

Chitin scar breaks in aged *Saccharomyces cerevisiae*

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Ageing in budding yeast is not determined by chronological lifespan, but by the number of times an individual cell is capable of dividing, termed its replicative capacity. As cells age they are subject to characteristic cell surface changes. *Saccharomyces cerevisiae* reproduces asexually by budding and as a consequence of this process both mother and daughter cell retain chitinous scar tissue at the point of cytokinesis. Daughter cells exhibit a frail structure known as the birth scar, while mother cells display a more persistent bud scar. The number of bud scars present on the cell surface is directly related to the number of times a cell has divided and thus constitutes a biomarker for replicative cell age. It has been proposed that the birth scar may be subject to stretching caused by expansion of the daughter cell; however, no previous analysis of the effect of cell age on birth or bud scar size has been reported. This paper provides evidence that scar tissue expands with the cell during growth. It is postulated that symmetrically arranged breaks in the bud scar allow these rigid chitinous structures to expand without compromising cellular integrity.

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INTRODUCTION

Senescence is a universal characteristic, exhibited by all eukaryotes. The yeast *Saccharomyces cerevisiae* has a limited lifespan, determined by its replicative capacity (Hayflick & Moorhead, 1961; Hayflick, 1965; Jazwinski, 1993; Sinclair *et al.*, 1998). Each cell within a population is only capable of a limited number of divisions prior to senescence and death. Yeast cells are subject to characteristic cellular modifications as a consequence of the ageing process (Jazwinski, 1990; Powell *et al.*, 2000). Such modifications may be genetic, metabolic, physiological and morphological in nature. Physiological changes associated with senescence are especially apparent at the cell surface; in particular, structural modifications to the cell wall can be readily visualized and provide an indication of cell age (Mortimer & Johnston, 1959; Sinclair *et al.*, 1998). The cell wall is a highly dynamic structure which is known to change constantly in its properties and functions as the cell grows and develops (Gooday, 1993; Klis, 1994; Cabib *et al.*, 1997). Age-associated modifications to the cell wall include an increase in size as a function of somatic growth (Bartholomew & Mittwer, 1953; Barker & Smart, 1996; Powell *et al.*, 2000), wrinkling of the cell surface due to loss of turgor (Mortimer & Johnston, 1959; Barker & Smart, 1996; Powell *et al.*, 2000) and a change in cell wall composition (Egilmez *et al.*, 1990; Cabib *et al.*, 1997). The cell wall is composed of glucan, mannan and comparatively

small amounts of proteins, lipids and chitin (Northcote & Horne, 1952; Cabib & Roberts, 1982; Valentin *et al.*, 1987; Bulawa, 1993; Klis, 1994; Cid *et al.*, 1995; Orlean, 1997). Chitin, a minor component of the cell wall, is predominantly located at the site of bud emergence (Bacon *et al.*, 1966; Cabib & Bowers, 1971; Cabib *et al.*, 1974, 1982; Roberts *et al.*, 1983; Osumi, 1998) and is produced immediately prior to and during cellular division by budding (Cabib *et al.*, 1974, 1982, 1997; Sloat & Pringle, 1978; Holan *et al.*, 1981; Yamaoka *et al.*, 1989). *S. cerevisiae* cells may divide as many as 30–50 times prior to death (Barton, 1950; Mortimer & Johnston, 1959) and as a consequence levels of chitin have been observed to increase throughout the lifespan (Egilmez *et al.*, 1990). Following cytokinesis the mother cell exhibits a crater-like ring of scar tissue known as the bud scar (Barton, 1950; Bacon *et al.*, 1966; Cabib *et al.*, 1997), which is composed almost solely of a chitin–glucan complex with a reinforced glucan–mannan layer (Seichertova *et al.*, 1973; Holan *et al.*, 1981; Kollar *et al.*, 1995). The daughter cell retains a less prominent structure known as the birth scar (Barton, 1950; Bacon *et al.*, 1966). It has been reported that the birth scar contains little or no chitin (Beran *et al.*, 1972; Shaw *et al.*, 1991), although the exact composition of this structure is unknown.

