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Biofilm-derived spores of Clostridioides (Clostridium) difficile exhibit increased thermotolerance compared to planktonic spores

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Abstract

Biofilm-derived spores of strains of four ribotypes (001, 020, 027 & 078) of Clostridioides (Clostridium) difficile were found to exhibit increased thermotolerance compared to spores produced in planktonic culture. In addition, 'thick' and 'thin' exosporium morphotypes described previously were visualised by electron microscopy in both biofilm and planktonic spores.

Keywords; C. difficile; heat treatment; spores; biofilm; germinants

Clostridioides (Clostridium) difficile is a pathogen of concern worldwide, causing C. difficile infection (CDI). CDI manifests in ranging severity, the most common presentation is diarrhoea (1). Although commonly associated with the nosocomial setting, studies have shown a lack of a clear transmission pathway in a substantial number of cases in the UK (2). A number of environmental reservoirs have been suggested including food, gardening products, lawns and animals (3-9). C. difficile has been isolated from cooked meats, food products (4), as well as raw meat products (10). In addition C. difficile spores have been isolated from raw 'ready to eat' vegetables in France (11). Spores produced within biofilms have been found to have increased heat resistance compared to planktonic spores in Bacillus cereus (12). Spore thermotolerance is of clinical importance as food is often heated at temperatures below that necessary to eradicate or inactivate C. difficile spores. The current study seeks to compare the thermoresistance of C. difficile spores produced in planktonic and biofilm cultures.

In this study, spores of four PCR ribotypes (001, 020, 027 & 078) were produced in liquid media; ~5 x 10^8 spores were aliquoted in to 500ml of BHI (supplemented with 0.1% taurocholate) and incubated anaerobically for 10 days. For all ribotypes, biofilm-derived and planktonically-derived spore populations were produced. For production of spores in planktonic culture, flasks were continuously shaken at 180RPM for the duration of incubation. Flasks for biofilm cultivation were not shaken.

After 10 days, the contents were centrifuged at 3750RPM. The spores were purified using a modified protocol utilising HistoDenz™ as previously described (13). Briefly, the pellet was resuspended in 400µl of 20% HistoDenz™ and layered on to 500µl of 50% HistoDenz™. The solution was centrifuged at 15000g for 15 minutes, after which the supernatant containing vegetative cells and cell debris was carefully removed. The pellet was washed three times in PBS and resuspended in 1ml of PBS. Spore suspensions were aliquoted in to 450µl of PBS in Eppendorfs. Eppendorfs were transferred to a heat plate and heated for 1 hour at 80 ℃ . At time points 0, 15, 30 and 60 minutes 20µl aliquots were serially diluted in PBS in a 96-well plate. Twenty-microlitres of the appropriate dilution were streaked on to CCEYL agar and incubated anaerobically for 48 hours.

Planktonic and biofilm produced spores of the RT 027 strain were visualised by TEM. Spores were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 150 minutes. Two subsequent washes in 0.1M phosphate buffer were performed. Osmium tetroxide (1%) was used to stain samples overnight. Sample dehydration was performed by incubation with an ascending alcohol series (20, 40, 60, 80, 100%) with each step consisting 60 minutes. These steps were performed in Eppendorf tubes, with samples centrifuged and resuspended after each stage. Spores were embedded in an epoxy resin using a accelerator and hardener left overnight to polymerise at 60℃ (14). Samples were cut in to thin sections (~80-100nm) using an ultramicrotome which were picked up on 3.05mm copper grids. Grids were stained with saturated uranyl acetate (120 minutes) and Reynolds lead citrate (30 minutes). Samples were visualised at a maximum of 10000X direct magnification in the bright field setting on a JEOL JEM1400 TEM at 120kV. Images were taken on an AMT 1k CCD using AMTv602 software.

Statistical analysis was carried out by IBM SPSS version 22. Data normality was assessed visually by histograms and statistically with Kolmogorov-Smirnov tests. Homogeneity of variance between groups was assessed using Levene's test. $\bar{x}$ represents the mean of two or more specified ribotypes. All means are reported with the standard error of the mean

(SEM). P values < 0.05 were considered significant, < 0.01 very significant and < 0.001 highly significant.

