multi-walled  
591 carbon nanotubes for plasmid delivery into Escherichia coli cells. *Lab Chip* 5: 536–539.  
592 Available: <http://www.ncbi.nlm.nih.gov/pubmed/15856091>. Accessed 9 March 2016.
- 593 41. Yoshida N, Kodama K, Nakata K, Yamashita M, Miwa T (2002) Escherichia coli cells  
594 penetrated by chrysotile fibers are transformed to antibiotic resistance by incorporation of  
595 exogenous plasmid DNA. *Appl Microbiol Biotechnol* 60: 461–468. Available:  
596 <http://www.ncbi.nlm.nih.gov/pubmed/12466888>. Accessed 1 July 2013.
- 597 42. Rodríguez-Beltrán J, Elabed H, Gaddour K, Blázquez J, Rodríguez-Rojas A (2012) Simple  
598 DNA transformation in Pseudomonas based on the Yoshida effect. *J Microbiol Methods* 89:  
599 95–98. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22405834>. Accessed 18 June 2013.
- 600 43. Cosenza MR, Krämer A (2016) Centrosome amplification, chromosomal instability and  
601 cancer: mechanistic, clinical and therapeutic issues. *Chromosom Res* 24: 105–126. Available:  
602 <http://link.springer.com/10.1007/s10577-015-9505-5>. Accessed 18 November 2016.
- 603 44. Barlow CA, Lievens L, Gross S, Ronk CJ, Paustenbach DJ (2013) The role of genotoxicity  
604 in asbestos-induced mesothelioma: an explanation for the differences in carcinogenic  
605 potential among fiber types. *Inhal Toxicol* 25: 553–567. Available:  
606 <http://www.ncbi.nlm.nih.gov/pubmed/23905972>. Accessed 15 April 2016.
- 607 45. Zhang Y, Yu L-C (2008) Single-cell microinjection technology in cell biology. *BioEssays* 30:  
608 606–610. Available: <http://doi.wiley.com/10.1002/bies.20759>. Accessed 18 November 2016.
- 609 46. Rodriguez-Beltran J, Elabed H, Gaddour K, Blazquez J, Rodriguez-Rojas A (2012) Simple  
610 DNA transformation in Pseudomonas based on the Yoshida effect. *J Microbiol Methods* 89:  
611 95–98. Available: [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?  
612 cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=22405834](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22405834).
- 613 47. Tan H, Fu L, Seno M (2010) Optimization of bacterial plasmid transformation using  
614 nanomaterials based on the yoshida effect. *Int J Mol Sci* 11: 4961–4972. Available:  
615 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?  
616 artid=3100829&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3100829&tool=pmcentrez&rendertype=abstract). Accessed 1 July 2013.
- 617 48. Mitsudome Y, Takahama M, Hirose J, Yoshida N (2014) The use of nano-sized acicular  
618 material, sliding friction, and antisense DNA oligonucleotides to silence bacterial genes.  
619 *AMB Express* 4: 70. Available: [http://www.pubmedcentral.nih.gov/articlerender.fcgi?  
620 artid=4230895&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4230895&tool=pmcentrez&rendertype=abstract). Accessed 6 April 2016.
- 621 49. Terry KL, Karageorgi S, Shvetsov YB, Merritt MA, Lurie G, et al. (2013) Genital powder  
622 use and risk of ovarian cancer: a pooled analysis of 8,525 cases and 9,859 controls. *Cancer  
623 Prev Res (Phila)* 6: 811–821. Available: <http://www.ncbi.nlm.nih.gov/pubmed/23761272>.  
624 Accessed 18 November 2016.

- 625 50. Muscat JE, Huncharek MS (2008) Perineal talc use and ovarian cancer: a critical review. *Eur*  
626 *J Cancer Prev* 17: 139–146. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18287871>.  
627 Accessed 18 November 2016.
- 628 51. Anderson EL, Sheehan PJ, Kalmes RM, Griffin JR (2016) Assessment of Health Risk from  
629 Historical Use of Cosmetic Talcum Powder. *Risk Anal*. Available:  
630 <http://doi.wiley.com/10.1111/risa.12664>. Accessed 18 November 2016.
- 631 52. Wu W-N, McKown LA (2004) In Vitro Drug Metabolite Profiling Using Hepatic S9 and  
632 Human Liver Microsomes. *Optimization in Drug Discovery*. Totowa, NJ: Humana Press. pp.  
633 163–184. Available: <http://link.springer.com/10.1385/1-59259-800-5:163>. Accessed 18  
634 November 2016.
- 635 53. Barrett JC, Lamb PW, Wiseman RW (1989) Multiple mechanisms for the carcinogenic  
636 effects of asbestos and other mineral fibers. *Environ Health Perspect* 81: 81–89. Available:  
637 <http://www.ncbi.nlm.nih.gov/pubmed/2667990>. Accessed 3 November 2016.
- 638 54. Foley-Comer AJ, Herrick SE, Al-Mishlab T, Prêle CM, Laurent GJ, et al. (2002) Evidence  
639 for incorporation of free-floating mesothelial cells as a mechanism of serosal healing. *J Cell*  
640 *Sci* 115: 1383–1389. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11896186>. Accessed  
641 18 November 2016.
- 642 55. Mendes GP, Vieira PS, Lanceros-Méndez S, Kluskens LD, Mota M (2015) Transformation of  
643 *Escherichia coli* JM109 using pUC19 by the Yoshida effect. *J Microbiol Methods* 115: 1–5.  
644 Available: <http://linkinghub.elsevier.com/retrieve/pii/S0167701215001517>. Accessed 3  
645 November 2016.
- 646 56. Hall BM, Ma C-X, Liang P, Singh KK (2009) Fluctuation analysis CalculatOR: a web tool  
647 for the determination of mutation rate using Luria-Delbruck fluctuation analysis.  
648 *Bioinformatics* 25: 1564–1565. Available:  
649 <http://www.pubmedcentral.nih.gov/articlerender.fcgi?>  
650 [artid=2687991&tool=pmcentrez&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2687991&tool=pmcentrez&rendertype=abstract). Accessed 1 May 2014.
- 651 57. Kaes C, Katz A, Hosseini MW (2000) Bipyridine: The Most Widely Used Ligand. A Review  
652 of Molecules Comprising at Least Two 2,2'-Bipyridine Units. *Chem Rev* 100: 3553–3590.  
653 Available: <http://dx.doi.org/10.1021/cr990376z>. Accessed 6 April 2016.
- 654 58. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image  
655 analysis. *Nat Methods* 9: 671–675. Available: <http://dx.doi.org/10.1038/nmeth.2089>.  
656 Accessed 9 July 2014.
- 657 59. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in  
658 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97: 6640–6645.  
659 Available: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?>  
660 [cmd=Retrieve&db=PubMed&dopt=Citation&list\\_uids=10829079](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10829079).
- 661 60. R Development Core Team (n.d.) R Development Core Team (2013). *R: A language and*  
662 *environment for statistical computing*. R Foundation for Statistical Computing, Vienna,