The number of bud scars present on the cell surface is directly related to the number of times a cell has divided and therefore enumeration of bud scars provides a means of determining the replicative age of cells within a population (Barton, 1950; Egilmez *et al.*, 1990; Sinclair *et al.*, 1998). It has been suggested that accumulation of chitin may be a

Abbreviations: SEM, scanning electron microscopy; WGA, wheat-germ agglutinin.

cause of senescence in *S. cerevisiae*. Mortimer & Johnston (1959) proposed that an increase in the number of bud scars may limit the availability of surface area for budding and for nutrient exchange, as scar tissue is thought to be less efficient than normal cell wall material at facilitating the transport of macromolecules into the cell. This theory has since been disproved (Johnson & Lu, 1975; Egilmez & Jazwinski, 1989), demonstrating that scarring is a result and not a cause of replicative ageing.

Previous analysis of cell wall composition has typically been performed using stationary-phase cultures, which characteristically consist of 50% virgin cells, 25% first-generation mothers, 12.5% second-generation mothers, etc. Studies have thus concentrated on the analysis of virgin cells and young mother cells and little is known about the structure or organization of the cell wall in aged individuals. In addition, chitin scar rings have previously been considered to be non-dynamic, rigid structures which persist throughout the lifespan without undergoing structural alterations or greatly influencing the yeast cell (Egilmez & Jazwinski, 1989; Chant & Pringle, 1995). Thus although the changes which occur at the cell surface as a consequence of cellular ageing are well documented, to our knowledge there has been no previous analysis of the effect of cell age on chitin scar tissue. Here the relationship between longevity in *S. cerevisiae* and bud and birth scar microarchitecture is investigated.

METHODS

Yeast strains and growth conditions. The polyploid brewing yeast strain BB11 was provided by Coors Brewers, Burton-on-Trent, UK. Strain KS1 was obtained from the Oxford Brookes University culture collection; KS1 has also been characterized as being a polyploid yeast strain (K. Smart, unpublished).

Each strain was propagated on YPD medium [2% (w/v) neutralized bacteriological peptone, 1% (w/v) yeast extract, 2% (w/v) glucose]. Cultures of each yeast strain were maintained on YPD slopes and YPD agar plates prior to use. YPD medium was sterilized immediately after preparation by autoclaving at 121 °C and 15 p.s.i. (103.5 kPa) for 15 min. All medium components were supplied by Oxoid.

Preparation of aged cell fractions. Cell fractions were isolated by sedimentation through sucrose gradients using a modified version of the protocol published by Egilmez *et al.* (1990). Sucrose gradients were prepared in 50 ml skirted (free-standing) centrifuge tubes by layering 22.5 ml 10% (w/v) sucrose onto a base consisting of an equal quantity of 30% (w/v) sucrose. Tubes were inclined at 4 °C for 48 h to produce 45 ml linear 10–30% gradients. Following separation cells were grown in standard YPD, rather than YPD glycerol as recommended by Egilmez *et al.* (1990). Cells propagated in YPD glycerol exhibited a reduction in viability and an increase in size, making this an inappropriate carbon source for this study.

Preparation of virgin cells. A 1 litre flask containing 500 ml YPD was inoculated from a 10 ml culture, previously propagated from a single colony of yeast, and incubated in an orbital shaker at 25 °C for 72 h. The resulting culture was sonicated at maximum power in a sonicating waterbath (Camlab), followed by gentle agitation for 30 s. This was repeated three times to ensure that the resulting culture contained only discrete individuals. Cells were recovered by

centrifugation (4000 r.p.m. for 10 min), washed twice in 0.1 M phosphate-buffered saline (PBS), pH 7.3, and cell number was determined using a haemocytometer. An optimum cell suspension of 5×10^8 cells ml⁻¹ in PBS was achieved by dilution and 1 ml aliquots layered onto the surface of sucrose gradients. Subsequently cells were maintained at 4 °C during age synchronization.

Gradients were centrifuged in an IEC Centra-EC 4R refrigerated centrifuge at 4 °C with a swing-out rotor attachment at 1300 r.p.m. for 5 min. This resulted in two layers of cells: a lower compacted region and a less-dense upper layer, containing virgin cells. The top two-thirds of the upper cell band were recovered from each gradient, pooled and pelleted. The cell pellet was washed twice and resuspended in PBS (4 °C). The resulting population was examined for age purity using confocal microscopy.

