Effect of Sterilization by Dry Heat or Autoclaving on Bacterial
Penetration through Berea Sandstone

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Received 1 July 1985/Accepted 16 October 1985

A study was undertaken to determine why bacteria could penetrate lengths of consolidated sandstone (Berea)

faster when the sandstone was sterilized by autoclaving than when dry heat (150°C, 3 h) was used. Changes in

permeability, porosity, and pore entrance size of the rock as a result of autoclaving were not sufficient to explain

the differences in penetration times observed, but electron dispersion spectroscopy and electron microscopy of

the rock revealed changes in mineral composition and clay morphology. Autoclaved cores contained more

chloride than dry-heated cores, and the clays of autoclaved cores were aggregated and irregularly shaped.

Therefore, the decreases in bacterial penetration rates caused by autoclave sterilization were probably the

result of a change in surface charge of the pores of the rock and of a reduction in surface area of clays available

for adhesion. The results implied that dry-heat sterilization was preferable to autoclaving when examining

biotic and abiotic interactions in a native-state rock model.

MATERIALS AND METHODS

Organisms and cultivation medium. Strain 140 was isolated from a subterranean sample underlying the Norman

Municipal Landfill. BCI-1NS was isolated from a Berea sandstone core (Cleveland Quarries, Amherst, Ohio). Both

isolates are morphologically similar to cells of the genus Bacillus (7).

The growth medium (medium E with 0.1% [wt/vol] NaNO3 and 0.05% [wt/vol] yeast extract) and cultivation

conditions for both strains have been described previously (7).

Core preparation. For experiments involving cell penetration, cores were prepared as described previously (7). The

sides of each core were coated with epoxy. Berea sandstone from Cleveland Quarries was used in all experiments. The

permeability of 3/4-in.-diameter (1.91 cm) cores was determined by using a permeameter and Darcy’s law as described

previously (7); the results are given in millidarcys.

Sterilization of cores. (i) Autoclave. Cores used in penetration studies were presaturated under vacuum with 5%

(wt/vol) NaCl solution. The saturated cores were submerged in the brine and autoclaved at 121°C and 15 lb/in2 for 30 min.

The initial permeability (K0) was determined by using 5% NaCl solution previously filtered through a 0.22-μm mem-

brane filter (Millipore Corp., Bedford, Mass.). The cores were then placed in growth chambers containing the growth

medium as described previously (7) and autoclaved for an additional 20 min.

(ii) Dry heat. Cores were sterilized by dry heat in a forced-draft oven (Allied Fisher Scientific Co., Dallas, Tex.)

at 150°C. The cores were saturated with the 5% NaCl solution and the K0 was determined. The cores were then

placed in a glass petri dish and heated. Dry-heat-sterilized cores were removed aseptically from the petri dish and

vacuum saturated with sterile medium E containing 0.1% (wt/vol) NaNO3 and 0.05% (wt/vol) yeast extract. They were

then aseptically placed in growth chambers.

Penetration time. Penetration time was the time (in hours) elapsed from inoculation of one flask of the growth chamber

until faint visible turbidity occurred in the other flask of the growth chamber (7).
TABLE 1. Effect of autoclaving or dry-heat sterilization of sandstone cores on the rate of microbial penetration through Berea sandstone cores

<table>
<thead>
<tr>
<th>Strain</th>
<th>Core no.</th>
<th>Dry heat</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Penetration time (h)</td>
<td>$K_s$ (mdarcy)</td>
<td>$K_r$ (mdarcy)</td>
</tr>
<tr>
<td>140d</td>
<td>132</td>
<td>103</td>
<td>213.8</td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>103</td>
<td>314.5</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>ND</td>
<td>245.9</td>
</tr>
<tr>
<td>BCI-1NS</td>
<td>132</td>
<td>96</td>
<td>365.6</td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>60-75</td>
<td>445.3</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>ND</td>
<td>429.3</td>
</tr>
</tbody>
</table>

- Each core was 7.4 cm long and 1.9 cm in diameter. The same cores were used throughout the experiment, with core 134 serving as an uninoculated control. The sequence in which the treatments were applied was as follows: dry-heat sterilization, inoculated with strain 140; dry-heat sterilization, inoculated with BCI-1NS; and autoclaved, inoculated with BCI-1NS.
- Both strains are motile and were grown at 50°C in medium E plus 0.1% NaNO3 and 0.05% yeast extract.
- Dry-heat sterilization was at 150°C for 3.0 h. Autoclaving was for 20 min at 121°C. $K_s$ and $K_r$ refer to core permeabilities measured before inoculation and after incubation, respectively.
- It was later found that strain 140 was capable of penetrating in 27.5 h a 6.1-cm-long Berea core ($K_s = 202.0$ mdarcys) that had been sterilized by autoclaving.
- ND, Not determined.