663 Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.

664 61. Ponder RG, Fonville NC, Rosenberg SM (2005) A switch from high-fidelity to error-prone  
665 DNA double-strand break repair underlies stress-induced mutation. *Mol Cell* 19: 791–804.  
666 Available: <http://www.cell.com/article/S1097276505015224/fulltext>. Accessed 3 April 2016.

667

668

## 669 **Figure Legends**

670

671 **Figure 1.** Changes in mutant frequency induced by sepiolite treatment in *E. coli* MG1655 stationary  
672 phase cells. The friction force is exerted by a glass spatula over agar surface spreading the mixture  
673 of sepiolite and bacteria during 1, 2 and 3 minutes. The increase of mutant frequency after friction  
674 treatment with sepiolite supports the notion that clays can be mutagenic for bacteria in this situation.  
675 Plotted values correspond to the median of five replications while error bars represent the  
676 interquartile ranges.

677

678 **Figure 2.** Box plotting of the survival of *E. coli* MG1655 to the action of friction with sepiolite  
679 during 1, 2 and 3 minutes of treatment as described in Material and Methods section. Groups with  
680 and without sepiolite gently spread with glass beads onto agar plates were used as controls. Note  
681 how as spreading time (time of friction) is increased, viability decreases. Stationary phase cells  
682 seem to be more resistant to the killing.

683

684 **Figure 3.** Plasmid pET-19b extracted from sepiolite-treated *E. coli* DH5 $\alpha$ , a *recA* deficient strain,  
685 during one minute (five extractions per treatment). Note the enrichment in linearised plasmid DNA  
686 molecules from bacteria treated with sepiolite under two minutes of friction applied in 1% agarose  
687 gel (A). The plasmid pET-19b digested with a single cut site enzyme NdeI was used as a control for  
688 the linear molecule migration rate and as a reference to calculate relative intensities using a  
689 densitometry analysis with the gel tool of ImageJ (B).

690

691 **Figure 4.** Inactivation of the *recA* gene suppresses the mutagenic effect of sepiolite under friction in  
692 *E. coli* MG1655. This indicates that the mutagenic effect is due to double-strand break repair of  
693 DNA that depends on RecA protein. Sepio 2' WT and Sepio 2' *recA* represent a treatment with  
694 sepiolite during two minutes for the WT and its derivative *recA* mutant respectively. Plotted values  
695 correspond to the median while error bars represent the interquartile ranges.

696

697

698 **Figure 5.** Samples of stationary phase *E. coli* MG1655 treated with sepiolite were prepared for a  
699 scanning electro microscopy examination. Note the dimensions of bacteria compared to the fibres  
700 and the penetration by sepiolite fibres when friction is applied. Red arrows represent potential sites  
701 of sepiolite fibre penetration. In contrast, this kind of interaction were not observed in samples that  
702 were friction was not applied.

703

704 **Figure 6.** Removal of sepiolite fibres longer than 1  $\mu\text{m}$  decreases fibre-induced mutagenesis to the  
705 level of the control. Filtered, dry and reconstituted sepiolite (in the graph long fibre-depleted  
706 sepiolite, lf-depleted sepiolite) was used in a mutagenesis experiment. Dry and reconstituted  
707 sepiolite (normal sepiolite) and bacterial cells (labelled as control) with no sepiolite were used to  
708 compare the effects of long fibre removal. The application of friction force was set in two minutes.  
709 Plotted values correspond to the median while error bars represent the interquartile ranges.

710

711 **Figure 7.** Asbestos (crocidolite fibres) show similar mutagenic properties that sepiolite, with an  
712 increase in mutant frequency around one order of magnitude bigger than control or asbestos without  
713 friction. Crocidolites, one of the most hazardous asbestos, contains high level of iron but the  
714 addition the chelating agent as 2-2' bipyridyl did not significantly decrease the mutagenesis  
715 indicating that at least in stationary phase bacteria, metals are not responsible for mutations. Values  
716 correspond to the median of five replications while error bars represent the interquartile ranges.

717

718 **Figure S1.** Treatment sepiolite and friction do not increase mutant frequency when *E. coli* MG1655  
719 grows exponentially. The lack of mutagenesis can be explained because double strand breaks are  
720 not mutagenic because in this phase [61]. Plotted values correspond to the median of five  
721 replications while error bars represent the interquartile ranges.

