RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

INTERNATIONAL MICROBIOLOGY (2015) 18:189-194
doi:10.2436/20.1501.01.249. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Dormancy in *Deinococcus* sp. UDEC-P1 as a survival strategy to escape from deleterious effects of carbon starvation and temperature

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Received 29 July 2015 · Accepted 20 September 2015

Summary. Dormancy is characterized by low metabolism and absence of protein synthesis and cellular division enabling bacterial cells to survive under stress. The aim was to determine if carbon starvation and low temperature are factors that modify the proportion of dormant/active cells in *Deinococcus* sp. UDEC-P1. By flow cytometry, RedoxSensor Green (RSG) was used to quantify metabolic activity and Propidium Iodide (PI) to evaluate membrane integrity in order to determine the percentage of dormant cells. Cell size and morphology were determined using scanning electronic microscopy. Under carbon starvation at 30°C, *Deinococcus* sp. UDEC-P1 increased its proportion of dormant cells from 0.1% to 20%, decreased the count of culturable cells and average cell volume decreased 7.1 times. At 4°C, however, the proportion of dormant cells increased only to 6%, without a change in the count of culturable cells and an average cellular volume decrease of 4.1 times and 3% of the dormant cells were able to be awakened. Results indicate a greater proportion of dormant *Deinococcus* sp. UDEC-P1 cells at 30ºC and it suggests that carbon starvation is more deleterious condition at 30ºC than 4ºC. For this reason *Deinococcus* sp. UDEC-P1 cells are more likely to enter into dormancy at higher temperature as a strategy to survive. [**Int Microbiol** 18(3):189-194 (2015)]

Keywords: *Deinococcus* · dormancy · metabolism · starvation · flow cytometry

Introduction

Bacterial metabolism depends directly on physiochemical environmental conditions, such as carbon availability [31]. Under nutrient-limited conditions, bacteria exhibit intermittent growth [24] and a bacterial population often contains cells in distinct physiological states: metabolically active, damaged, dead, or dormant [17]. Dormant cells are characterized by low metabolism, halted DNA and protein synthesis and non-proliferation [22]. Lennon and Jones [21] proposed that dormant bacteria serve as a "seed bank," permitting the later recuperation of the population once conditions improve. In fact, metabolic dormancy can be induced in response to unfavorable environmental changes, triggering modifications in gene expression and protein synthesis, which in turn reduce bacterial metabolism [5,12]. In low temperature environments, for example, bacterial cell have almost undetectable metabolic activity [25,27].

It has been described that bacteria belonging to the genus *Deinococcus* have a greater ability to resist and survive under rigorous conditions [11,30]. Considering that limited nutrients and temperatures close to the freezing point of water are known to decrease the rate of bacteria growth and lead to metabolic dormancy [2,16,27], the aim of this work was to determine if carbon starvation and low temperature are factors that modify the proportion of dormant/active cells in *Deinococcus* sp. UDEC-P1.

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Material and methods

Bacterial strain. *Deinococcus* sp. UDEC-P1 was isolated from Témpanos Lake, an oligotrophic lake in the Chilean Patagonia [7] and *Escherichia coli* HB101 was used as control.

Carbon starvation. The bacterial strains were cultured at 30°C in R2A broth for 36 h with constant shaking (120 rpm). Subsequently, 30 ml of the culture was centrifuged at 1500 *g* for 30 min at 4°C, the supernatant was removed, and the pellet was resuspended in Mineral Saline Medium (MSM) [1], a procedure which was repeated three times. The washed bacterial cells were then inoculated in 100 ml of MSM without carbon source at a cellular density of ca 1.0 $\times 10^6$ cells/ml in Erlenmeyer and incubated at 30°C or 4°C for 20 days. The experiment was carried out three times at each temperature. Additionally, bacterial cells from the R2A broth were inoculated at a cellular density of *ca*. 1.0 $\times 10^6$ cells/ml in 300 ml of R2A broth and incubated at 30ºC with constant shaking (120 rpm) until the end of the exponential phase (48 h). At the beginning of the experiment, and after 20 days of carbon starvation, culturable cells were counted in agar R2A [10]. The number of cells with intact or damaged membrane was assessed by fluorescence microscopy using the Live/Dead *Bac*Light Bacterial Viability Kit (Molecular Probes, Oregon, USA). Bacterial counts were compared by means of an ANOVA statistical analysis, with a 95% confidence interval ($P < 0.05$).