Significant decreases in spore recovery were observed in all spores after 60 minutes of 80℃ heat treatment (Figure 1). Biofilm spores had increased viability at the 60 minute time point versus planktonic produced spores ($\bar{x}$ = 5.62 ± 0.07 vs 4.49 ± 0.05 $\log_{10}$ CFU/ml; P < 0.001). The greatest decrease in spore viability in both biofilm and planktonic spores was present after 15 minutes ($\bar{x}$ = 7.47 ± 0.02 vs 5.79 ± 0.07 $\log_{10}$ CFU/ml & 7.42 ± 0.08 vs 4.96 ± 0.10 $\log_{10}$ CFU/ml; P < 0.001). A gradual decline in spore recovery was observed in planktonic spores of the RT 020 and RT 027 strain between 15 and 60 minutes (4.69 ± 0.02 vs 4.47 ± 0.03 $\log_{10}$ CFU/ml & 4.45 ± 0.04 vs 4.13 ± 0.02 $\log_{10}$ CFU/ml). In contrast, biofilm spores of three strains (RT 020, RT 027 & RT 078) showed no significant difference in spore recovery at 15 vs 60 minutes ($\bar{x}$ = 5.72 ± 0.09 vs 5.68 ± 0.08 $\log_{10}$ CFU/ml; P = 0.73). The most heat resistant spores of any type were the RT 078 biofilm spores (6.18 ± 0.03 $\log_{10}$ CFU/ml). Planktonic RT 078 spores were also more heat resistant than planktonic spores of other strains (4.84 ± 0.06 vs $\bar{x}$ = 4.38 ± 0.20 $\log_{10}$ CFU/ml; P < 0.001).

Endospores were observed in both biofilm and planktonic culture produced RT 027 samples (Figure 2). Two morphotypes of spore with differing exosporium sizes were observed in both sets of spores. In addition, detached exosporium was visible in in both samples.

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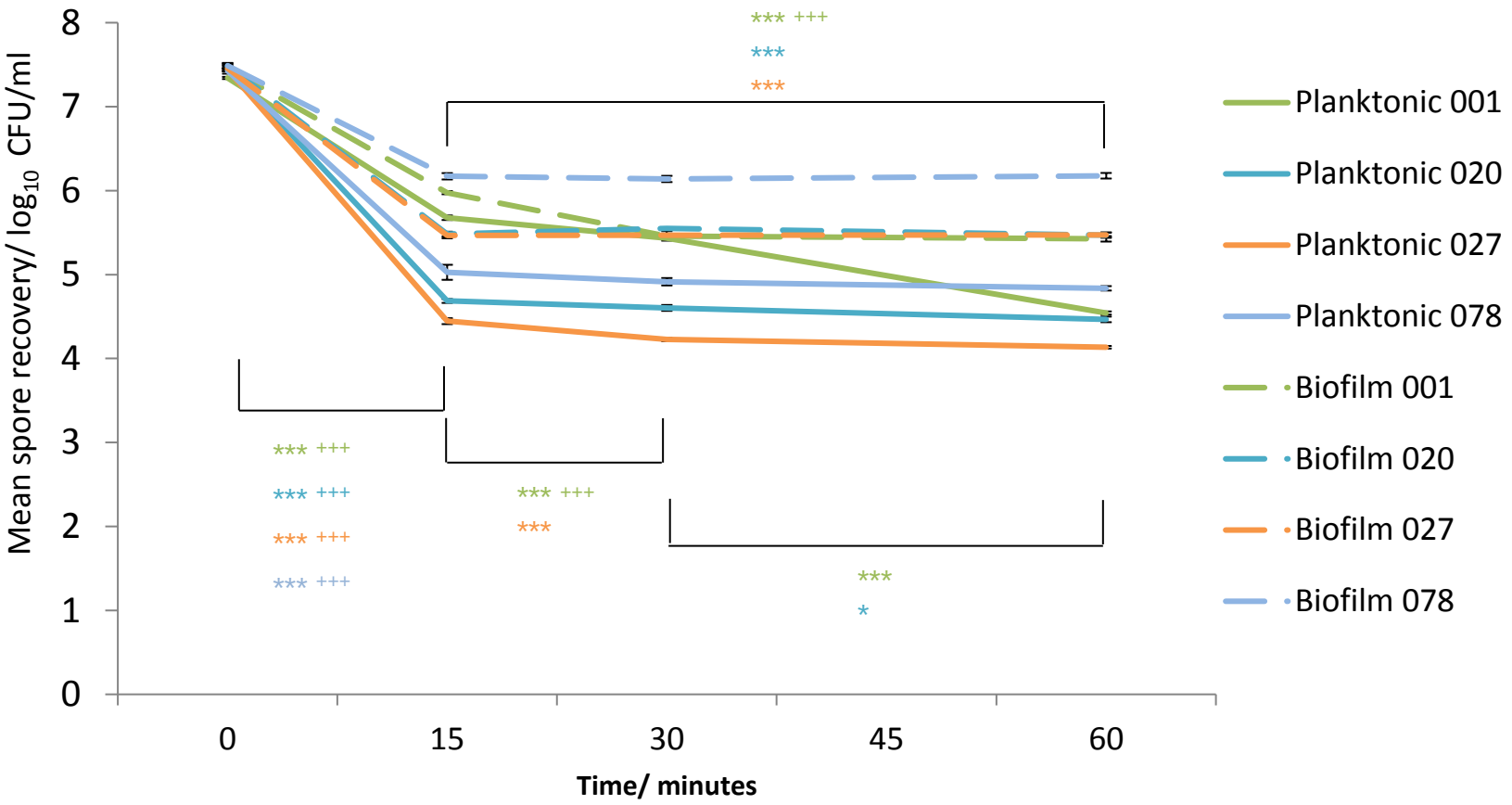