722

723 **Figure S2.** Although sepiolite and other minerals contain metals such as iron or aluminum, the  
724 addition of a chelating agent as 2-2' bipyridyl does not significantly suppress or diminish the  
725 mutagenic effect of sepiolite. Note also that in stationary phase bacteria, there is a high level of  
726 proteins such as DPS intended to control free iron inside the cell.

727

728 **Figure S3.** Direct observation of sepiolite fibres by scanning electro microscopy with two different  
729 magnifications. Note the heterogeneity in sizes, the fibrous nature and the small diameter  
730 compatible with the penetration capability.

731

732 **Figure S4.** Scanning electro microscopy of asbestos fibres used in mutagenesis experiment with  
733 two different magnifications. In comparison with sepiolite, asbestos (crocidotile in this case) show  
734 longer and sharper fibres.

735

736 **Figure S5.** A proposed approach to testing genotoxicity of asbestos or any industrial nano-fibres.  
737 The design is intended to resemble physiological conditions of the pleural cavity, one of the most  
738 frequently affected tissue by asbestosis. PDMS sheets can be colonised by human or animal cell  
739 lines (ideally mesothelial or epithelial cells) in appropriate conditions. For the experiments, two  
740 sheets of colonised PDMS pieces can be attached with both cell monolayers facing each other  
741 forming a 'sandwich', place it in a tissue culture dish (containing culture medium or ideally pleural  
742 liquid) and incubate with the desired amount of fibres. The same procedure without fibres can be  
743 used to generate a control group. The PDMS sheets can be removed at a given time, and both,  
744 attached and free cells can be analysed using different genotoxicity tests such as the formation  
745 micronucleus or other types of chromosome aberration. PDSM offer the advantage that is  
746 chemically inert, gas permeable and the surface can be easily modify or treated to meet the required  
747 properties for cell adhesion and growth. This system could be useful to study cytotoxicity of fibres  
748 in vitro.

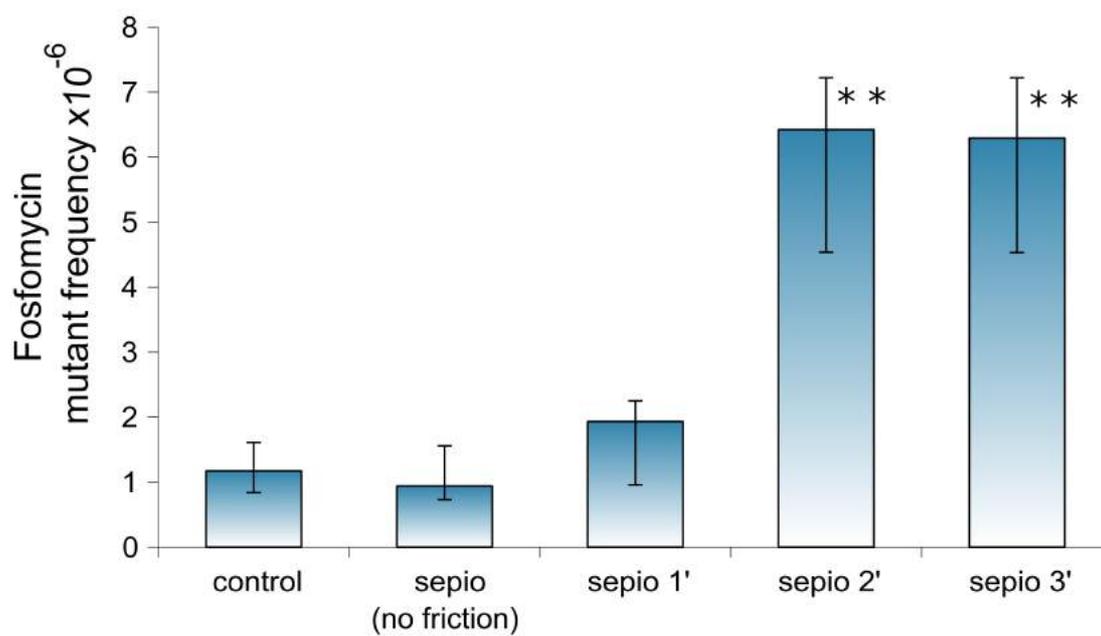


Figure 1.