Ageing protocol. Virgin cells prepared as described above were resuspended to a final concentration of 1.5×10^7 cells ml⁻¹ in YPD and incubated at 25 °C in an orbital shaker until the culture reached a density of 4.5×10^7 cells ml⁻¹. Cells were then harvested by centrifugation (13 000 r.p.m. for 1 min) and prepared for size fractionation using sucrose gradients as described above. The gradients were centrifuged at 1300 r.p.m. for 5 min and the top half of the cell band removed and discarded. The remainder of the band (containing two-division-old mothers) was recovered from each tube. These mother cells were resuspended at 1.5×10^7 cells ml⁻¹ in YPD and again incubated at 25 °C to achieve a density of 4.5×10^7 cells ml⁻¹. By repeating rate zonal sedimentation and incubation in YPD a further three times, the mother cells could be sequentially aged. In this way fractions containing second-, fourth-, sixth- and eighth-generation mothers could be achieved.

Determination of cell age. The age of individuals within a population was determined by enumerating bud scars on the cell surface using confocal microscopy. For age purity analysis the bud scars on 100 individuals were enumerated. Cell number was determined using a haemocytometer, after which cells were pelleted and resuspended in sterile PBS at a concentration of 5×10^7 cells ml⁻¹. Aliquots of 500 µl were washed twice in PBS and resuspended in 500 µl FITC-labelled wheat-germ agglutinin (lectin from *Triticum vulgare*; Sigma-Aldrich, UK), at a concentration of 1 mg ml⁻¹. Cells were gently agitated at room temperature for 15 min, harvested by centrifugation (13 000 r.p.m. for 1 min) and washed three times in PBS. The stained cell culture was resuspended in 250 µl PBS with an equal quantity of Citifluor (Agar Scientific) and examined ($\times 100$ oil-immersion lens) using an LSM 410 inverted laser scanning confocal microscope (Carl Zeiss) with a 488 nm argon ion laser for fluorescence imaging and transmission detector for differential interference contrast (DIC). Images were stored on optical disc and subsequently printed using a Tektronix Phaser 440 dye sublimation printer.

Scanning electron microscopy (SEM). Cells were prepared for electron microscopy by critical-point drying (Barker & Smart, 1996). They were resuspended in osmium tetroxide (2%, w/v), incubated statically for 60 min at room temperature, and harvested by centrifugation (13 000 r.p.m. for 1 min). The fixed and stained cells were dehydrated by 10 min incubations with gentle agitation in a graded ethanol series of 10, 20, 30, 50, 70, 100 and 100% dried absolute ethanol. The cells were then critical-point dried using a Tousimis Samidri 780 dryer and mounted on aluminium stubs. Gold sputter coating of the stub surface was achieved using a Bio-Rad polaron division SEM coating system. The stubs were examined using a Hitachi HS800 scanning electron microscope.

Determination of cell and scar dimensions. BB11 cell dimensions were determined using the confocal microscope measure function. Cell volume and surface area were calculated from cell

diameters (width and length), assuming that cells were prolate ellipsoidal with a smooth surface. For each age fraction 30 individuals were examined. All values are expressed \pm standard error. Significance of results was determined using the two-tailed Student *t*-test at the 5% confidence level. Pearson's correlation coefficient was used to determine the presence of a linear relationship between two datasets.

RESULTS AND DISCUSSION

Cell size increases with age

Previous investigations into the ageing morphology of yeast have demonstrated that there is an increase in cell size with age (Bartholomew & Mittwer, 1953; Mortimer & Johnston, 1959; Lorincz & Carter, 1979; Woldringh *et al.*, 1993; Barker & Smart, 1996). BB11 aged cell fractions were prepared using sucrose gradients to separate cells on the basis of size. The age purity of each population was determined by bud scar enumeration using confocal microscopy. A population of virgin cells was estimated to consist of approximately 93.8% discrete unbudded cells; the remaining 6.2% comprised aged or budding individuals. Separation of further fractions resulted in a progressive deterioration in purity; however, age-synchronized populations (purity) of second- (83.3%), fourth- (66.8%), sixth- (49.2%) and eighth- (27.8%) division-old cells were readily obtained.

Analysis of cells using confocal microscopy enabled the size of individual cells within a population to be determined. The relationship between divisional age and cell size is shown in Table 1. Virgin cells had a mean cell diameter of $6.4 \pm 0.2 \mu\text{m}$, while the mean diameter for eighth-generation cells was $9.0 \pm 0.1 \mu\text{m}$. Virgin cells had a mean cell volume of $153.6 \pm 16.5 \mu\text{m}^3$, while cells which had divided eight times reached a mean volume of $390.1 \pm 12.0 \mu\text{m}^3$ (Table 1). This represents an increase of approximately 150% in volume from virgin to eighth-division-old cells. The values obtained are representative of the size of polyploid yeast (Barker & Smart, 1996). However, it has been demonstrated that haploid and

diploid strains are considerably smaller, possibly due to repression of G₁ cyclins in polyploid individuals (Galitski *et al.*, 1999). Lorincz & Carter (1979) estimated the mean cell volumes of haploid C4,2 cells with 0, 1, 2 and ≥ 3 bud scars to be 38.7, 43.4, 47.6 and $62.1 \mu\text{m}^3$ respectively. Woldringh *et al.* (1993) reported that virgin cells of the diploid strain X2180 exhibited a cell volume between 25.9 and $74.3 \mu\text{m}^3$, with fourth-generation cells displaying a cell volume of approximately $97.5 \mu\text{m}^3$.

