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Thermal inactivation of *Enterococcus faecium*: effect of growth temperature and physiological state of microbial cells

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ABSTRACT

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Aims: To provide data on the effects on culture temperature and physiological state of cells on heat resistance of *Enterococcus faecium*, which may be useful in establishing pasteurization procedures.

Methods and Results: The heat resistance of this *Ent. faecium* (ATCC 49624 strain) grown at different temperatures was monitored at various stages of growth. In all cases, the bacterial cells in the logarithmic phase of growth were more heat sensitive. For cells which had entered in the stationary phase, D_{70} values of 0·53 min at 5°C, 0·74 min at 10°C, 0·83 min at 20°C, 0·79 min at 30°C, 0·63 min at 37°C, 0·48 min at 40°C and 0·41 min at 45°C were found. By extending the incubation times cells were more heat resistant as stationary phase progressed, although a different pattern was observed for cells grown at different temperatures. At the lower temperatures heat resistance increased progressively, reaching D_{70} values of 1·73 min for cells incubated at 5°C for 50 days and 1·04 min for those grown at 10°C for 16 days. At other temperatures assayed heat resistance became stable for late stationary phase cells, reaching D_{70} values of 1·05, 1·08 and 1·01 min for cultures incubated at 20, 30 and 37°C. Heat resistance of cells obtained at higher temperatures, 40 and 45°C, was significantly lower, with D_{70} values of 0·76 and 0·67 min, respectively. Neither the growth temperature nor the growth phase modified the *z*-values significantly.

Conclusions: D_{70} values obtained for *Ent. faecium* (ATCC 49624) varies from 0·33 to 1·73 min as a function of culture temperature and physiological state of cells. However, *z* values calculated were not significantly influenced by these factors. A mean value of 4·50 ± 0·39°C was found.

Significance and Impact of the Study: Overall results strongly suggest that, to establish heat processing conditions of pasteurized foods ensuring elimination of *Ent. faecium*, it is advisable to take into account the complex interaction of growth temperature and growth phase of cells acting on bacterial thermal resistance.

Keywords: *Enterococcus faecium*, food safety, growth phase, growth temperature, heat resistance, pasteurization.

INTRODUCTION

Enterococcus faecium is widely distributed in foods and due to high thermal resistance (Hardie and Whiley 1997; Morrison *et al.* 1997) and its ability to grow at wide range of temperatures (Murray 1990; Devriese *et al.* 1993) in presence of salt and in low pH values (Sanz Pérez *et al.* 1982; Flahaut *et al.* 1997; Giraffa *et al.* 1997), it has been frequently considered as reference micro-organism for thermal treatments to be applied in pasteurized meals or 'sous vide' type foods (Smith *et al.* 1990; Ghazala *et al.* 1991). It is well known that a great number of factors can affect bacterial heat resistance. Several studies have

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demonstrated that micro-organisms thermal inactivation is strongly influenced by the growth conditions, specially by the incubation temperature and the age of cultures, but information concerning the effect of these factors on thermal resistance parameters is contradictory. In some cases, it has been found out that cells grown at higher temperatures are more heat resistant than those grown at lower temperatures (Dega *et al.* 1972; Ng 1982; McKey and Derrick 1987), but it has been also observed the opposite effect (Juneja *et al.* 1998). However, the influence of age of cultures on heat resistance is not clear. Generally, it is assumed that bacteria became more resistant to heat as they progress from exponential to stationary phase (Loewen and Hengge-Aronis 1994; Foster and Spector 1995; Fang *et al.* 1996; Beck Hansen and Knochel 2001). In some cases it has been found that *D*-values obtained for cells in the early stationary phase were higher than those found in the late stationary phase (Beuchat and Lechowich 1968; Gaze *et al.* 1989; Kornacki and Marth 1989), whereas in others an opposite effect was found (Hurst *et al.* 1974; Olson and Nottingham 1980). Furthermore, no correlation between age and heat resistance has been described (Ng 1982). This lack of conformity may perhaps be due to species and strain differences or differences in methodology, although it is possible that acquired thermoresistance can be influenced by other factors. It has been pointed out that the incubation temperature influences the degree of increase in the heat resistance during the bacterial growth (Elliker and Frazier 1938) but, as far as we know, no further studies have been made to clarify this finding. In the particular case of *Ent. faecium*, there does not appear to be literature available concerning the effects of culture temperature and physiological state of cells on its heat resistance. Hence, this study was carried out to provide data on the effect of these factors on thermal resistance of this micro-organism, which may be useful in establishing pasteurization procedures.

