Growth at high pH increases *Enterococcus faecalis* adhesion to collagen

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Abstract

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Aim To evaluate the effect of growth at pH levels from 7.1 to 9.5 on the adherence of *Enterococcus faecalis* to bovine serum albumin (BSA) and collagen type I.

Methodology *Enterococcus faecalis* strain A197A was grown in broth of adjusted pHs varying between 7.1 and 9.5. Aliquots of bacterial suspensions were added to wells coated either with BSA or with collagen type I. Bacteria adhering to the surfaces were stained with crystal violet. Spectrophotometric measurements of the dissolved stain were used to assess the number of bacteria adhering to the surfaces. The data obtained were analysed using the Kolmogorov–Smirnov test, Levene's test and Student's *t*-test, with $\alpha = 0.05$ as the level for statistical significance.

Results The adhesion of *E. faecalis* to BSA-coated surfaces decreased inversely with alkalinity of the growth medium. The pH 7.1-grown bacteria bound to BSA significantly more than the other BSA groups. On the contrary, the adhesion to collagen type I-coated surfaces of bacteria grown at pH 8.0 and 8.5 was significantly greater than for those grown at pH 7.1.

Conclusions A minor increase in pH up to 8.5, which may be a consequence of insufficient treatment with alkaline medicaments such as calcium hydroxide, increases the collagen-binding ability of *E. faecalis*, *in vitro*. This can be a critical mechanism by which *E. faecalis* predominates in persistent endodontic infections.

Keywords: adhesion, albumin, bacteria, calcium hydroxide, collagen, stress.

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Introduction

Enterococci are Gram-positive facultative anaerobic bacteria which can cause a wide variety of diseases in humans, infecting the urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds and indwelling foreign devices (Jett *et al.* 1994). Enterococci have also been implicated in endodontic infections. Although they constitute only a small proportion of the initial flora of untreated teeth with necrotic pulps (Sundqvist 1992, Siqueira *et al.* 2002, Baumgartner *et al.* 2004, Rôças *et al.* 2004), enterococci, particularly *Enterococcus faecalis*, have been frequently found in filled root canals with signs of chronic apical periodontitis. It has been isolated in 23–70% of positive cultures from such teeth (Engström 1964, Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, Hancock *et al.* 2001), often occurring in monoculture (Sundqvist *et al.* 1998, Dahlén *et al.* 2000, Peciuliene *et al.* 2000, Hancock *et al.* 2001).

Enterococcus faecalis can withstand harsh environmental conditions, including high alkalinity levels. As described by Sherman (1937), *E. faecalis* can grow at pH 9.6 and can tolerate pH levels as high as 11.9

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(Flahaut *et al.* 1997). Previous studies have shown that *E. faecalis* is resistant to calcium hydroxide treatment (Byström *et al.* 1985, Haapasalo & Ørstavik 1987, Ørstavik & Haapasalo 1990, Haapasalo *et al.* 2000), probably partly due to its proton pump mechanism which maintains ideal cytoplasmic pH levels (Evans *et al.* 2002).

Adhesion to the host by microorganisms is the first step in most infectious diseases, after which pathological changes occur. Adherence of *E. faecalis* to extracellular matrix proteins, including collagen type I, has been demonstrated (Zaręba *et al.* 1997, Xiao *et al.* 1998, Rich *et al.* 1999, Nallapareddy *et al.* 2000, Love 2001, Rozdzinski *et al.* 2001). Adhesion to collagen type I by bacteria is of particular importance as this is the main organic component of dentine (Linde & Goldberg 1993). Some strains of *E. faecalis* were found to bind to collagen more after they were grown under stressful condition, which was described as growth at 46 °C; and this was termed 'conditional adherence' (Xiao *et al.* 1998). In a pilot test prior to this study, using a clinical isolate of *E. faecalis* (strain A197A), increased adhesion to collagen type I was found, but not to bovine serum albumin (BSA), after bacterial growth at 46 °C (unpublished data, Fig. 1). It was decided, therefore, to examine the effects on the adherence of *E. faecalis* of other stressful growth conditions found in medicated root canals. As calcium hydroxide fails to eliminate *E. faecalis* in the root canal, it may be speculated that its alkaline effect increases the adhesiveness of the bacterium.