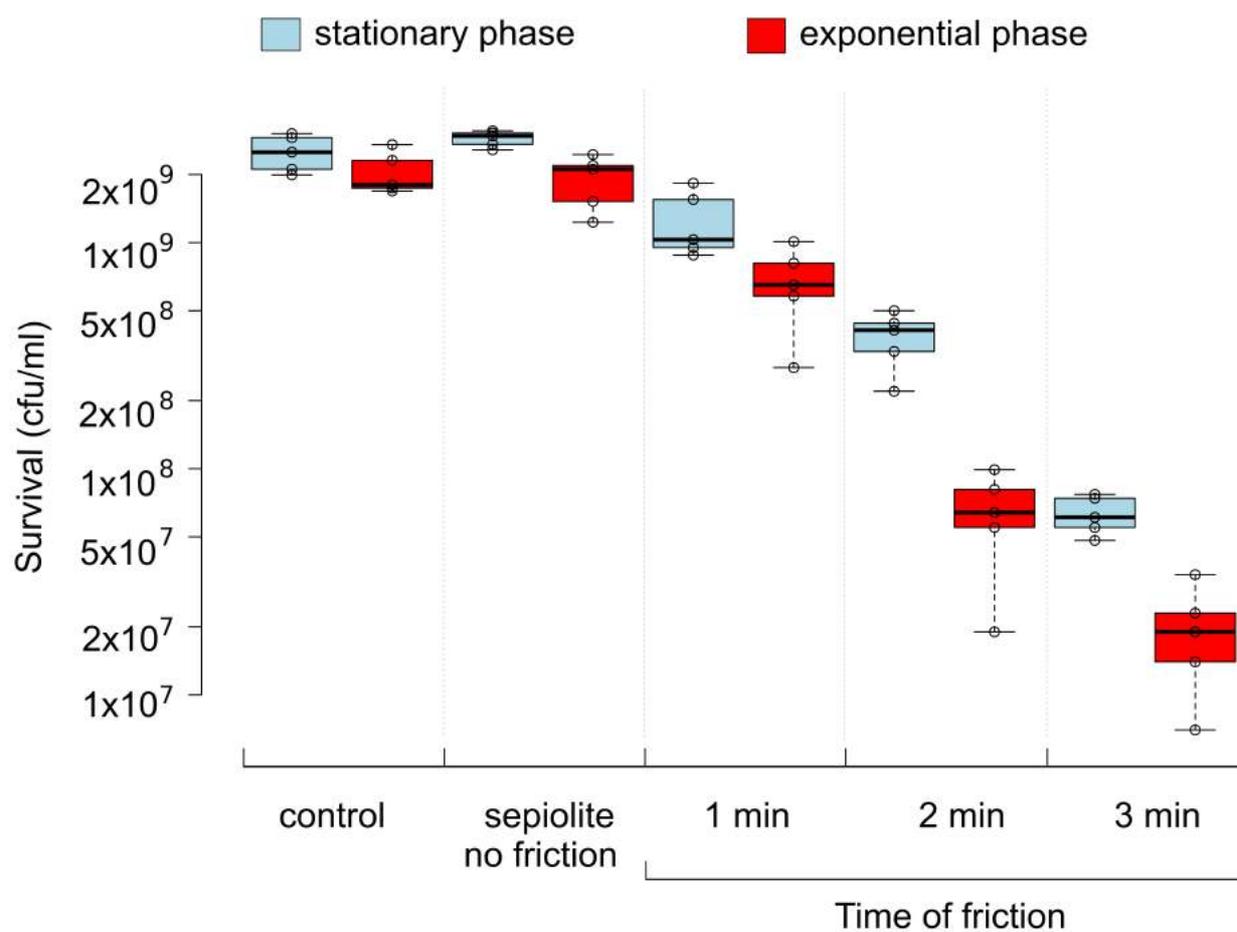


Figure 2.

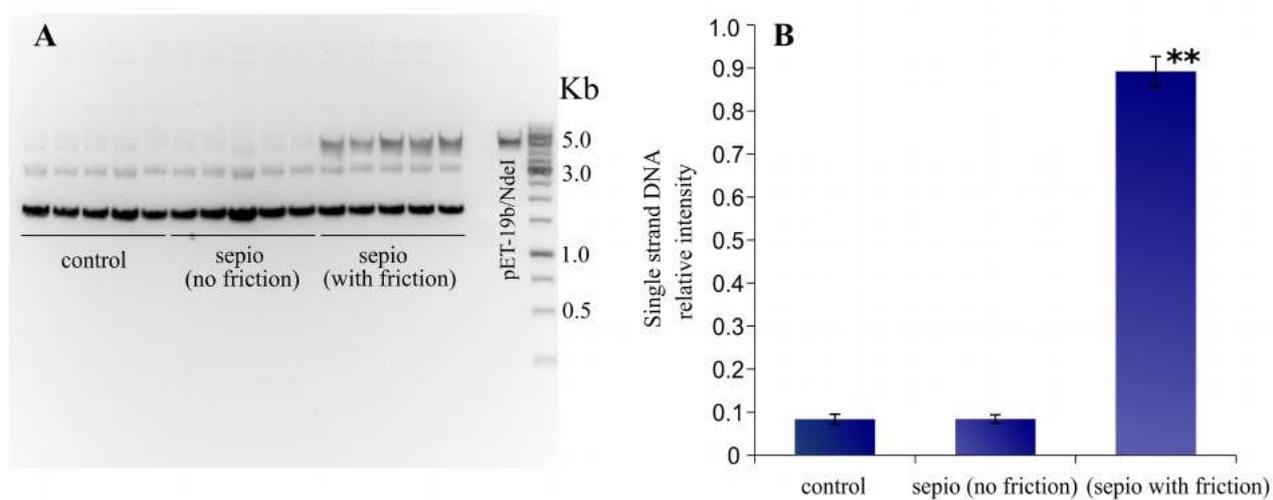


Figure 3.