MATERIALS AND METHODS

Bacterial culture and media

The strain of *Ent. faecium* (ATCC 49624) used in this study was obtained from American Type Culture Collection. The lyophilized culture was revived in brain heart infusion broth (BHI, Oxoid) and incubated for 24 h at 37°C. Pure cultures of *Ent. faecium* were maintained on BHI agar plates at 4°C. Subcultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 ml of sterile BHI followed by incubation at 37°C for 24 h. From this subculture seven 250-ml flasks containing 50 ml of BHI, equilibrated at different growth temperatures (5, 10, 20, 30, 37, 40 and 45°C) were inoculated to a final concentration of approx. 10^6 cells ml^{-1}. At different times samples were removed in order to obtain cells in different stages of growth and cell concentration and heat resistance were determined.

Growth curves and calculation of growth parameters

Samples (1 ml) of the cultures obtained at different assayed conditions were decimally diluted in sterile 0·1% (w/v) peptone solution (Oxoid) and appropriate dilutions were plated in duplicate on BHI agar. Plates were incubated at 37°C for 48 h, and the numbers of colonies (CFU ml^{-1}) were enumerated. Viable counts were converted to $\log_{10}$ values. Growth curves generated by fitting the data to the Gompertz equation as previously described (Gibson *et al.* 1988; Buchanan *et al.* 1997) were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density.

Heat treatments

Heat treatments were carried out in a thermoresistometer TR-SC as described elsewhere (Condón *et al.* 1993). An inoculum of each culture was injected into the heating menstruum (Sorensen buffer, pH 7) previously stabilized at treatment temperature (65–72°C). During heating, samples of 0·2 ml were removed at predetermined time intervals and were collected in tubes containing melted sterile BHI agar and pour plated. Then plates were incubated at 37°C for 72 h and survivors were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Condón *et al.* 1996).

Heat resistance parameters

D-values (min) were calculated from the negative inverse slope of the straight portion of survival curves obtained from a plot of the log number of survivors vs their corresponding heating times. Only survivor curves with more than six values in the straight portion and descending more than 3 log cycles were used. D_{70} values were obtained at least in triplicate and compared using Student's *t*-test (Steel and Torrie 1986a). *z*-Values were determined from the line obtained by plotting the log of the *D*-values against exposure temperatures. In order to compare *z*-values homogeneity test of slopes was used worked out as described by Steel and Torrie (1986b).

RESULTS AND DISCUSSION

Viable counts of *Ent. faecium* ATCC 49624 were monitored on BHI (pH 7·2) during growth at different temperatures (5, 10, 20, 30, 37, 40 and 45°C). Growth was initiated after about 3 days at 5°C, 32 h at 10°C, 10 h at 20°C, 4 h at

30°C, 0·35 h at 37°C and immediately at 40–45°C. Although this micro-organism is able to grow at lower temperatures (5–10°C), low storage temperatures could be a means of controlling *Ent. faecium* growth, with generation times of 37 and 30 h at 5 and 10°C, respectively, compared with a generation time of 6·5 h at 20°C, 1·5 h at 30°C, 0·81 h at 37°C, 0·49 h at 40°C and 0·43 h at 45°C. The stationary phase was attained after around 13 days at 5°C, 6 days at 10°C, 48 h at 20°C, 18 h at 30°C, 8 h at 37°C, 5 h at 40°C and 4 h at 45°C. Maxima populations were achieved at temperatures between 30 and 45°C, ranging values of log CFU per millilitre from 8·90 to 9·10. The cell concentration reached in the suspensions grown at 5, 10 and 20°C was around one log cycle lower.