Determination of metabolic activity and membrane integrity. Flow cytometry was used to evaluate the metabolic state of bacterial cells after 20 days of carbon starvation at 30°C or 4°C. First, aliquots were filtered through polycarbonate membrane filters, pore size 0.2 µm, and washed with PBS, after which the cells were resuspended in 0.5 ml of PBS and adjusted to a cellular density of 1.0 $\times 10^7$ cells/ml. Bacterial suspensions were stained with LIVE/DEAD *Bac*Light RedoxSensor Green Viability Kit (Molecular Probes, Oregon, USA) containing RedoxSensor Green (RSG), to quantify metabolic activity, and Propidium Iodide (PI), to evaluate membrane integrity. Analysis of the flow cytometry data was performed using FlowJo v.10 software, as recommended by MIFlowcyt [20]. In order to discriminate cells from the background debris, a gate for cell detection was applied. Bacterial cells were grouped into gates, by those stained with RSG, those with PI, and those not stained neither with RSG nor PI.

Bacterial cell size and volume. Using the cytometry data obtained in the previous step, the size variation of *Deinococcus* sp. UDEC-P1 cells stained with RedoxSensor Green (RSG+), Propidium Iodide (PI+), and those without staining (RSG-PI-) were compared using the *Forward Scatter* (FSC) parameter [34]. To determine the volume of cells exposed to carbon starvation at 30°C or 4°C, the length and width of 300 cell images, taken with a Scanning Electron Microscope (Jeol, JF12M6380LUV), were measured. The average cellular volume in each treatment was calculated after using the formula $V = \pi/4d^2(L - d/3)$ for each cell [8].

Cell awakening. *Deinococcus* sp. UDEC-P1 cells incubated without carbon source at 4°C for 20 days were stained with the Kit RedoxSensor Green Viability and separated by cell sorting into events stained with RSG and those without neither RSG nor PI dye. The separated cells were adjusted to a density of *ca.* 2.0 $\times 10^5$ cells/ml, and in each group, the culturable cell counts were determined by plating 0.1 ml on R2A agar.

To evaluate the awaking of the unstained cells, considered as dormant, 150 µL of this cellular suspension were mix with either 500µl of R2A broth or 250 µl of that broth plus 250 µL of a supernatant from a 24 h *Deinococcus* sp. UDEC-P1 culture [7]. These mixtures were incubated for 1 h at 30°C and culturable cell counts were determined by spreading 0.1 ml on R2A agar or on R2A agar supplemented with 1 ml of supernatant, as suggested by Ayrapetyan [2]. In addition, an aliquot of 500µl of the unstained events was stained with 1µl (1.67 mM) of SYTO 9, Green Fluorescent Nucleic Acid Stain (Molecular Probes, Oregon, USA), in order to detect DNA by flow cytometry in cells considered as dormant.

Flow cytometry and cell sorting. Flow cytometry was conducted with a BD FACSAria III sorter (BD, New Jersey, USA) equipped with a 20 mW 488 nm argon solid state laser. Bacterial samples were suspended in PBS buffer with 6.0 µm diameter microspheres at a concentration of approximately 10^6 beads/ml, and 100,000 events were analyzed with the Forward Scatter (FSC) and *Side Scatter* (SSC) detectors with a 500 to 5000 events/s sampling rate. Thresholds in FSC were set at 1000 and 200 for *Deinococcus* sp. UDEC-P1 and *E. coli* HB101, respectively. Bacteria stained with the RSG and SYTO 9 dyes were detected with a 530/30 band-pass filter, and the red fluorescent emission of the PI stain with a 616/23 band-pass filter. Fluorescence signals of stains were compensated using controls with only RSG or PI for a each experiment. The PI single-stained controls were performed using dead cells previously treated with ethanol at –20°C.

Cell sorting was carried out to obtain dormant bacterial subpopulations, for this purpose cells that emitted fluorescence (RSG or PI) were separated from the non-fluorescent cells and collected in a sterile flask contained PBS buffer.