Pore entrance size distribution. The centrifugation method of Slobod et al. (10) as modified by Torbati et al. (11) was used to determine the capillary pressure profiles of the sandstone cores at increasing centrifugal forces. An ultracentrifuge (model L5-50B; Beckman Instruments, Inc., Fullerton, Calif.) was used. Pore entrance size distribution was generated from the capillary pressure curve and from physical data on the length, diameter, dry weight, and wet weight of the core by using a computer program (E. C. Donaldson, personal communication).

EDS and SEM. Both electron dispersion spectroscopy (EDS) and scanning electron microscopy (SEM) of sandstone were done as previously described by Crocker et al. (4). Three Berea cores (2- by 6-in. (5- by 15-cm) cylinders) were cut from a 12- by 12- by 6-in. (30- by 30- by 15-cm) block of sandstone and steam cleaned for 2 weeks to remove humic substances as described previously (F. D. Sutterfield, M. S. thesis, University of Tulsa, Tulsa, Okla., 1973). Each core was saturated with 5% (wt/vol) NaCl solution under vacuum. Core 1 was sterilized by autoclaving at 121°C and 15 lb/in² for 1 h while submerged in brine. Core 2 was sterilized by heating in a forced-draft oven at 150°C for 3 h. Core 3 was steam cleaned but not brine saturated or sterilized. Samples for analysis were taken from both ends of each core and from a point midway between the two ends.

RESULTS AND DISCUSSION

The penetration time for strains BCI-1NS and 140 was dependent on the method of core sterilization used (i.e., dry heated or autoclaved). The same cores (132, 133, 134) were used for all penetration experiments shown in Table 1 to eliminate variations between different cores. Cores that were sterilized by dry heat had longer penetration times compared with autoclaved cores of the same length. Both sterilization methods increased the permeability of inoculated and uninoculated cores which indicated that changes in permeability cannot explain the differences in penetration times observed with the two sterilization methods. The times for the penetration of 7.4 cm of dry-heat-sterilized rock were 103 h for both cores inoculated with strain 140, whereas, strain BCI-1NS took from 60 to 75 or 96 h to penetrate the same cores after additional dry-heat sterilization. When these same cores were then autoclaved, the time for penetration by BCI-1NS was four to six times faster. These penetration times for autoclaved cores are well within the times previously reported for strain BCI-1NS under similar conditions (7).

Successive autoclaving decreased the penetration times of strain BCI-1NS through Berea cores of 7.4 to 7.6 or 11.2 to 11.5 cm in length (Table 2). The penetration times of 7.4- to 7.6-cm-long cores in Table 2 are similar to the penetration times reported in Table 1 after autoclaving. The 3.6- to 3.7-cm-long cores had faster penetration times that were unaffected by successive autoclaving.

Strain 140 penetrated a 6.1-cm-long autoclaved Berea core ($K_s = 202.0$ mdarcys) in 27.5 h or approximately 3.5 times faster than it took to penetrate a 7.4-cm-long core that was dry-heat-sterilized. Jenneman et al. (7) previously showed that penetration rate was independent of length for cores with permeabilities above 200 mdarcys by using autoclaved Berea cores and strain BCI-1NS. So the small difference in length (6.1 versus 7.4 cm) cannot account for the large increase in penetration time observed when strain 140 was grown in an autoclaved core. The presence of either a dry-heat-sterilized core or an autoclaved core did not inhibit growth of BCI-1NS in liquid culture (results not shown). Therefore, it was concluded that some change occurred in the sandstone during autoclaving.

The modest increases in permeability observed after autoclaving previously dry-heat-sterilized cores (Table 1) did not seem sufficient to account for the large decreases observed in penetration times because the penetration rate of BCI-1NS in Berea cores with permeabilities above 200 mdarcys is not affected by increasing permeability (7). Therefore, it was necessary to examine what other physical changes in the rock occurred as a result of autoclaving and dry-heat sterilization.

The autoclaving process might have increased the sizes of pore entrance in the rock, thereby allowing the cells easier penetration.