Samples were removed at various stages of growth (exponential and stationary phase and during extended incubation) from cultures at different temperatures and after variable intervals, which depended on the culture temperature. *D* values (time in minutes for survival count to drop 1 log cycle) at 70°C were determined. Logarithms of surviving cells (in CFU per plate) were plotted against heating time and *D* values were calculated by linear regressions from the linear portion of the survivor curves. The coefficient of correlation (r^2) ranged between 0·97 and 0·99. An example of survival curves obtained at 70°C for cells when stationary phase was attained are shown in Fig. 1.

D values were reported as the mean of three independent experiments ± the standard deviation (Table 1). In Fig. 2 changes in heat resistance during the growth of *Ent. faecium* (ATCC 49624) at different culture temperatures are shown. Data obtained showed that the heat resistance of *Ent. faecium* (ATCC 49624) was dependent on culture age and on the incubation temperature. In all cases, the bacterial cells in the logarithmic phase of growth were more heat sensitive. Heat resistance of *Ent. faecium* initially increased as cells from the inocula entered exponential growth. No significant differences were observed among D_{70} values determined for cultures obtained at different temperatures during early exponential growth, with a mean value of 0·37 ± 0·04 min. For cells that had entered in the stationary phase, cultures incubated at 10°C (144 h), 20°C (48 h) and 30°C (18 h) were the most resistant to heat, with *D*-values of 0·74 ± 0·08, 0·83 ± 0·08 and 0·79 ± 0·07 min, respectively. At higher temperatures there was a progressive decrease in heat resistance. Decimal reduction times of cultures at this stage of growth incubated at 40°C (5 h) and 45°C (4 h) decreased about a 40–50%. Cells grown at 5°C (312 h), showed a similar thermotolerance than those grown at 40°C (5 h) and 45°C (4 h). Numerous workers have shown for several species of bacteria that stationary phase cells are more resistant than exponential phase cells (Hansen and Riemann 1963; Beuchat and Lechowich 1968; Magnus *et al.* 1988; Condón *et al.* 1992; Kaur *et al.* 1998; Pagán *et al.* 1999). The structural and physiological changes that occur in this stage could be critical for the survival of the bacterial cell during heat exposure. In some cases these differences have been ascribed to a programmed adaptation mediated by alternative sigma factors, including *Rpos* in Gram-negative bacteria, i.e. active in the stationary phase, directing RNA polymerase to transcribe a large number of genes that encode proteins designed to respond to and protect cells from a variety of environmental stresses (Loewen and Hengge-Aronis 1994; O'Neal *et al.* 1994; Schellhorn *et al.* 1998; Hengge-Aronis 1999).

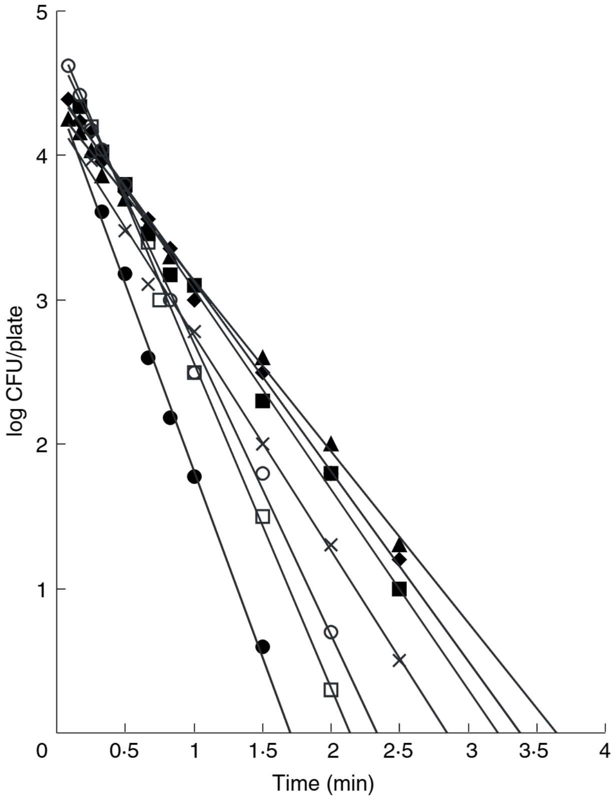

Fig. 1 Survival curves at 70°C for *Enterococcus faecium* (ATCC 49624) grown at different temperatures (● 5°C; ◆ 10°C; ■ 20°C; ▲ 30°C; × 37°C; ○ 40°C; □ 45°C) when cells had entered in stationary phase