Thus, the aim of this study was to evaluate, *in vitro*, the effect of growth at pH levels from 7.1 to 9.5 on the adherence of *E. faecalis* to collagen type I- and BSA-coated surfaces.

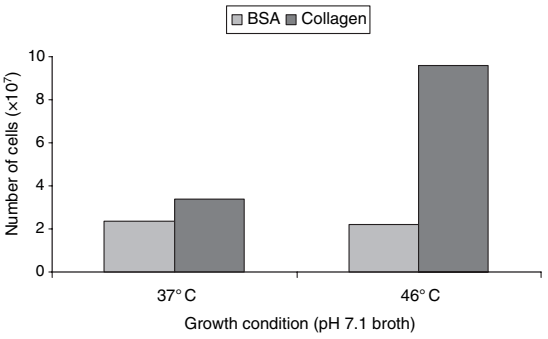

Figure 1 Number of cells of *E. faecalis* A197A adhering to bovine serum albumin- or collagen type I-coated surfaces following growth at 37 or 46 °C (the experimental conditions were identical to those used in the reported study).

Materials and methods

Bacterial strain and culture conditions

The bacterial strain used in this experiment was *E. faecalis* A197A, which is an isolate from a persistent endodontic infection (Sirén *et al.* 1997). Tryptone soya broth (TSB; Oxoid Ltd, Basingstoke, UK) was used as the basic growth medium. It had a pH of 7.1 when prepared according to the manufacturer's instructions. Media of pH 7.5, 8.0, 8.5, 9.0 and 9.5 were prepared by the addition of 1 mol L^{-1} NaOH to TSB. Colonies were picked up from solid media (TSB agar) and inoculated into each medium. Incubation was at 37 °C overnight. The cultures were harvested by centrifugation at 10 000 ***g*** for 10 min, washed three times with equal amounts of phosphate-buffered saline (PBS, pH 7.4) and finally resuspended in PBS. The purity of the bacterial cultures was checked routinely during the experiments by Gram-staining and by observing colony morphology.

Adherence assay

Three hundred microlitres of BSA (100 μg mL^{-1} PBS) (Sigma Chemical Co., St Louis, MO, USA) or collagen type I (50 μg mL^{-1} PBS) (Sigma) was added to 96-well microtitre plates (Sarstedt Inc., Newton, NC, USA) and incubated at 4 °C overnight. The wells were emptied, rinsed three times with sterile PBS, blocked with 300 μL of BSA (100 μg mL^{-1} PBS) at 4 °C for 1 h, and rinsed again.

Bacterial adhesion to the coated surfaces was quantified employing an assay described by O'Toole & Kolter (1998) with some modifications. Briefly, 200 μL of bacterial suspension of an optical density $OD_{540} = 1.0$ (approximately 2×10^9 bacteria mL^{-1}) were added to each well and incubated at 37 °C for 2 h. Wells coated with BSA or collagen, and not filled with the bacterial suspensions served as controls. The wells were rinsed three times with PBS to remove unbound bacteria and the plate was dried in an inverted position. The wells were stained with 325 μL of 1% crystal violet for 20 min, rinsed with PBS and then dried again. Bound crystal violet was solubilized by addition of 200 μL of ethanol–acetone (4 : 1, v/v) and the optical density at 570 nm of the dissolved dye was measured (Multiskan EX; Labsystems, Helsinki,

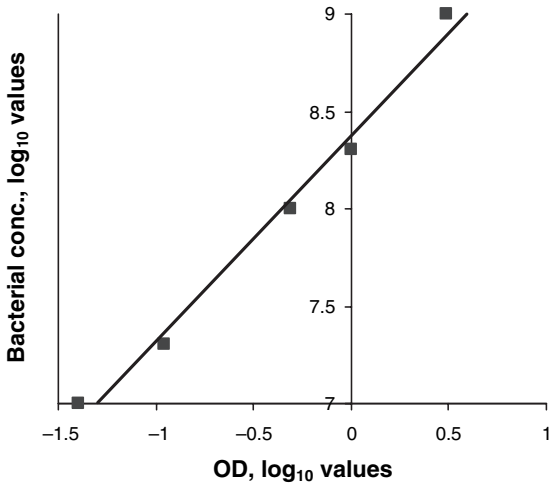

Figure 2 The standard curve constructed by intersecting known concentrations of bacteria and OD readings at 570 nm (for collagen-coated wells).