Results

Cellular response to carbon starvation. At the beginning of the experiment, 89% of the *Deinococcus* sp. UDEC-P1 cells inoculated in MSM without carbon were stained with RSG, 10% with PI and 0.1% were unstained (Table 1). After 20 days of carbon starvation at 30°C, the number of culturable cells and cells with an intact membrane decreased one order of magnitude ($P < 0.05$), (Fig. 1A). Flow cytometry analysis showed that 68% of the *Deinococcus* sp. UDEC-P1 population incorporated PI, 25% were stained with RSG, and 7% were non-stained and therefore considered as dormant (Table 1). When the analysis did not included PI stained cells [3], dormant cells accounted for 20%. Under the same conditions, *E. coli* also significantly decreased its cell count ($P < 0.05$) (Fig. 1B), besides RSG stain cells decreased from 83% to 5% after carbon starvation while unstained cells increased from 5% to 71% (Table 1). When only cells with an intact membrane were taken into account, this proportion increased to 93%.

Under carbon starvation at 4°C for 20 days, *Deinococcus* sp. UDEC-P1 showed no change in culturable cells counts (Fig 1a). Flow cytometry showed that 76.4% of the cells stained with RSG and there was a 5% of dormant cells (Table 1), but representing 6% if only cells with intact membrane are considered. Under identical conditions, the *E. coli* significantly reduced the number of culturable cells ($P < 0.05$) (Fig. 1B). Approximately 61% of the *E. coli* cells were stained with PI, 5% with RSG, and 34% were unstained (Table 1). If PI stain cells are disregarded, unstained cells (dormants) account for 87%.

Table 1. Metabolic activity of *Deinococcus* sp. UDEC-P1 and *E. coli* HB101 performed by flow cytometry using the RedoxSensor Green Vitality Kit

	Deinococcus sp. UDEC-P1 (% cells)			*Escherichia coli* HB101(% cells)		
	RSG(+)[a]	RSG(–)[b]	RSG(–)[c]	RSG(+)[a]	RSG(–)[b]	RSG(–)[c]
Treatment	PI(–)	PI(+)	PI(–)	PI(–)	PI(+)	PI(–)
Control R2A broth	89.3%	10.5%	0.1%	83.4%	11.3%	5.3%
Carbon starvation at 30ºC	25.3%	67.8%	6.6%	5.3%	23.5%	71.3%
Carbon starvation at 4ºC	76.4%	18.6%	5.0%	5.0%	61.3%	33.6%

[a]Cells stained with RedoxSensor Green (RSG). [b]Cells stained with Propidium Iodide (PI). [c] Unstained cells .

Awakening. *Deinococcus* sp. UDEC-P1 cells that underwent carbon starvation at 4°C were separated, by cell sorting, and RSG+ cells were adjusted to ca $2.0x10^5$ ml^{-1} and 50% of these bacteria with an active metabolism were culturable (Table 2). Regarding unstained events (dormant cells), also adjusted to ca 2.0×10^5 cells/ml only 6.2×10^3 CFU/ml were culturable (3.1%) (Table 2). Incubating these cells for 1h in R2A broth supplemented with *Deinococcus* UDEC-P1 supernatant did not increased the number of culturable cells (Table 2). Furthermore, application of SYTO 9 to the unstained events separated by cell sorting indicated the presence of DNA in dormant cells.

Granularity, relative cell size and volume. Carbon starved *Deinococcus* sp. UDEC-P1 cells stained with RSG were larger than those unstained (or dormant cells) when cultured at 4°C or 30°C (Fig. 2) and both showed a decrease in cytoplasmic granularity, indicated by a lower Side Scatter (SSC) median value (1,163 at 30ºC and 1,152 at 4ºC) when compared to the control (1,589) after the analysis of 5,000 events per group. Cellular volume before carbon starvation ranged from 0.6 to 15.52 μm^3, with an average of 3.85 ± 2.1 μm^3 (Fig. 3A). After 20 days of carbon starvation at 30°C, the cells decreased their volume 7.1 times, with an average length of 1.05 ± 0.24 μm and showed a coccobacillar morphology (Fig. 3B). Cells incubated at 4°C were only 4.1 times smaller than their initial condition, with bacillar morphology and an average length of 1.27 ± 0.31 μm (Fig. 3C).