When the resistance of cultures of *Ent. faecium* (ATCC 49624) to heat was monitored during more extended periods of ageing, a different pattern was observed for cells grown at different temperatures (see Fig. 2). At the lower temperatures (5–10°C), heat resistance increased progressively as incubation was prolonged, reaching D_{70} values of 1·73 and 1·04 min for cells incubated at 5°C for 1200 h and at 10°C for 384 h, respectively, whereas at the other temperatures tested heat resistance only increased during the first stage of stationary phase and afterwards remained constant throughout all period studied, reaching D_{70} values of 1·05, 1·08 and 1·01 min for cultures incubated at 20, 30 and 37°C for 240

Table 1 Effects of growth temperature and age of cultures on D_{70} values (min) of *Enterococcus faecium*

	Temperature (°C)						
Growth time (h)	5	10	20	30	37	40	45
1						0·40 ± 0·06^{a}	0·33 ± 0·07^{a}
2					0·36 ± 0·05^{a}	0·45 ± 0·03^{a}	0·39 ± 0·03^{a}
4					0·40 ± 0·07^{a}	0·47 ± 0·07^{ab}	0·41 ± 0·04^{a*}
5				0·40 ± 0·08^{a}	0·61 ± 0·08^{b}	0·48 ± 0·05^{ab*}	
8				0·55 ± 0·08^{a}	0·63 ± 0·05^{b*}	0·52 ± 0·08^{acb}	
12			0·37 ± 0·05^{a}	0·76 ± 0·08^{b}	0·67 ± 0·04^{b}	0·56 ± 0·10^{abcd}	0·45 ± 0·08^{ab}
18			0·48 ± 0·07^{a}	0·79 ± 0·07^{b*}	0·76 ± 0·07^{b}		
24			0·68 ± 0·08^{b}	0·86 ± 0·08^{b}	0·92 ± 0·06^{c}	0·62 ± 0·08^{cb}	0·57 ± 0·06^{bc}
30			0·72 ± 0·05^{b}				
36		0·45 ± 0·04^{a}	0·79 ± 0·06^{bc}	0·85 ± 0·07^{b}	0·97 ± 0·06^{c}	0·71 ± 0·10^{cd}	0·62 ± 0·06^{c}
48		0·55 ± 0·05^{ab}	0·83 ± 0·08^{bc*}	0·89 ± 0·09^{b}	0·96 ± 0·08^{c}	0·73 ± 0·11^{d}	0·63 ± 0·07^{c}
56		0·62 ± 0·05^{bc}					
72		0·64 ± 0·06^{bc}	0·94 ± 0·09^{cd}	0·94 ± 0·08^{bc}	0·98 ± 0·09^{c}	0·74 ± 0·09^{d}	0·64 ± 0·90^{c}
96			0·98 ± 0·08^{cd}		0·99 ± 0·10^{c}		
120	0·33 ± 0·05^{a}	0·71 ± 0·07^{c}	1·08 ± 0·10^{d}	1·12 ± 0·08^{c}	0·97 ± 0·11^{c}	0·75 ± 0·06^{d}	0·66 ± 0·10^{c}
144		0·74 ± 0·08^{cd*}					
168			1·02 ± 0·10^{d}	1·12 ± 0·10^{c}	0·99 ± 0·10^{c}	0·76 ± 0·05^{d}	0·67 ± 0·08^{c}
216	0·32 ± 0·06^{a}	0·87 ± 0·08^{d}	1·08 ± 0·13^{d}	1·13 ± 0·13^{c}	1·01 ± 0·14^{c}		
240	0·40 ± 0·05^{ab}		1·05 ± 0·11^{d}	1·08 ± 0·09^{c}			
312	0·53 ± 0·08^{b*}	0·94 ± 0·06^{e}					
384	0·74 ± 0·07^{c}	1·04 ± 0·10^{e}					
480	0·75 ± 0·09^{c}						
720	1·14 ± 0·15^{d}						
960	1·49 ± 0·16de						
1200	1·73 ± 0·12^{e}						

*D_{70} values for cells that had entered in the stationary phase.