TABLE 2. Effect of successive autoclave treatments on penetration time of BCI-1NS through Berea sandstone cores

<table>
<thead>
<tr>
<th>Core no. and length (cm)</th>
<th>$K_s$ (mdarcy)</th>
<th>Penetration time after indicated autoclave treatment (h)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>B60, 3.6</td>
<td>269</td>
<td>8.5</td>
</tr>
<tr>
<td>B61, 3.7</td>
<td>205</td>
<td>9.5</td>
</tr>
<tr>
<td>B62, 3.6</td>
<td>405</td>
<td>10.5</td>
</tr>
<tr>
<td>B57, 7.5</td>
<td>521</td>
<td>21.0</td>
</tr>
<tr>
<td>B58, 7.4</td>
<td>478</td>
<td>17.5</td>
</tr>
<tr>
<td>B59, 7.6</td>
<td>484</td>
<td>21.0</td>
</tr>
<tr>
<td>B63, 11.4</td>
<td>379</td>
<td>37.5</td>
</tr>
<tr>
<td>B64, 11.5</td>
<td>423</td>
<td>24.0</td>
</tr>
<tr>
<td>B65, 11.2</td>
<td>367</td>
<td>29.5</td>
</tr>
</tbody>
</table>

$^a$ Permeabilities were measured after the initial 30-min autoclaving period (see Materials and Methods).

$^b$ Each core was first autoclaved for 30 min, flushed with medium E with 0.05% yeast extract and 0.1% NaNO3, mounted in the growth chamber, and inoculated with BCI-1NS. After growth was observed in flask B, the treatment was repeated, except the core was autoclaved for 20 min before mounting and inoculating.
FIG. 1. Effect of autoclaving and dry heat on pore entrance size distribution of Berea sandstone. The pore entrance size distribution of four cores cut from a block of Berea sandstone (400 mdarcys) was determined before heating. (A) Two cores were autoclaved at 121°C for 30 min and then dry heated for 3 h at 150°C. (B) The other two cores were dry heated and then autoclaved as described in panel A. Pore entrance size distribution was determined after each heating step. Data obtained from only one of the replicate cores is shown because similar results were obtained for each core.

access through the core. However, the results from pore entrance size determinations of four different cores indicated that neither autoclaving nor dry-heat sterilization altered the distribution of pore entrance sizes in the sandstone (Fig. 1). Furthermore, the order of treatment (autoclave then dry heat [Fig. 1A] or dry heat then autoclave [Fig. 1B]) had little effect on the pore entrance size distribution. However, there were differences in distribution of pore entrance sizes between the different cores which is indicative of the inherent heterogeneity of consolidated rock samples.

Another physical property of the rock that might have been affected was porosity. Porosity is a measure of the fraction of void space to bulk volume in a porous medium. The effect of autoclaving and dry-heat sterilization on static and nonstatic porosity is shown in Table 3. Nonstatic porosity refers to the porosity determined after 5% NaCl solution was flushed through the core under a differential pressure of about 2.5 atm (253.2 kPa), whereas static porosity was measured without fluid flow through the core. Dry-heat sterilization did not appear to significantly change the static or nonstatic porosity of the cores tested, nor did autoclaving have a significant effect on the static porosity of these same cores. However, the nonstatic porosity of autoclaved cores increased by an average of 7.3% above values for the dry-heat-sterilized cores (Table 3). However, the increase in nonstatic porosity of autoclaved cores was not statistically significant at P < 0.1 when a two-tailed t test for the difference between two means was used (results not shown). Additional autoclaving had little or no effect on subsequent nonstatic porosity measurements (Table 3).

Crocker et al. (4) showed that the clay minerals in Berea

### Table 3. Effect of dry heat and autoclaving on the static and nonstatic porosity of Berea sandstone cores

<table>
<thead>
<tr>
<th>Core length (cm)</th>
<th>Porosity (mdarcy) by treatment:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Static&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>3.7</td>
<td>0.214</td>
</tr>
<tr>
<td>3.6</td>
<td>0.214</td>
</tr>
<tr>
<td>3.6</td>
<td>0.214</td>
</tr>
<tr>
<td>2.8</td>
<td>0.227</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cores used for static porosity measurements were not epoxied and were 2.54 cm in diameter. All cores were cut from a block of Berea sandstone (400 ± 50 mdarcy).

<sup>b</sup> Cores used for nonstatic porosity were epoxied on the sides and were 1.90 cm in diameter. Flow through cores was performed after each treatment at a differential pressure of 1.0 atm (103.3 kPa). Porosities were determined after flowing brine (5% NaCl) through core.

<sup>c</sup> Dry-heat treatment was for 3 h at 150°C, and autoclaving was for 20 min at 121°C while the core was submerged in 5% NaCl brine.

### Table 4. Effect of dry heat and autoclaving on elemental composition of the pore surface of Berea sandstone as determined by EDS

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SiO&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>88.7 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dry heat</td>
<td>84.9 ± 0.2</td>
</tr>
<tr>
<td>Autoclave</td>
<td>72.8 ± 10.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each core was prepared as described in the Materials and Methods section.