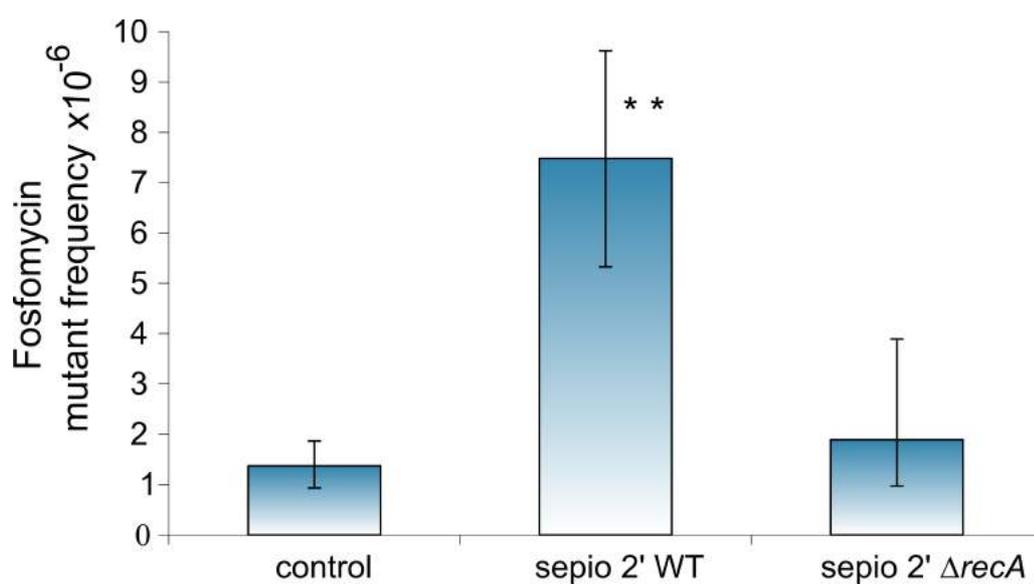


Figure 4.

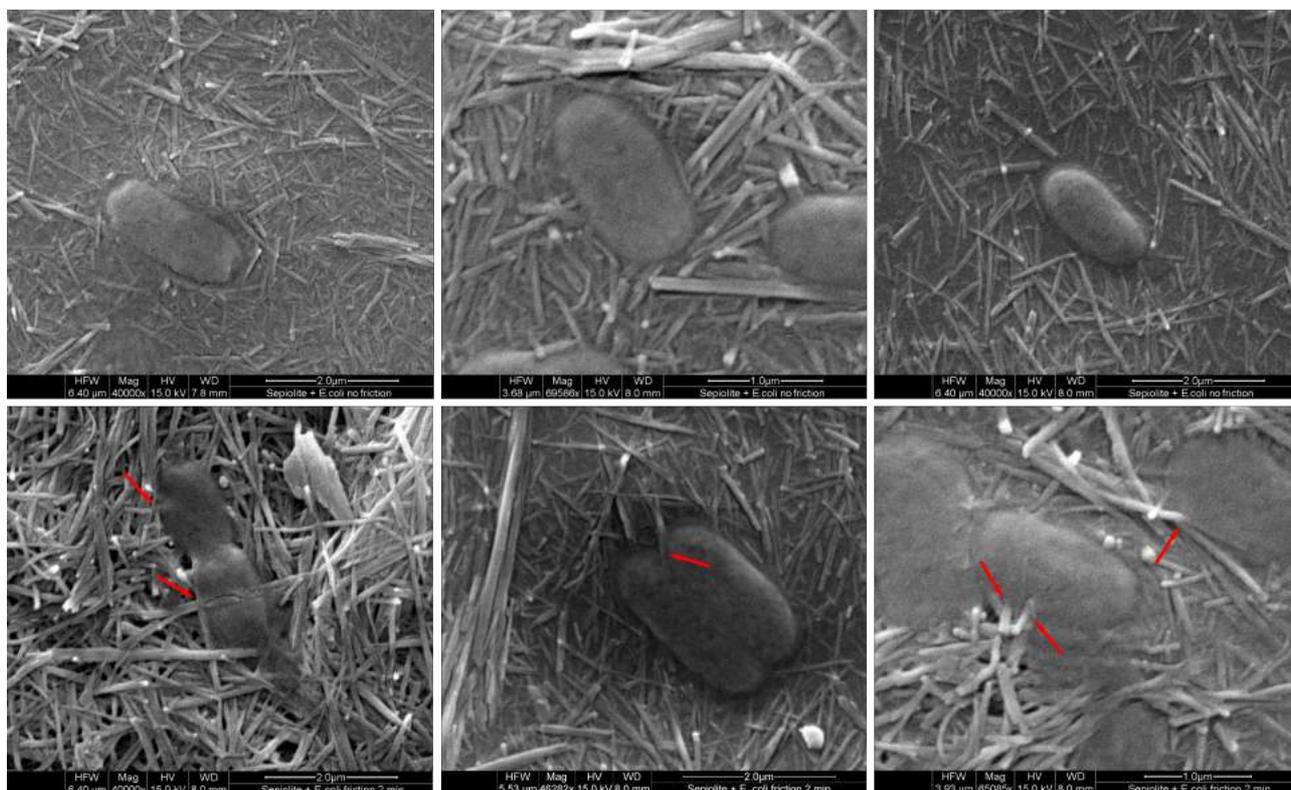


Figure 5.