$^{a-e}D_{70}$ values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P < 0{\cdot}05$).

and 216 h. Heat resistance of cells obtained at higher temperatures, 40 and 45°C, was significantly lower, with D_{70} values of 0·76 and 0·67 min, respectively (see Table 1). It is noteworthy that the most resistant cells were obtained by incubating at 5°C, although it was necessary to prolong incubation time up to 50 days. Also of interest was the fact that to obtain cells grown at 5°C with a thermoresistance similar to the maxima attained for cells grown at 10, 20 and 30°C it was necessary to extend incubation time up to 30 days.

Previous studies on heat resistance of bacterial cultures throughout stationary phase gave controversial results (Beuchat and Lechowich 1968; Hurst *et al.* 1974; Olson and Nottingham 1980; Ng 1982; Gaze *et al.* 1989; Kornacki and Marth 1989). Lack of agreement between data reported might be the result of the bacterium growing at different temperatures. Evidence provided in the present study clearly demonstrates that the culture temperature influences the heat resistance of cells in different stages of growth. The acquired heat resistance of *Ent. faecium* (ATCC 49624) in early and late stationary phase cultures was significantly lower for cells obtained at higher temperatures (40–45°C).

Generally, it is assumed that micro-organisms grown at higher temperatures show greater resistance to heat (Ng *et al.* 1969; Dega *et al.* 1972; Pagán *et al.* 1999). It has been demonstrated that the environmental conditions present during growth are responsible for qualitative and quantitative changes in the membrane fatty acid profile of bacteria. At higher temperatures, the saturated fatty acid content increases and unsaturated fatty acids decrease (Nagamachi *et al.* 1991). These changes in the composition of the membrane lipid affect mainly the fluidity of the cellular membrane. An increase in the fluidity of the bacterial membrane corresponded to a decrease in thermal resistance (Cornelis *et al.* 1987; Tsuchiya *et al.* 1987; Nagamachi *et al.* 1991; Goverde *et al.* 1994; Bodnaruk and Golden 1996; Logue *et al.* 2000). However, with *Ent. faecium* (ATCC 49624) heat treatment was more effective for cells incubated at higher temperatures (40 and 45°C). A similar behaviour was described by Juneja *et al.* (1998) for *Listeria monocytogenes* grown in BHI acidified to pH 5·4 at temperatures between 10 and 37°C. This opposite trend was observed although these authors demonstrated that the fatty acid profile of cells obtained at lower temperatures was modified

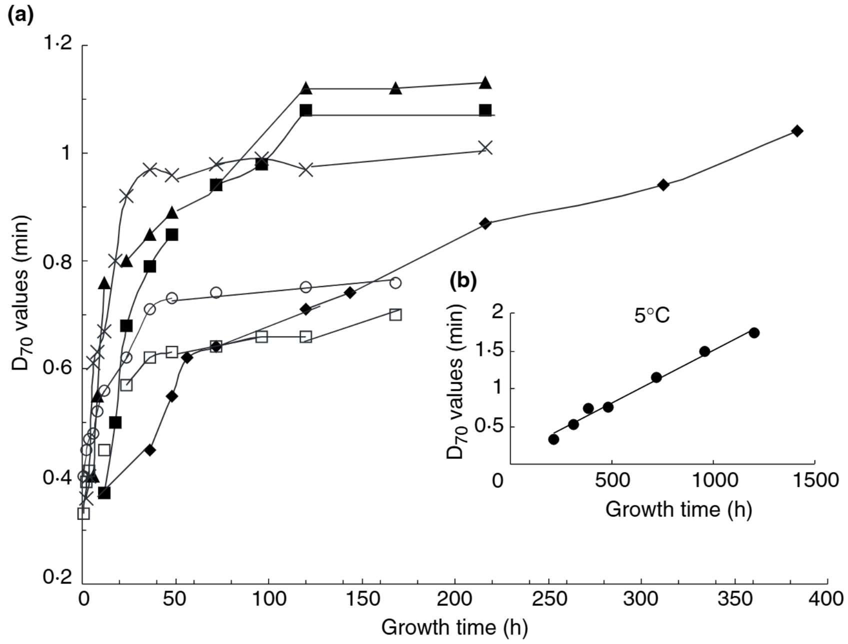

Fig. 2 Relation between heat resistance of *Enterococcus faecium* (ATCC 49624) and age of cultures at different growth temperatures. (a) ◆ 10°C, ■ 20°C, ▲ 30°C, × 37°C, ○ 40°C, □ 45°; (b) ● 5°C