Discussion

It has been reported that during carbon starvation, some bacteria modify their morphology and cell size, decrease the synthesis of DNA, mRNA and proteins, and enter a reversible state of low or undetectable metabolic activity known as

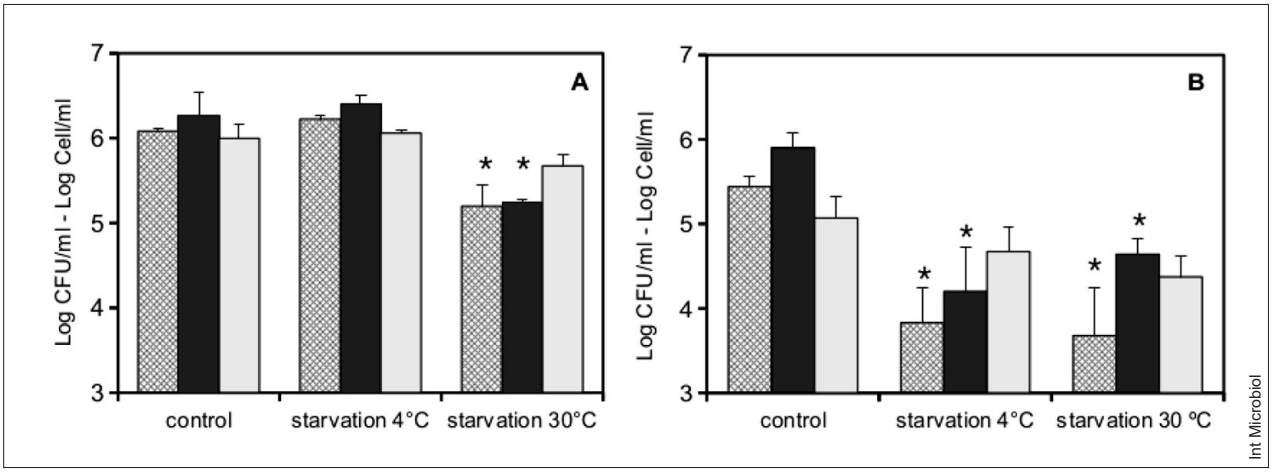

Fig. 1. Survival during carbon starvation. (**A**) Bacterial counts of *Deinococcus* sp. UDEC-P1 and (**B**) *Escherichia coli* HB101, at the beginning of the experiment (control) and after 20 days of carbon starvation at 4°C or 30°C. Culturable cells (dotted gray bar), (Log CFU/ml); cells with intact membrane (black bar), and cells with damaged membrane (gray bar), (Log Cell/ml). *Indicates significant differences ($P < 0.05$) between bacteria exposed to carbon starvation and those without carbon starvation. The data was obtained in three independent experiments.

Table 2. Awakening of dormant *Deinococcus* sp. UDEC-P1 cells, previously incubated under carbon starvation at 4ºC, and separated by cell sorting (adjusted at 2.0×10^5 cells/ml) non-stained events

	Cultivable cells (UFC/ml)	
Treatment	Agar R2A	Agar R2A + Supernatant[a]
Non-stained events	6.23×10^3	–
Non-stained events incubated 1h in R2A broth	6.51×10^3	7.28×10^3
Non-stained events incubated 1h in R2A broth supplemented with supernatant	6.73×10^3	7.49×10^3

[a]*Deinococcus* sp. UDEC-P1 culture in R2A broth without cells.

dormancy [9]. Kalyuzhnaya et al [14] indicated that bacterial cells non-stained by both IP and RSG are alive but dormant, having an intact membrane. Furthermore, Zacharias et al, [35] using a monoculture of *Eschericha coli,* compared the results of flow cytometry associated with the Live/Dead *Bac*Light Bacterial Viability Kit with those of qPCR, obtaining similar results with both methodologies with respect to viable bacteria. However, it has been described that high concentrations of PI (over 20 µg/mL) modifies the proportion of dormant/inactive cells [23] but PI concentrations used in the present work did not reached this concentration. Our results indicated that *Deinococcus* sp. UDEC-P1 increased the percentage of cells in dormancy under carbon starvation at 30°C. This increase was concomitant with a decreased count of culturable cells and an increase in the percentage of cells with a damaged membrane, indicative of a deleterious effect on this cellular structure [26]; while only 5% of *E. coli* HB101 cells under carbon starvation were metabolically active. This last percentage is consistent with that described by Rezaeinejad & Ivanov [29] also for *E. coli*. Additionally these authors determined that metabolically inactive cells preserve their protein motor force and are, therefore, potentially viable.