Finland). The assay was performed in four parallels and repeated twice.

The relationship of the OD measurements of the dissolved crystal violet to bacterial numbers was assessed in a separate methodological experiment: suspensions of known bacterial concentrations were dried onto the sides of wells prior to the staining procedure. The ensuing OD readings were used to construct standard curves for OD vs. bacterial cell concentrations. A near-linear relationship was found for a log–log plot of the results (Figs 2 and 3), which was used to transform the OD measurements to bacterial cell concentrations. The data obtained were analysed using a statistical package program, SPSS version 8.0, using the Kolmogorov–Smirnov test for normality, Levene's test for equality of variances and Student's *t*-test, with $\alpha = 0.05$ as the level for statistical significance.

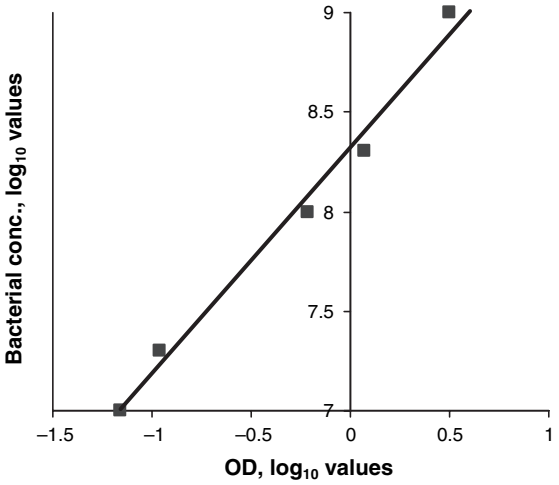

Figure 3 The standard curve constructed by intersecting known concentrations of bacteria and OD readings at 570 nm (for BSA-coated wells).

Scanning electron microscopy

A separate plate not stained with crystal violet was used for scanning electron microscopy examinations. The wells were kept in 10% neutral buffered formaldehyde solution overnight, rinsed thoroughly with distilled water and dried at 50 °C for an hour. The bottoms of the wells were separated using a diamond disc at low speed. The specimens were mounted on aluminium stubs with conducting carbon cement, sputter coated with gold-palladium and observed in scanning electron microscope (Phillips XL 30 ESEM, Eindhoven, The Netherlands). The pattern of adherence in terms of the presence of chains and clusters of bacteria, as well as area coverage, was examined.

Results

No difference was found between the background staining of BSA and collagen as determined by the comparison of BSA and collagen-coated wells free of bacteria.

The experimental results are summarized in Fig. 4.

Adherence to BSA-coated surfaces

Bacterial adherence to BSA-coated surfaces decreased steadily from pH 7.1 group down to pH 9.5 group. The pH 7.1-grown bacteria bound to BSA significantly more than the other pH groups.

Adherence to collagen-coated surfaces

Bacterial adherence to collagen-coated surfaces increased gradually from pH 7.1 group up to pH 8.5 group, which had the maximum binding level observed. Bacteria grown at pH 8.0 and pH 8.5 bound to collagen significantly more than those grown at pH 7.1, 9.0 and 9.5. Adherence to collagen of bacteria grown at pH 9.0 and 9.5 were not significantly different from that of bacteria grown at pH 7.1.