in the direction of lower melting point fatty acids that help maintain membrane fluidity and, consequently, these cells should be more sensitive to heat. These authors suggested that the parallel decrease in heat resistance with increasing temperature implies an involvement of general stress proteins in the induction of thermotolerance and cross protection. Nevertheless, this reduction in heat resistance induced by high growth temperatures could perhaps also be explained by other changes induced by the incubation temperature. According to Neidhardt *et al.* (1990), temperatures higher than the optimum inactivate some biosynthetic routes, increasing the nutritional requirements of microorganisms. Furthermore, Pagán *et al.* (1999) demonstrated with *Yersinia enterocolitica* that the outer membranes of cells grown at 4 and at 37°C were different, as only those cells grown at 37°C were sensitized to lysozyme and nisin after a heat treatment.

The results of our investigation for *Ent. faecium* (ATCC 49624) also showed that the cells grown at lower temperatures during the late stages of the stationary phase (50 days at 5°C) survived heating better than the ones grown at higher temperatures, which reflect the response of cells to the stress imposed by cold. It is assumed that all stressing conditions to some extent cause protein denaturation and increase the concentration of unfolded proteins in cell. Such event may be the trigger for inducing synthesis of stress proteins, heat-shock proteins that would increase heat resistance by repairing the cellular damages. However, as the mechanisms by which growth temperature modify bacterial heat resistance are unclear, no definitive conclusions can be drawn.

The thermal death time curves obtained for *Ent. faecium* (ATCC 49624) grown at different temperatures when cells had entered in stationary phase are shown in Fig. 3. No statistically significant differences ($P < 0{\cdot}05$) could be found among the *z* values calculated at seven temperatures, which ranged from 4·03 (for cells grown at 40°C) to 5·07°C (for cells grown at 5°C), with a mean value of 4·48 ± 0·40°C. Also of interest was the fact that the phase of growth did not affect this parameter either. *z*-Values calculated for cultures obtained at 37°C in the exponential phase (4 h) and early (8 h) and late (24 and 72 h) stationary phase were 4·38, 4·94, 4·64 and 4·47°C, respectively (data not shown). There are no available information on the effect of incubation temperature on *z*-values of *Ent. faecium* and, as far as we know, only

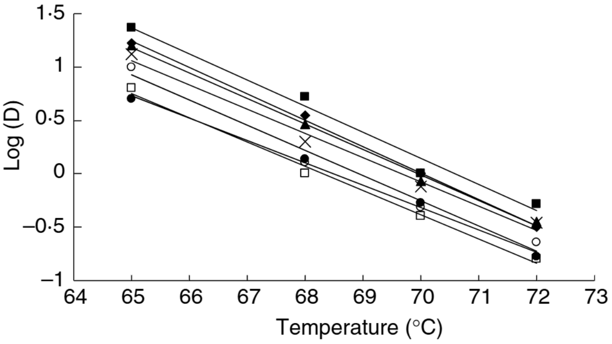

Fig. 3 Thermal death time curves for *Enterococcus faecium* (ATCC 49624) grown at different temperatures (● 5°C; ◆ 10°C; ■ 20°C; ▲ 30°C; × 37°C; ○ 40°C; □ 45°C) when cells had entered in stationary phase

Houben (1982) reported that z-values obtained for strain E_{20} of *Ent. faecium* to prolong incubation time at 37°C from 24 to 48 h increased around 30%, but he did not offer any statistical support.

In conclusion, thermal resistance of *Ent. faecium* (ATCC 49624) was shown here to be dependent on growth temperature and growth phase. The complex interaction of these factors acting on bacterial thermal resistance should be take into consideration to forecast the time/temperature required to process mild heat foods in order to ensure destruction of vegetative bacteria that cause spoilage or poisoning.

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