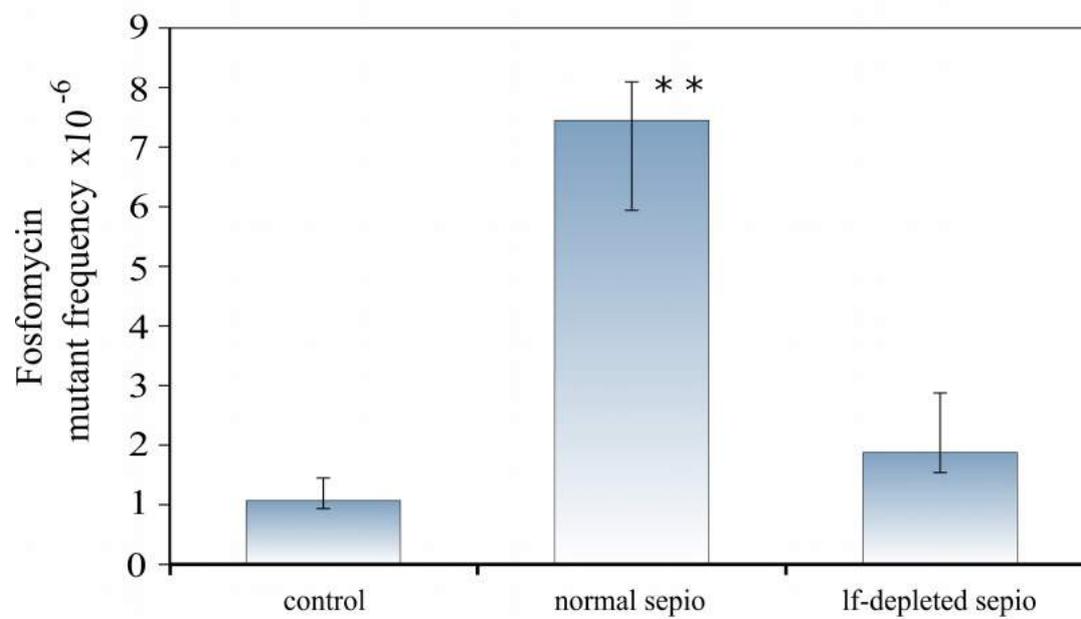


Figure 6.

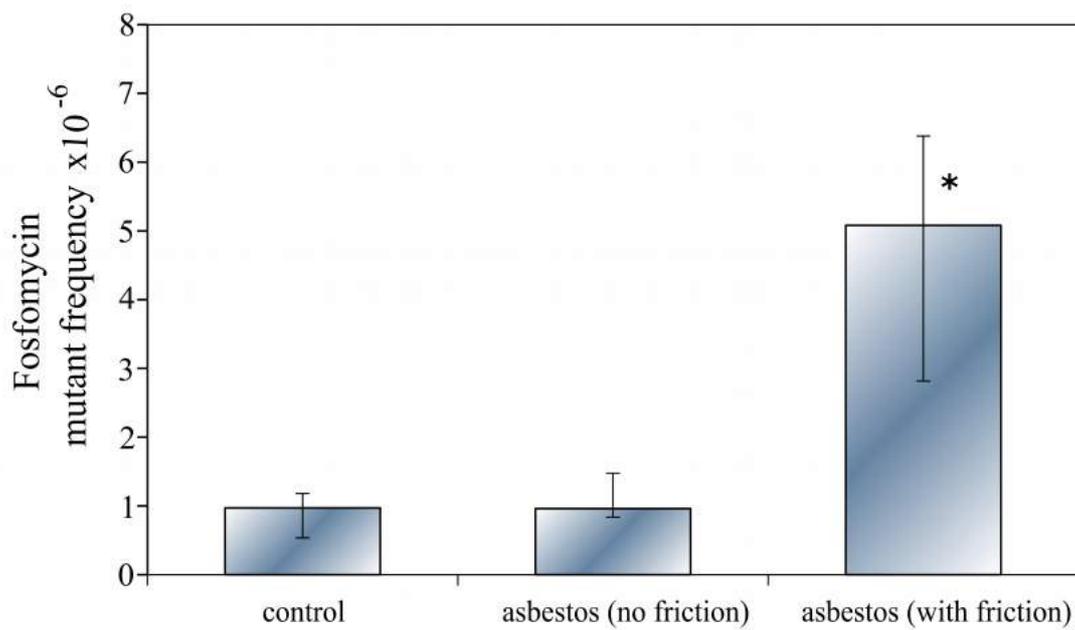


Figure 7.

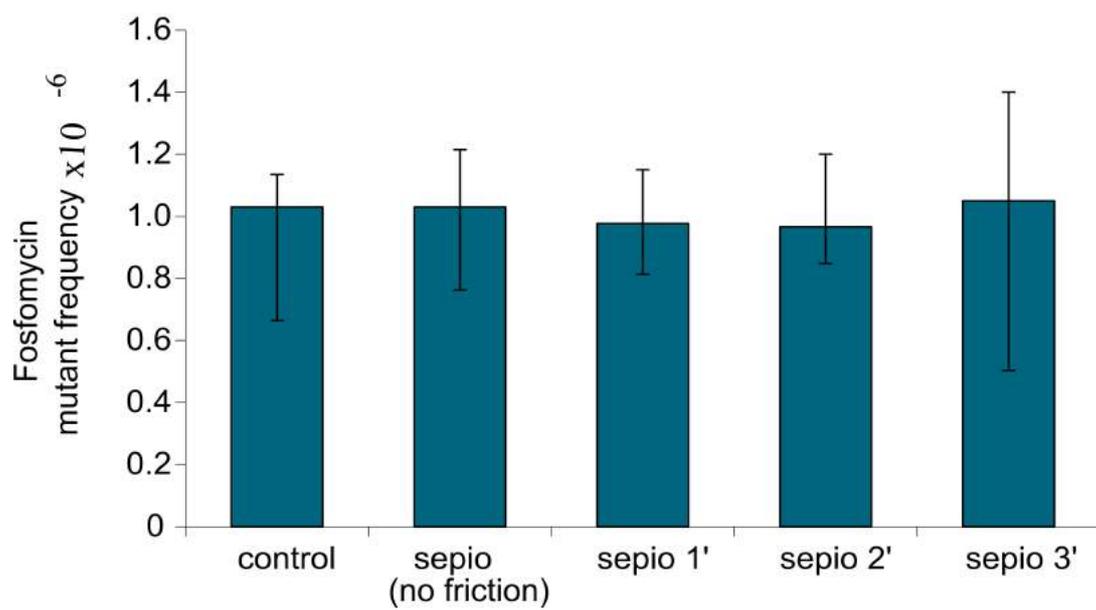


Figure S1.