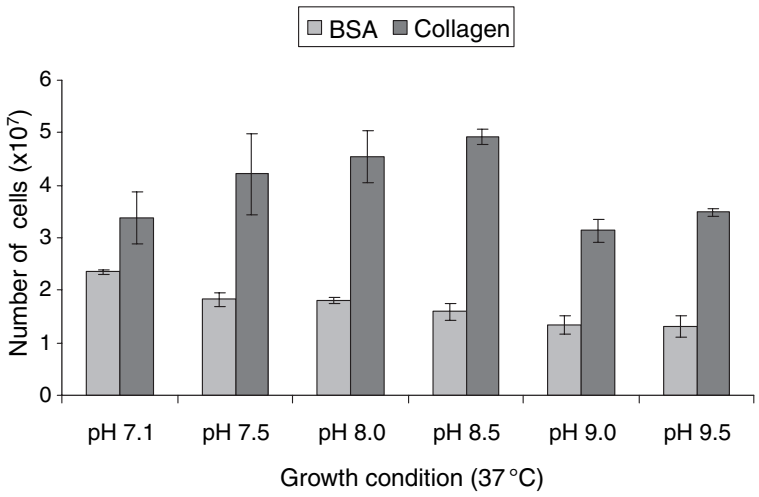

Figure 4 Number of cells of *E. faecalis* A197A adhering to bovine serum albumin or collagen type I-coated surfaces following growth at different pHs. The data are means and standard deviations (bars) for three experiments.

Difference between the number of bacteria binding to BSA and collagen separately for each pH group was also statistically analysed. Except for the pH 7.1 group, the number of bacteria binding to collagen was significantly higher than those binding to BSA.

Scanning electron microscopy

Presence of bacteria was evident for all specimens examined except the control wells. Whilst bacteria adhering to collagen were observed as single cells, pairs, chains or clusters of varying size evenly spread out over the surface (Figs 5–7), bacteria adhering to BSA surfaces were more irregularly distributed and occupied limited areas, with bands or strands of cells criss-crossing the surface (Figs 8 and 9).

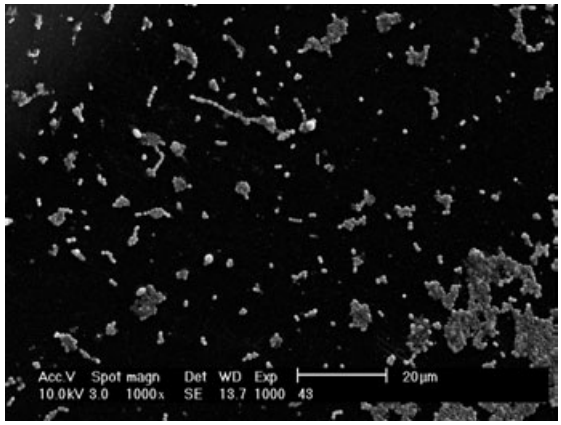

Figure 5 Binding of the bacteria grown at pH 7.1 to the collagen-coated surface. Original magnification 1000×.

Discussion

The major finding in this study was that the pH increase in the growth medium led to decreased bacterial adherence to BSA, and increased adherence to collagen type I, with a peak at pH 8.5.

The method used in this study to quantify bacterial adherence was based on staining of the adhering bacteria with crystal violet, followed by solubilizing the absorbed dye and measuring the optical density. Crystal violet is also used during Gram-staining procedure in the microbiology laboratory. Crystal violet enters the cytoplasm of both Gram-positive and Gram-negative cells and stains them purple (Tortora *et al.* 2004). Adherence assays based on crystal violet staining have been previously done for both types of cells (O'Toole & Kolter 1998, Toledo-Arana *et al.* 2001).

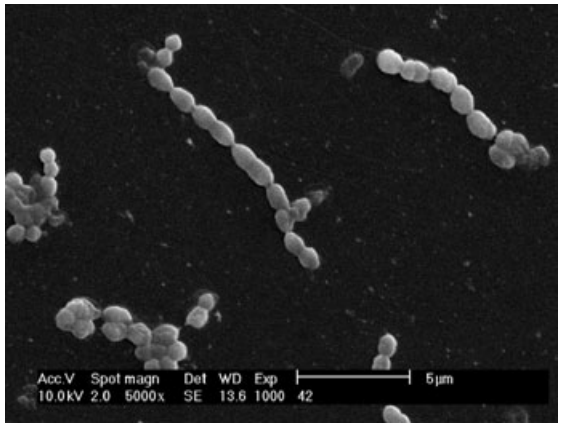

Figure 6 Binding of the bacteria grown at pH 7.1 to the collagen-coated surface. Original magnification 5000×.