The lower proportion of dormant cells in *Deinococcus* sp. UDEC-P1, when compared to *E. coli* HB101, may be attributed to the fact that dormancy plays a central role in ensuring survival in adverse conditions for *E. coli* [17,19,32]. On the other hand *Deinococcus* sp. UDEC-P1 cells, contain cytoplasmic inclusions such as polyphosphate and carbohydrate granules [7,29,33], that could prolong metabolic activity in *Deinococcus* sp. UDEC-P1delaying the entrance into dormancy, as it has been previously suggested in other

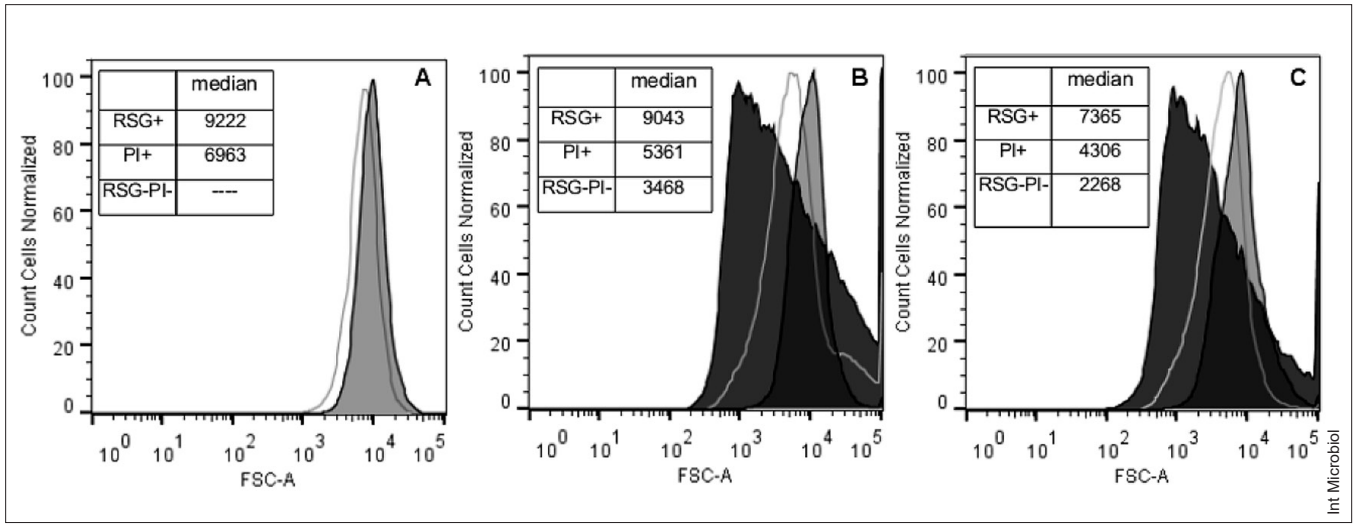

Fig. 2. Comparison of cell sizes of the different metabolic states of *Deinococcus* sp. UDEC-P1. Cells stained with RSG (white curve) or PI (gray curve) and those not stained (black curve) were grouped in different gates and expressed in function of *Forward Scatter* (FSC), proportional to cell size. These results were compared using the median value of the cells at the beginning of the experiment.