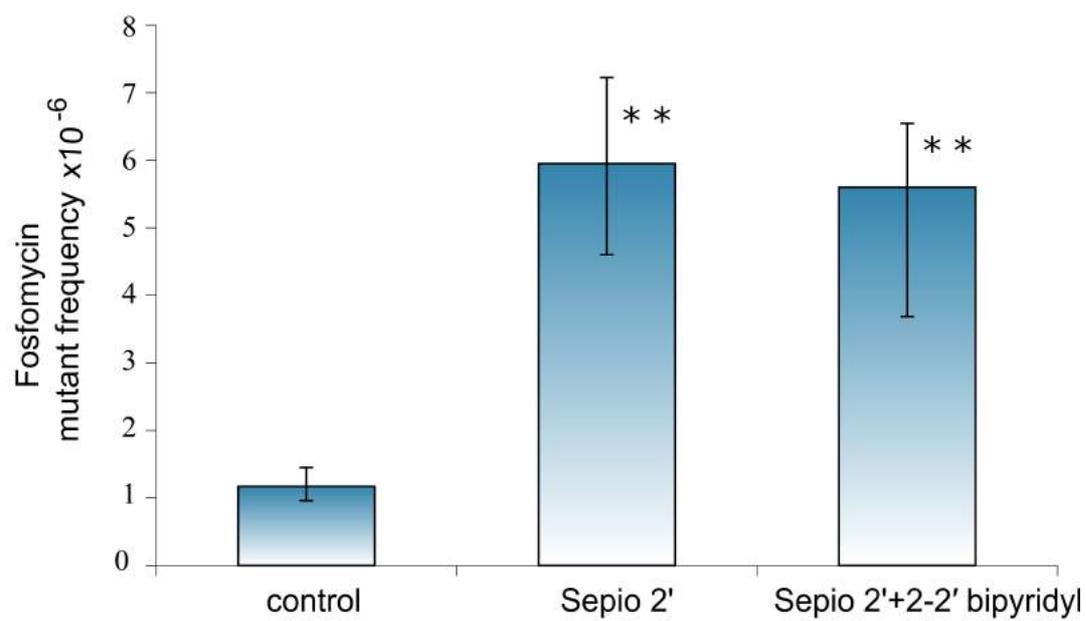


Figure S2.

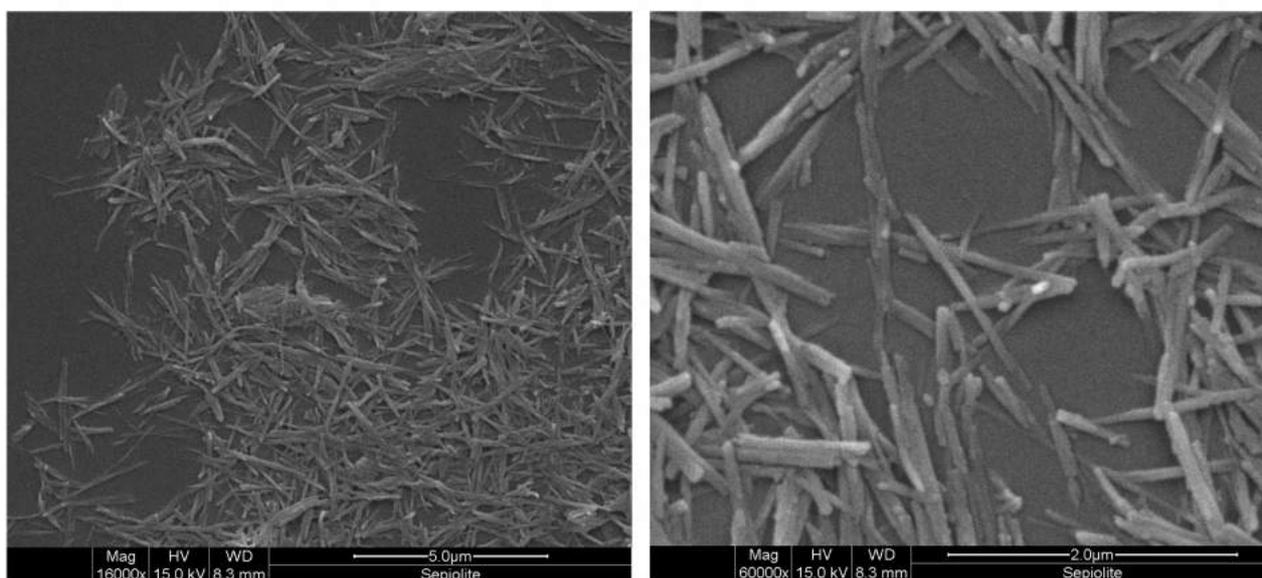


Figure S3.