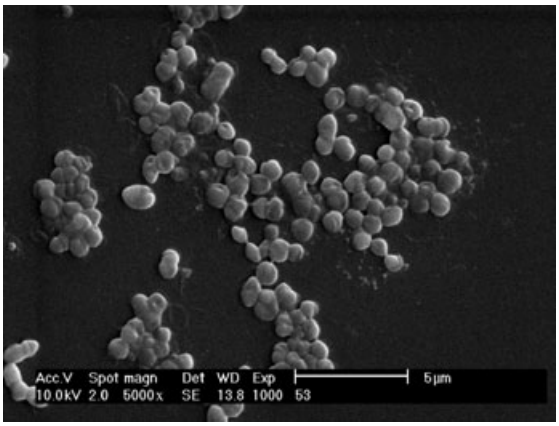

Figure 7 Binding of the bacteria grown at pH 8.5 to the collagen-coated surface. Original magnification 5000×.

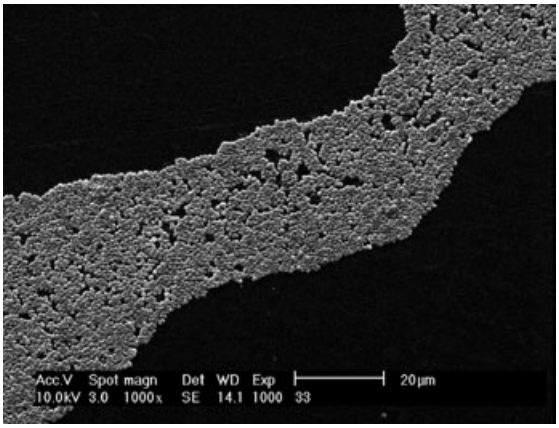

Figure 8 Binding of the bacteria grown at pH 7.1 to the BSA-coated surface. Bacteria adhering in large sheets in coated surface. Original magnification 1000×.

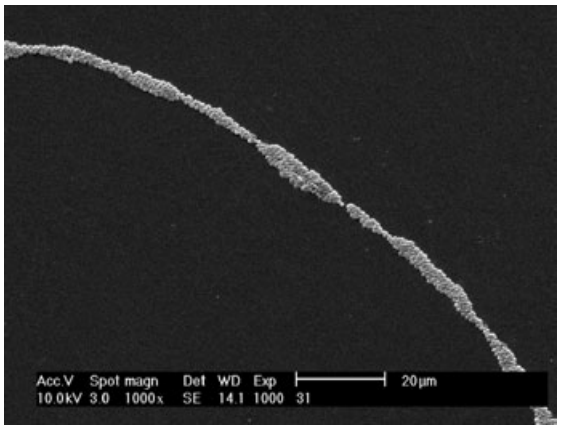

Figure 9 Binding of the bacteria grown at pH 7.1 to the BSA-coated surface. Thin strands of bacteria adhering to coated surface. Original magnification 1000×.

Enterococcus faecalis has been shown to invade the dentinal tubules in several studies (Akpata & Blechman 1982, Haapasalo & Ørstavik 1987, Ørstavik & Haapasalo 1990, Love 2001) and this has been related to its ability to adhere to collagen (Love 2001). It has been reported that adhesins on the cell surface, such as 'aggregation substance' and 'Ace' mediate adhesion of *E. faecalis* to collagen (Rich *et al.* 1999, Rozdzinski *et al.* 2001). The importance of Ace as an adhesin that aids *E. faecalis* to bind to dentine was shown in a recent study (Hubble *et al.* 2003). Whilst these are the well-characterized adhesins of *E. faecalis*, approximately 200 different proteins are found following exposure of *E. faecalis* to various stressful conditions, including alkaline stress (Flahaut *et al.* 1997). The function of most of these proteins, however, is still unknown (Rince *et al.* 2000, Giard *et al.* 2001); and perhaps some of them may be adhesins.

Besides adhesins, physicochemical characteristics of the bacterial cell surface such as surface tension, surface charge and hydrophobicity may act in the context of bacterial adhesion to solid surfaces. In one study, the collagen adhesion of *E. faecalis* was linked to its cell surface hydrophobicity (Zaręba *et al.* 1997). Change in the environmental pH as a stimulus has been known to alter the physicochemical characteristics of bacteria as well as to trigger expression of various genes associated with virulence or metabolic regulations (Olson 1993). Thus, in addition to the possibility of synthesis of adhesins by the bacterium, it is also possible that change in the bioenergetic parameters of the cell surface might have increased its adherence to the collagen-coated surfaces.