Figure 1. Mean (± SE) spore recovery of four ribotypes (001, 020, 027 & 078) of C. difficile heated for 60 minutes at 80℃. Both biofilm and planktonic culture produced spores are present. Spores were enumerated at 0, 15, 30 & 60 minutes. Experiments were carried out in biological duplicate and processed in technical triplicate. Spore recovery was compared between time points using RM-ANOVA with Tukey's multiple comparisons. Statistically significant (P < 0.05) results are highlighted by *, very significant (P < 0.01) by ** and highly significant (P < 0.001) by *.The $^{+}$ symbol is used for biofilm derived spores, * for planktonic culture derived spores.**

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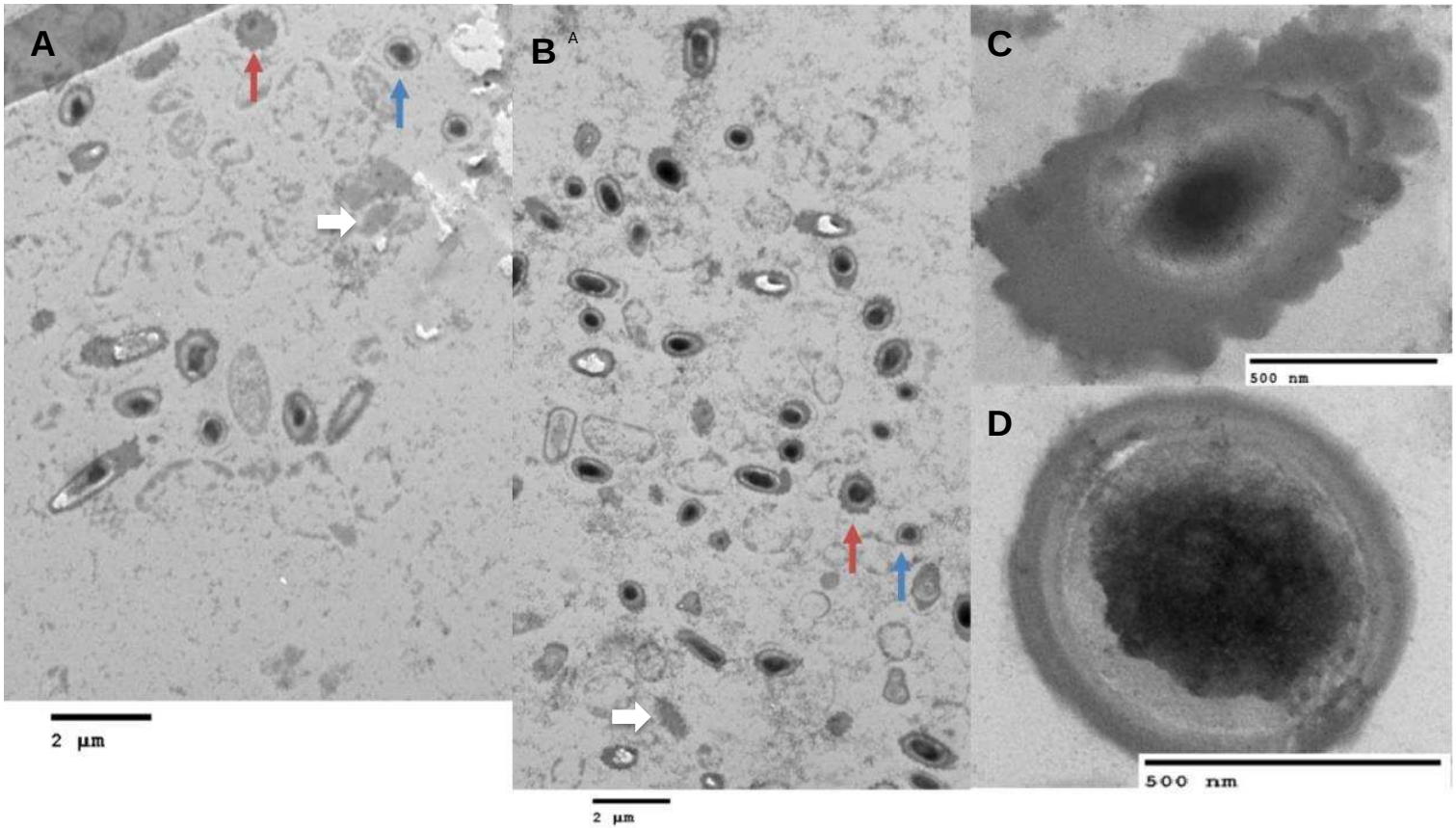