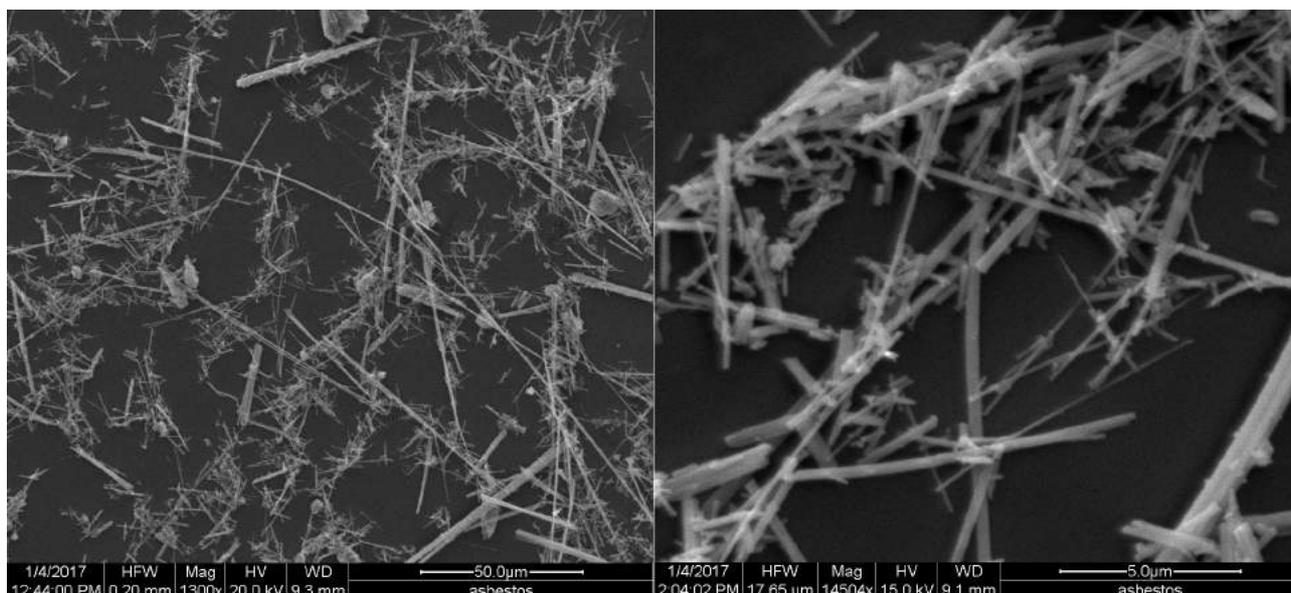


Figure S4.

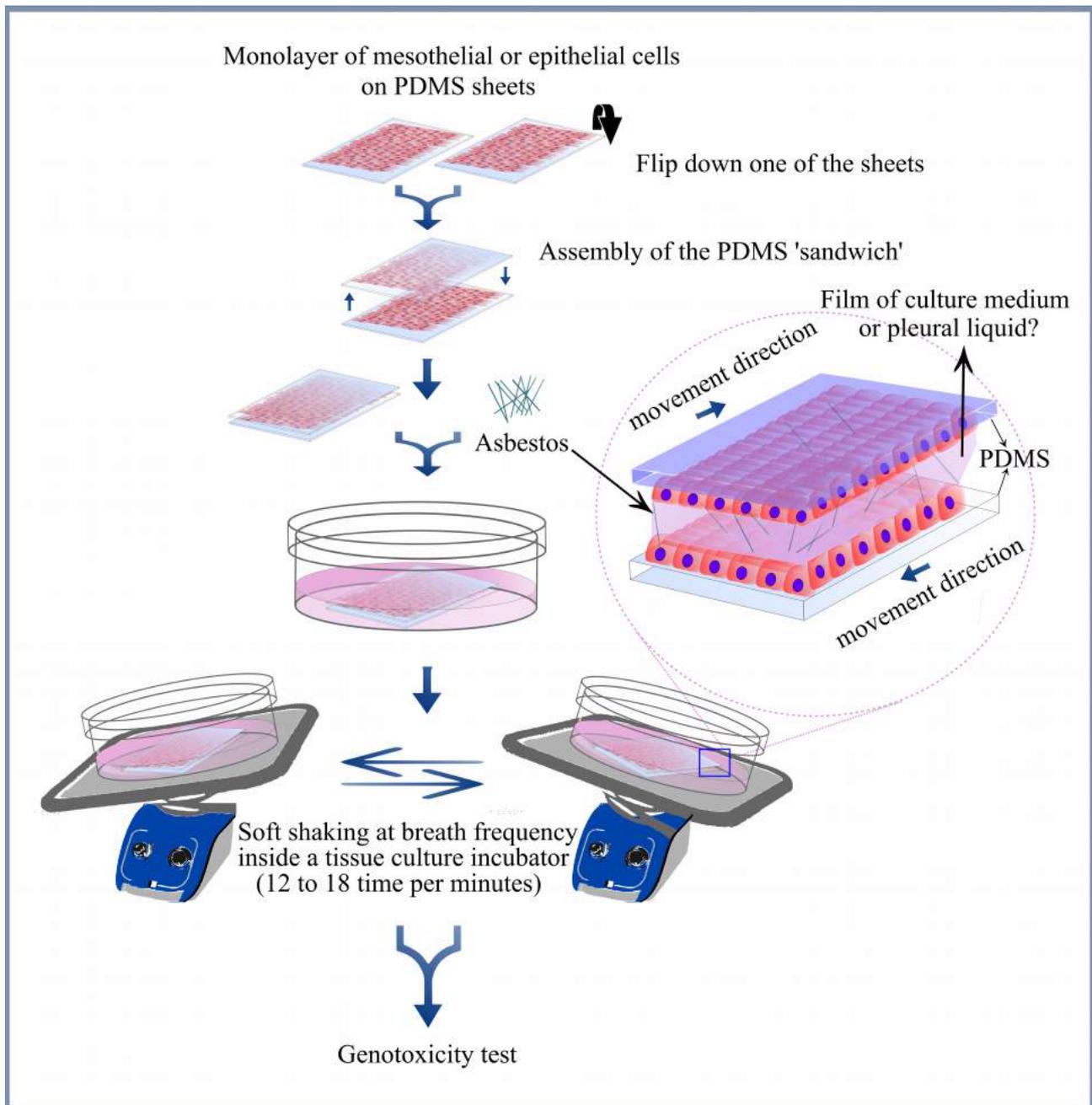


Figure S5.