In accordance with the increase in cell volume, cell diameter and surface area were estimated to increase by approximately 41% and 92% respectively from virgin to eighth-division cells. The large standard error calculated from virgin population samples (Table 1) is attributed to the rapid increase in size previously observed in newly formed daughter cells (Barton, 1950; Kennedy *et al.*, 1994) prior to achieving the critical cell volume required for division (Hartwell & Unger, 1977; Carter & Jagadish, 1978; Wheals, 1987; Futcher, 1993). It is suggested that cells which have achieved a critical size and have therefore met the requirements to pass through START (Lorincz & Carter, 1979; Pringle & Hartwell, 1981; Kuntzel *et al.*, 1996) may be more homogeneous in size than a virgin population, which comprises newly formed and therefore diminutive daughters.

Analysis of each fraction revealed a linear increase in volume between successive age-synchronized populations (Table 1) according to Pearson's correlation coefficient, supporting previous observations (Barker & Smart, 1996). The results obtained add further support to the evidence suggesting that an increase in size is directly related to cell age and therefore constitutes a biomarker for the cell's divisional age. However, the rate of increase in cell size may not continue throughout the lifespan, but may decrease in older individuals in accordance with the diminution in metabolic rate previously reported for aged cells (Motizuki & Tsurugi, 1992). Previous studies in our laboratory have indicated that extremely aged individuals display a decrease in size; however, this typically accompanies the final divisions of the individual cell (unpublished data) and may result from loss of turgor.

Table 1. Relationship between age and cell size in BB11

Values were calculated from analysis of 30 individuals selected at random from each age fraction. Cell surface area and volume were calculated assuming that cells were prolate ellipsoidal spherical with a smooth cell surface. All values are means \pm SE.

Generation	Cell diameter (μm)	Cell surface area (μm^2)	Cell volume (μm^3)
Virgin	6.4 ± 0.2	133.9 ± 9.5	153.6 ± 16.5
Second	7.4 ± 0.1	172.1 ± 5.0	214.3 ± 9.6
Fourth	8.2 ± 0.1	212.7 ± 5.8	294.0 ± 12.1
Sixth	8.7 ± 0.1	239.1 ± 4.3	348.9 ± 9.2
Eighth	9.0 ± 0.1	257.4 ± 5.1	390.1 ± 12.1

Chitin scar tissue and age

The impact of cell ageing on bud and birth scar parameters was determined using SEM and confocal microscopy in conjunction with FITC-labelled wheat-germ agglutinin (WGA). The advantage of using confocal rather than traditional fluorescence microscopy is the ability to visualize the entire cell surface. This enables accurate analysis of all scars present on the surface of each cell. WGA is highly specific to *N*-acetylglucosamine, the major component of chitin, although it does react weakly with *N*-acetylneuraminic acid, *N*-acetylgalactosamine and Man- β -(1 \rightarrow 4)-GlcNAc- β -(1 \rightarrow 4)-GlcNAc- β -*N*-Asn (Goldstein & Poretz, 1986; Bulawa, 1993). Gold-conjugated WGA has previously been used to study the surface of yeast cells using SEM (Horisberger & Volanthen, 1977; Shaw *et al.*, 1991) and WGA has also been used successfully to determine levels of non-bud-scar chitin within the cell wall (Molano *et al.*, 1980); however, FITC-WGA has not previously been utilized to examine bud scar morphology. Analysis of BB11 age-synchronized fractions, using both SEM and confocal microscopy, enabled bud and birth scars to be identified and their morphology to be analysed. Bud scars were clearly apparent and were observed to protrude extensively from the surface of the mother cell (Fig. 1). Interestingly, a portion of the cell wall within the bud scar was often observed to extend further than the scar itself (Fig. 1); the constrictive nature of the chitin ring may cause such protrusions to occur. Although the composition of the cell wall material at the centre of the ring is unknown, this observation also suggests that it may be similar in arrangement to other areas of the cell wall, and therefore is able to expand at a similar rate to the rest of the cell. In contrast to the bud scars, birth scars projected to a much lesser degree, tending instead to lie flat upon the cell surface (Fig. 1). Although it has been reported that birth scars contain little or no chitin (Beran *et al.*, 1972; Shaw *et al.*,