Figure 2. Transmission electron microscopy (TEM) images (1000X magnification) of biofilm produced spores (A) and planktonic culture produced spores (B). Both sets were produced from the RT 027 strain used previously in this study. Two spore morphotypes are visible in both; thick-exosporium spores are designated by red arrows, thin-exosporium morphotype spores by blue arrows. Detached exosporium was visible in micrographs of both samples (white arrows). Higher magnification (10000X) example images of thick (C) and thin-exosporium (D) spores are presented.

After 60 minutes, the viability of biofilm produced spores was ~1 $\log_{10}$ CFU/ml higher than spores produced in planktonic culture. Transmission electron microscopy showed the presence of thin and thick-exosporium spores in both samples. These observations were made previously in the R20291 strain (15). Unfortunately the processing of samples from other strains used in this study was not practicable. No quantitative measurement of exosporium size or spore morphotype number was possible. Despite purification of spores by density gradient centrifugation using HistoDenz™, detached exosporium was present in both samples. These results are congruent with a previous study highlighting the presence of cellular debris in spore preparations following purification by density gradient centrifugation (16). An increased presence of exosporium/extracellular matrix in the biofilm produced spores could result in a more heat-resistant population.

One study also found that C. difficile spores produced in biofilms began to accumulate a surrounding ‘shroud’ that attached to the spore after 7-14 days of incubation (17). This ‘layer’ was found to consist of dead cellular debris, and it is hypothesised C. difficile spores accumulate this layer after mother cell lysis (17). In addition, biofilm generated spores were found to be less responsive to germinants and exhibited decreased germination. If the increased heat resistance of biofilm produced spores is due to an extracellular matrix/ shroud or an intrinsic spore property, biofilm spores in non-laboratory conditions are likely to retain this resistance. Biofilm produced spores are still likely to exhibit increased heat resistance in non-laboratory scenarios. On the other hand, in the first 15 minutes of 80℃ heat treatment biofilm spores exhibited log-linear inactivation kinetics. Previously, it has been suggested the ‘shoulder’ seen in some heat inactivation models is due to an extracellular matrix buffering the effects of heat (18).

Further work providing quantitative measurements and exploring a range of ribotypes is needed to strengthen conclusions. Despite these limitations, this study supports the existence of two distinct C. difficile spore morphotypes. Building on previous work, it is suggested biofilm produced C. difficile spores are more environmentally robust and could

exhibit increased levels of heat resistance. Further work will need to clarify the importance of both detached and attached exosporium in relation to spore thermotolerance.

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