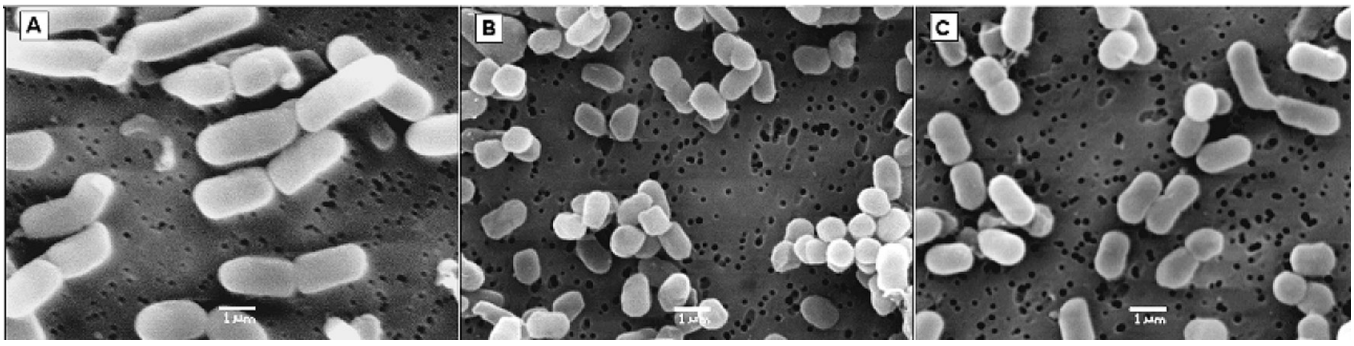

Fig. 3. Scanning electronic microscopy of *Deinococcus* sp. UDEC-P1. Image at the beginning of the experiment (**A**), and after carbon starvation for 20 days at 30°C (**B**) and 4°C (**C**).

bacterial strains [13,28]. Our results indicated that under carbon starvation, the percentage of survival of *Deinococcus* sp. UDEC-P1 cells was larger at 4°C than at 30ºC. Furthermore, at 4ºC a higher proportion of cells showed active metabolism in comparison to 30°C. Perhaps, the detrimental effects of carbon starvation at 30ºC, due to a more active metabolism, results in a more rapid dead and damaged to cells [15].

Epstein [6] described that low levels of recovery of dormant cells could be due to a deep state of dormancy, with undetectable metabolic activity. Indeed, it has been proposed that the reduction of bacterial metabolism is gradual, with metabolically active cells existing alongside those with low metabolic activity and others in a state of deep dormancy [32]. These dormant cells are not immediately culturable, and the recuperation of their metabolic activity is gradual, or stochastic, according to the microbial scout hypothesis [4,6]. In concordance with this hypothesis, the presence of molecules able to stimulate the waking of dormant cells was not detected in the supernatant of *Deinococcus* sp. UDEC-P1. However, previous studies in this strain have described mechanisms of Quorum Sensing associated with stress [2,7]. For this reason, we suggest that future studies investigating the relationship between Quorum Sensing signals and the entry into and exit from dormancy in *Deinococcus* sp. UDEC-P1 should be done.

Carbon starvation causes morphological and cellular volume changes in *Deinococcus* sp. UDEC-P1. A smaller cell size was detected in bacteria incubated at 30°C than at 4°C, reaffirming the more intense effect of carbon starvation at 30ºC than at 4ºC. By flow cytometry analysis of the *Deinococcus* sp. UDEC-P1 cells considered dormant showed a smaller size than metabolically active cells, similar to that described by Kim et al., [18]. Moreover, our results indicate that *Deinococcus* sp. UDEC- P1 decrease the cytoplasmic granularity upon carbon starvation at both temperatures, indicating the consumption of intracellular material [26].

The dormant cells detected in *Deinococcus* sp. UDEC-P1 under carbon starvation at 4°C showed only a 3% culturability**,** this could be related to a low metabolic activity and reduced growth rate described in dormant cells [9,22]. Potential toxicity of RSG stain upon *Deinococcus* sp UDEC-P1 was discarded because cells stained with RSG were 52% culturable [14]. Furthermore, staining dormant events with SYTO 9 revealed the presence of DNA, indicating that the unstained events correspond to cells.

Our results indicate that the strain *Deinococcus* sp. UDEC-P1 is less affected during carbon starvation at 4ºC. On the other hand, the increased proportion of dormant cells at 30ºC could reflect a cellular survival response to deleterious conditions, allowing them to maintain their genetic material and restart growth once conditions improve. These findings also help to explain *Deinococcus* ubiquity in extreme conditions.

Acknowledgments. This study was supported by grants Fondecyt 1100462 and Enlace VRDI No. 214.036.041-1.0. Additionally, the authors would like to thank Ruth Contreras for her technical support and Sara Schilling for her editing support and Dr. Carlos T. Smith for the critical review of the manuscript

Competing interests. None declared.

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