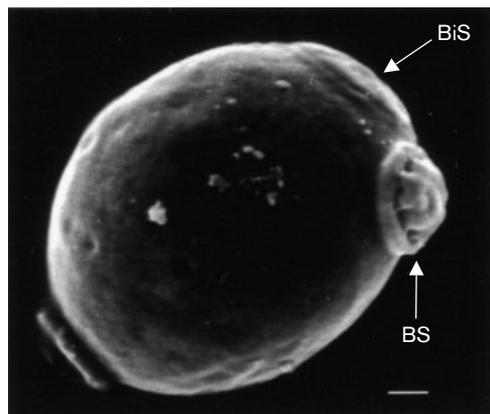


Fig. 1. SEM image demonstrating bud (BS) and birth (BiS) scar morphologies. Bar, 1 μ m.

1991) confocal microscopy of BB11 cells labelled with WGA clearly demonstrates large chitinous birth scar ring structures (Fig. 2a).

BB11 cells taken from each age fraction displayed a specific number of bud scars, corresponding to the generation number (Fig. 2a–e). In addition, individuals from virgin, second-, fourth-, sixth- and approximately 25% of eighth-generation cells were observed to display a birth scar. Birth scars closely resembled bud scars in appearance; however, these scars could be distinguished due to their large size and dispersed fluorescence (Fig. 2c). In comparison, bud scars were identifiable due to their highly defined band of fluorescence, indicating a denser ring of chitin.

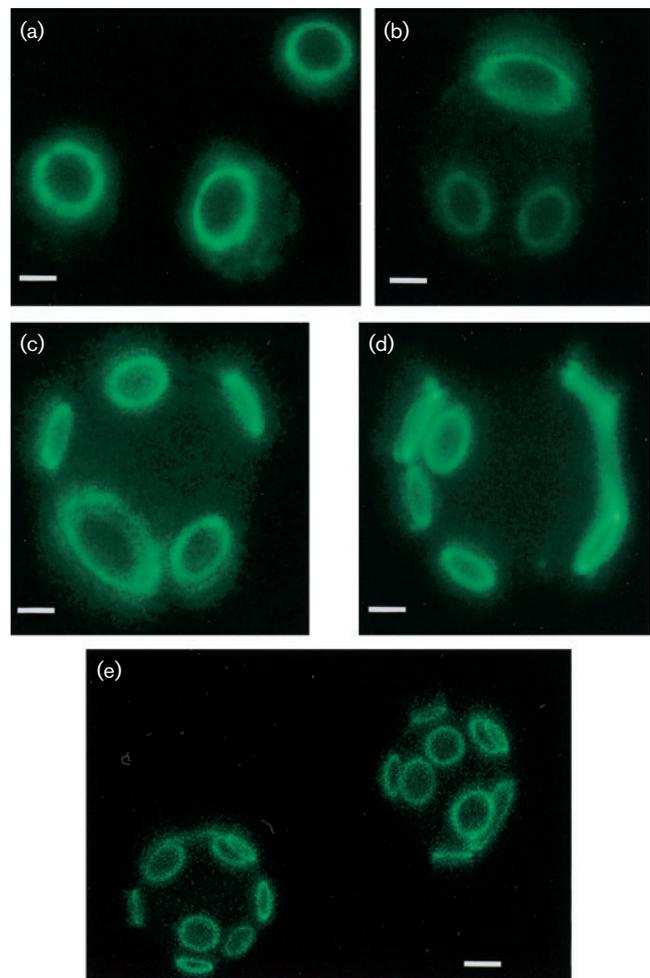


Fig. 2. Age-synchronized populations of virgin (a), and second- (b), fourth- (c), sixth- (d) and eighth- (e) generation BB11 cells illustrating birth (a–e) and bud scar (b–e) morphology. Birth scars are distinct due to their large size. Bars, 3 μ m (a), 1.5 μ m (b, c), 2.3 μ m (d), 3.5 μ m (e). Eighth-generation cells only occasionally exhibit a birth scar (e). Occasionally birth scars would be observed in the form of a double ring of chitin (c).