<sup>b</sup> Values obtained from only one or two subsamples taken along the length of the core.

<sup>c</sup> Mean ± standard deviation of the values of the three subsamples taken along the length of the core.

<sup>d</sup> ND, Not determined.
sandstone act to cement together the silica grains lining the pores as opposed to other sandstones in which the clays are dispersed freely within the pores. Therefore, it is possible that heating the cores in the presence of a hydrating phase could cause a dissolution of the clay and silica particles which would result in a change in the degree of cementation and hence porosity. This effect would be especially pronounced if the cores were subjected to a subsequent brine flush as was done when measuring nonstatic porosity. Baking sandstone cores (usually at 600°C) tends to stabilize clays and fines and reduces their movement in the presence of a flowing fluid (4). This may explain why nonstatic porosity did not increase with dry-heated cores.

EDS analysis of the surface of the dry-heat-sterilized cores and the autoclaved cores revealed some interesting differences (Table 4). The surfaces of the nontreated and dry-heated Berea sandstone cores had higher silica, aluminum, and potassium contents than the autoclaved cores. The dry-heated core had a much lower chloride content than the autoclaved core. Other ions (Fe$^{3+}$, Ca$^{2+}$, and Ti$^{4+}$) made up only a small or negligible part of the surface components. In all cases, the total amount of elemental oxides detected was near 100% of the total oxide measured in the sample so that if other ions were present they would be in very small quantities. These data are consistent with a change in porosity after autoclaving because a reduction in silica content indicates a loss in cementation between clays and the grain particles (4). Furthermore, decreases in the amounts of aluminum and potassium in autoclaved core samples are also consistent with a loss in clay content since clays are composed of these elements.

More importantly, these analyses suggest that a change in surface charge resulted from autoclaving. The large increase in chloride content and the reduction in the content of aluminum and potassium could have shifted the surface to a more negatively charged state which could cause a greater electrostatic repulsion between negatively charged bacterial cells and mineral surfaces. Chang and Yen (2) found that the addition of the anion pyrophosphate to cell suspensions decreased the degree of cell retention inside Berea sandstone. They attributed this to a higher repulsive energy induced at the surface of either the bacterial cell or the particle surface, thereby reducing the possibility of irreversible adhesion of cells.

SEM of untreated, dry-heated, and autoclaved Berea cores also showed that autoclaving changed the nature of the clay particles inside the core (Fig. 2). The untreated and dry-heated cores contained clay particles that were flat and platelike with irregularly shaped edges. The clay particles in the autoclaved core were clumped, with smooth and rounded edges.

The effect of autoclaving is manifold. Increases in the porosity of the autoclaved cores along with decreases in the amount of silica, aluminum, and potassium, which are elements often associated with clays, imply that autoclaving causes the removal of clay particles. However, no changes were observed in pore entrance size distribution, which would argue that these losses in cementation are not significant with respect to pore geometry. On the other hand, EDS of the surface of the autoclaved core showed a large increase in the chloride content compared with dry-heat-sterilized and nontreated cores. This could result in an increased negative charge at the surface of the rock which would cause a greater electrostatic repulsion between the negatively charged surfaces of the bacterial cells and the rock. SEM, although it does not indicate any quantitative change in clay content, shows that the clays have been structurally altered from their natural sharp-edged appearance to a more rounded edge and aggregated arrangement. These aggregated particles would have less surface area available for adhesion of cells than the flat, platelike clay particles observed in the nontreated and dry-heated samples. Therefore, it is likely that autoclaving resulted in an alteration of clays and of the surfaces lining the pores. In turn, these alterations
probably resulted in an increase in the content of negatively charged ions at the surfaces and in a reduction in surface area available for cell adhesion.

Understanding the effect of sterilization on the physical properties of porous rock and other porous media is crucial in explaining the role of biotic and abiotic factors in cellular penetration or retention. The information provided by this work should prove useful in future comparisons of bacterial penetration times and rates in different porous media. In addition, the results suggest that dry-heat sterilization may be a better method than autoclaving to determine the penetration rate of bacteria in native-state cores. This information will be useful for future studies of processes involving the cleanup of toxic wastes polluting groundwater and of microbially enhanced oil recovery, both of which may require transport of injected microorganisms through consolidated rock.

ACKNOWLEDGMENTS
This work was supported by Department of Energy contract no. DE-AC19-80BC10300 and by the Energy Resources Institute of the University of Oklahoma.

We thank H. M. Torbati and E. C. Donaldson for assistance in determining pore throat size distribution and D. E. Menzie and E. C. Donaldson for helpful comments.

LITERATURE CITED