Calcium hydroxide has a strong antimicrobial effect in direct contact with microorganisms, due to its pH of 12.5. However, there exist some limitations with the efficient use of calcium hydroxide in the root canal. First, dentine has an inhibitory effect on calcium hydroxide (Haapasalo *et al.* 2000). When dentine is included in experimental designs, the effectiveness of calcium hydroxide dramatically decreases (Haapasalo & Ørstavik 1987, Ørstavik & Haapasalo 1990). Secondly, variations in alkalinizing potential of various formulations of calcium hydroxide exist (Schäfer & Al Behaissi 2000). Thirdly, efficient delivery of calcium hydroxide into the root canal, especially to the apical third, may be difficult to obtain (Sigurdsson *et al.* 1992, Rivera & Williams 1994, Deveaux *et al.* 2000). Moreover, the pH distribution through dentine after delivery of calcium hydroxide has been reported to decrease from the pulpal dentine outwards and also to be lower

in the apical than in the cervical regions (Tronstad *et al.* 1981, Nerwich *et al.* 1993). Therefore sufficient alkalinity cannot always be achieved in all parts of the root canal. The pH rise may not be sufficient for growth inhibition, not to mention killing, of *E. faecalis*. It may, on the contrary, promote the adhesion of the bacterium to collagen which in turn may lead to increased dentinal tubule invasion, and the invading bacteria will become less sensitive to further disinfection procedures as they will be shielded in deeper locations.

In teeth with necrotic pulps, there may be ingress of tissue fluids through the apex from periradicular tissues. Albumin is a major protein component of tissue fluids. *Enterococcus faecalis* has previously been found to bind to albumin with the involvement of protein- and/or carbohydrate-containing components as the surface receptor (Shorrock & Lambert 1989). In this study, no significant difference was observed between the adherence of the bacteria to albumin- or collagen-coated surfaces after growth at neutral pH, but this changed in favour of adherence to collagen as the pH of the growth medium increased. This was most remarkable after growth at pH 8.5. This suggests that *E. faecalis* growing at increased pH binds to the collagen moiety of the dentine and is less inhibited by the albumin component of serum. However, it should be noted that our experimental design lacked many of the *in vivo* factors (e.g. other organic macromolecules, hydroxylapatite) and also was set to investigate binding to each organic substrate separately. The effect of the simultaneous presence of both substrates on bacterial adhesion may be more complicated. For example, in one study, albumin added to the growth medium of *E. faecalis* JH2-2 did not inhibit the dentinal tubule invasion of the bacterium; but when dentine was treated with albumin prior to incubation with bacteria, dentinal tubule invasion was significantly reduced. And in the microtitre plate adherence assay, binding to collagen-coated wells by bacteria incubated simultaneously with albumin was somehow significantly increased (Love 2002).

In this study, testing a range of pH levels for growth condition, an increase in binding to collagen to the extent the bacteria exhibited after growth at 46 °C (Fig. 1) was not observed. However, there was still a pronounced increase in binding to collagen when the bacterium was grown at mildly alkaline media. Growth at pH 8.5 was found to be stressful for *E. faecalis* A197A in pilot tests, as deduced from growth yields. Whilst 46 °C is not a physiological temperature to affect the bacterial growth in the root canal, it is representative of a stressful condition. It has been shown that various stress conditions can induce production of same proteins by *E. faecalis* (Rince *et al.* 2000, Giard *et al.* 2001). Therefore, stress conditions other than growth at 46 °C may similarly induce increased adherence by *E. faecalis*. As this study has evaluated the effect of a limited range of pH which allowed the growth of the species, the effect of exposure to higher pH levels on the adhesiveness of the bacterium deserves further investigation.

Conclusions

A minor increase in pH up to 8.5, which may occur in the root canal after treatment with alkaline medicaments such as calcium hydroxide, increases the collagen-binding ability of *E. faecalis*, *in vitro*. As adherence of the microorganism to host tissue is an essential step in the establishment of an infection, this may contribute to the predominance of *E. faecalis* in persistent endodontic infections.

Acknowledgements

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