Birth scars expand and fade with age

Age-synchronized populations of BB11 cells were analysed for birth scar characteristics. Staining of chitin using FITC-WGA enabled birth scars to be detected on all individuals obtained from virgin, second-, fourth- and sixth-generation fractions. Previous observations of birth scars have identified them as being single circular rings (Barton, 1950); however, birth scars were occasionally noted to display a 'double ring' of chitin (Fig. 2c), although the occurrence of this phenomenon was extremely rare (approx. 2–3% of the population). The relationship between cell age and birth scar size parameters is shown in Table 2. Birth scars were readily identifiable on virgin cells due to the absence of any other chitin scar structures (Fig. 2a); however, the ease with which the birth scar could be identified decreased with age. This was due to an apparent thinning or fading of the birth scar with age, rather than misidentification caused by the increasing number of bud scars. Fading of the birth scar became significantly noticeable when examining sixth- (Fig. 2d) and eighth-division individuals, where approximately 75% of cells did not exhibit a birth scar (Fig. 2e).

Age-heterogeneous populations of BB11 cells exhibited birth scars 3.1–3.7 μm in diameter. This compares with previous measurements of birth scars, which have indicated a diameter of approximately 3 μm (Barton, 1950). When comparing cell populations containing virgin, second- and fourth-generation cells it was observed that the mean birth scar size increased with age (Table 2). The mean birth scar diameter of BB11 increased from $3.1 \pm 0.1 \mu\text{m}$ in virgin cells to $3.7 \pm 0.1 \mu\text{m}$ in fourth-generation cells. The increase in diameter between virgin and second-generation and between second- and fourth-generation cells was significant ($P < 0.5$). The reason for this observation is believed to be expansion of the cell. However, despite an increase in size, the proportion of the mother cell covered by the birth scar remained constant (Table 2). Although the mother cell surface area increases with age (Table 1), the birth scar consistently occupies approximately 5% of the cell surface (Table 2). This indicates that the increase in birth scar size is correlated with an increase in cell size, suggesting that the birth scar is an integrated component of the cell wall and not a distinct structure.

Although it was anticipated that the birth scar would continue to expand with age, cells of sixth-divisional age exhibited birth scars of a similar size to fourth-generation individuals. The reason for this is not known, although it is possible that wrinkling and crenellation of the cell surface, which occur with age (Mortimer & Johnston, 1959; Muller, 1971; Barker & Smart, 1996), cause the birth scar to appear smaller. Alternatively cell growth may cause the outer portion of the birth scar to stretch at a faster rate than the inside, being closer to more readily expandable wall material. If the outer section of the ring were to stretch faster it would be subjected to reintegration into the cell wall at an earlier stage of development than the middle, causing fading and a stabilization of birth scar size. In a similar fashion, further increases in cell surface area may eventually lead to the birth scar being completely reintegrated into the cell wall, thus explaining scar fading and the absence of birth scars on aged individuals.

Bud scars expand with age

The presence of bud scars was noted on the surface of each aged BB11 cell, corresponding to the divisional age of the sample (Fig. 2b–e). Virgin cells did not display bud scars as they had yet to produce daughter cells. Bud scars characteristically displayed a higher density of fluorescence than birth scars, indicating the higher levels of chitin in these structures.

Bud scars present on BB11 cells were randomly distributed over the entire cell surface, indicating a pattern of bud site selection closely resembling bipolar division (Freifelder, 1960; Streiblova, 1970; Chant & Pringle, 1995). However, KS1 exhibited an axial budding pattern (Chant & Pringle, 1995), with buds being produced at the cell poles. Bud scars were not observed to overlap in any way, supporting the observations of Barton (1950) and Streiblova (1970). However chitin scars located on KS1 individuals were occasionally observed to be located very close to one another, to the extent that the edges touched. Analysis of the characteristics of bud scars in BB11 revealed that an increase in size with age occurs in a similar fashion to that observed for birth scars. The mean bud scar diameter increased from $1.9 \pm 0.2 \mu\text{m}$ in second-generation cells to

Table 2. Relationship between cell age and birth scar size in BB11

Values (means \pm SE) were calculated from 30 individuals selected at random from each age fraction.

Generation	Mean birth scar diameter (μm)	Percentage of cell surface covered by birth scar	Mean bud scar diameter (μm)	Mean bud scar surface area (μm^2)	Percentage of cell surface covered by bud scars
Virgin	3.1 ± 0.1	5.9	–	–	–
Second	3.2 ± 0.1	4.8	1.9 ± 0.1	3.0 ± 0.1	3.6
Fourth	3.7 ± 0.1	5.2	2.1 ± 0.1	3.7 ± 0.1	6.9
Sixth	3.6 ± 0.1	4.4	2.2 ± 0.1	3.9 ± 0.1	10.0
Eighth	ND*	ND*	2.3 ± 0.1	4.2 ± 0.1	13.4

*For eighth-division-old cells birth scars were infrequently or not detected (ND).

2.3 ± 0.1 µm in eighth-division-old cells (Table 2). Barton (1950) and Woldringh *et al.* (1995) estimated bud scar tissue to be approximately 2 µm and 1 µm in diameter respectively; these values are comparable to the observed scar diameters for BB11. Sixth-generation individuals displaying birth scars of reduced size did not show a similar reduction in bud scar characteristics. Sixth- and eighth-generation individuals continued to display an increase in bud scar size when compared to younger individuals. In addition, bud scars were not observed to fade in the manner of birth scars displayed on aged individuals. Each age fraction was analysed statistically to compare bud scar size between successive cohorts of aged cells. The results indicated that bud scar size was unique for each age fraction ($P < 0.5$ in each instance). An increase in bud scar diameter in accordance with cell size was first predicted by Barton (1950), although there has been no study to support this original hypothesis. It is postulated that cell wall assembly occurring both outside and within the chitin ring may result in an increase in bud scar diameter, causing the scar to appear stretched. If this is the case, it would be reasonable to assume that a degree of 'thinning' would also be observed for bud scars. However we did not observe bud scar thinning in any individual. Despite the increase in mean bud scar size, the size of the smallest (most recent) scar remained constant, regardless of the age of the mother cell. It is known that chitin synthase 2 (Chs2) (Sburlati & Cabib, 1986) is responsible for primary septum formation, while chitin synthase 3 (Chs3) is responsible for production of chitin in the ring at bud emergence (Shaw *et al.*, 1991; Valdivieso *et al.*, 1991). The genetic regulation of the production and position of chitin indicates that there may also be control over the exact size of the chitin ring immediately prior to bud emergence. This would suggest a relationship between the size of the smallest bud scar on the mother and the birth scar on virgin cells. However, no correlation was observed between the size of these two structures. It is known that daughter cell growth occurs rapidly immediately after septum formation, while the speed of cell wall carbohydrate production in mothers is reduced immediately before division (Hayashibe *et al.*, 1977). It is suggested that a combination of these factors may have resulted in the discrepancy in scar size observed. Interestingly the mean size of the smallest bud scar was not

observed to exceed 2.0 µm in diameter (Table 3), indicating that despite variation in the dimensions of the mother cell, daughter cell size may remain constant. Although Woldringh *et al.* (1993) demonstrated that daughters produced from second-generation mothers were actually smaller than those generated from one-division-old cells, it has been observed in haploid (Jazwinski, 1990) and polyploid (Barker & Smart, 1996) yeast strains that daughter cell size does indeed remain relatively stable, regardless of the size or age of the mother cell.

Bud scars exhibit breaks in their structure

Although accumulation of non-bud-scar chitin has been shown to have no adverse effects on cell lifespan (Egilmez & Jazwinski, 1989), it is possible that localized highly concentrated regions of chitin may influence cellular vitality to a greater extent than dispersed quantities. Such regions may restrict cell wall expansion, leading to reduced elasticity and increased cell fragility. However, the robustness of the cell wall is not influenced by age (Egilmez *et al.*, 1990), implying that specific events may occur to ensure cellular function. Barton (1950) suggested that bud and birth scars may expand with cell growth; however, the mechanisms of scar expansion have not previously been investigated. In addition there has been no analysis of the impact of cell size or age on the structure of chitin scar material.

The morphology of chitin scar rings was analysed for each age fraction. As previously described, bud scars were observed to increase in diameter with each subsequent division of the mother cell. However, unlike birth scars, bud scars were not observed to fade with age. A possible explanation is that this is prevented due to the higher levels of the rigid cell wall component – chitin. This hypothesis does not explain the expansion of bud scars during replicative ageing, which would be impeded by the rigidity of chitin. A close examination of scar microarchitecture in strain BB11 revealed that bud scar rings contain small breaks in their structure (Fig. 3). Bud scar microarchitecture in strain KS1 was also examined for the occurrence of breaks and these could be readily visualized even in individuals isolated from a mixed-age population, suggesting that bud scar breaks represent a universal phenomenon. Bud scar breaks have not been previously

Table 3. Relationship between smallest bud scar size and cell age in BB11

Values (means ± SE) were calculated from 30 individuals selected at random from each age fraction.

Generation	Cell surface area (µm ²)	Mean bud scar diameter (µm)	Mean smallest bud scar diameter (µm)
Virgin	133.9 ± 52.1	–	–
Second	172.1 ± 27.5	1.9 ± 0.2	1.8 ± 0.3
Fourth	212.7 ± 31.6	2.1 ± 0.1	1.9 ± 0.1
Sixth	239.1 ± 23.4	2.2 ± 0.1	1.9 ± 0.2
Eighth	257.4 ± 28.2	2.3 ± 0.1	2.0 ± 0.1

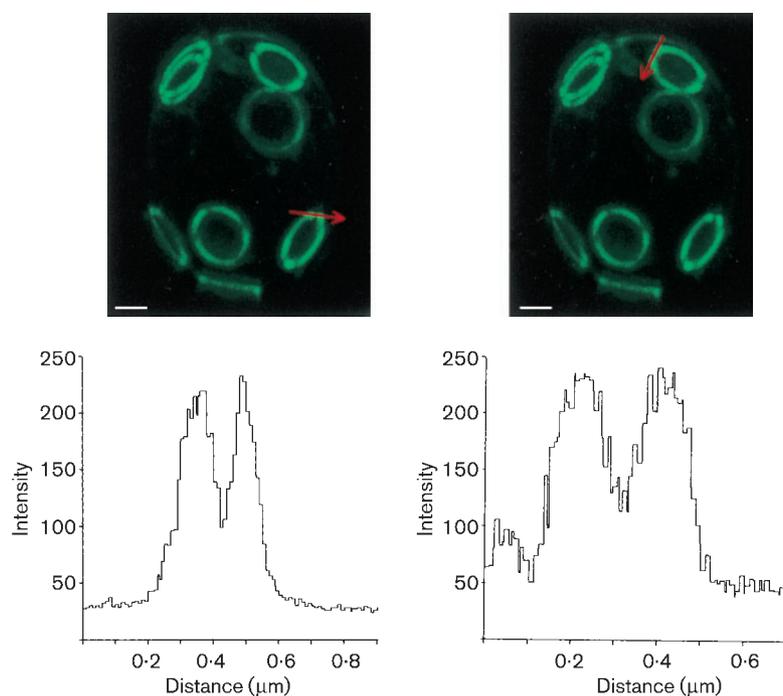


Fig. 3. Eighth-generation BB11 cell exhibiting bud scar breaks. Bar, 1.25 μm . Note that the two bud scars which appear to overlap are actually situated adjacent to one another, one on the 'front' of the cell and the other on the 'rear' of the cell. It is possible to confirm this by digital rotation of the reconstructed image. The large faint ring present at the top of the cell is believed to be the birth scar. The distribution of chitin around the bud scar rings is indicated by the fluorescence intensity plots. The arrow indicates the field of analysis displayed in the corresponding graph. Each bud scar break is clearly indicated by a decrease in fluorescence intensity.

reported despite detailed analysis using powerful tools such as SEM. It is suggested that shrinking of the cell wall caused by dehydration during critical-point drying or freeze drying sample preparation may have masked scar breaks when analysing cells by SEM. In addition the greater specificity of WGA for GlcNAc compared to alternative fluorescent stains such as calcofluor, Congo red and primulin, which bind many other β -linked polysaccharides (Pringle *et al.*, 1989; Bulawa, 1993), may have allowed breaks to be identified more clearly. For both BB11 and KS1 bud scar breaks occurred in a specific pattern, occupying opposing sites on the chitin ring in groupings of either two or four.

Barton (1950) proposed that bud scars produced prior to the maximum size of the cell being attained would be subject to stretching. We suggest that chitin rings possess breaks in their structure which only become apparent on expansion of the cell wall. It is possible that these morphological characteristics result purely as a function of cellular growth. However, due to their uniform structure, arrangement and positioning around the ring, we suggest that bud scar breaks are an essential genetically predetermined structural design feature. It is proposed that the opposing position of breaks may function to permit the bud scar ring to stretch, despite the inelasticity of chitin, at a constant rate in each direction, thus preventing an uneven distribution of scar tissue and serving to eliminate a potential source of damage to the cell wall.

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