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Spontaneous mutation results in lower cellulose production by a *Gluconacetobacter xylinus* strain from Kombucha

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ABSTRACT

This study aimed to investigate the characteristics of a spontaneous mutation that results in lower cellulose production by a *Gluconacetobacter xylinus* strain isolated from Kombucha. The growth of this mutant was initially observed in Hestrin Schramm (HS) static and agitated cultures but not in modified (higher nutrient) HS cultures, indicating different nutrient conditions may play a role in the selection of the spontaneous mutant. The mutant produced a significantly ($P < .05$) lower amount of cellulose than the wild-type in HS static culture. Nuclear magnetic resonance and scanning electron microscopy indicated that the cellulose produced by the mutant had the same crystalline structure as that produced by the wild-type but had a lower density of fibrils. Two-dimensional gel electrophoresis of total proteins demonstrated the presence of the enzyme deoxythymidine diphosphate (dTDP)-4-dehydrorhamnose 3,5-epimerase in the mutant but not in the wild-type. This enzyme could be involved in the synthesis of acetan, which may reduce cellulose synthesis and be the basis for the characteristics of the mutant.

1. Introduction

Gluconacetobacter xylinus (formerly *Acetobacter xylinum*) is an aerobic bacterium which can produce relatively high amounts of extracellular cellulose (Iguchi, Yamanaka, & Budhiono, 2000). The production of this cellulose is thought to play an important role in this species by providing a means of floatation and thus access to air/liquid interfaces where the supply of oxygen is abundant (Ross, Mayer, & Benziman, 1991). Under artificial static culture conditions *G. xylinus* produces cellulose as a relatively large and uniform pellicle on the surface of the medium. However, in agitated culture most strains yield a lower amount of cellulose in the form of globules of different sizes (Schramm & Hestrin, 1954). The lower cellulose yield has been shown in some strains to be due to the selection of spontaneous mutants which do not produce cellulose (Krystynowicz et al., 2002). These non-cellulose mutants have a mucoid (wet and flat) colony morphology, which is different from the non-mucoid (dried and convex) colonies of the wild-type strains. It has been proposed that the abundance of oxygen created by sufficient and uniform aeration of agitated cultures is a crucial factor resulting in selection for these non-cellulose mutants (Valla & Kjosbakken, 1982). Cellulose deficiency in the mutants described above has been subsequently suggested to be due to lack of production of two enzymes, phosphoglucomutase and uridine diphosphoglucose (UDP-glucose) pyrophosphorylase. These enzymes are required to synthesize UDP-glucose which is a critical substrate for cellulose synthesis (Krystynowicz et al., 2005; Valla et al., 1989).

Although most spontaneous mutations have been reported from agitated cultures, they also occur in static cultures. The mutants in this case displayed different characteristics from those appearing in agitated cultures. In particular, a spontaneous mutant of *G. xylinus* ATCC 23769 has been isolated from medium without agitation and found to maintain the ability to produce cellulose (Kuga, Takagi, & Brown, 1993). However, this type of cellulose consists of anomalous band-like structures of native folded-chain cellulose II which is different from the crystalline ribbon-like microfibrils of cellulose I usually produced by the wild-type strain.

We have isolated a bacterial cellulose producing strain of *G. xylinus* from Kombucha (Nguyen, Flanagan, Gidley, & Dykes, 2008a), a traditional fermented tea-based beverage with reported health benefits (Dufresne & Farnworth, 2000). We have also used bacterial cellulose produced by the strain for the development of a nisin-containing film to inhibit the growth of *Listeria monocytogenes* on processed meat (Nguyen, Gidley, & Dykes, 2008b). In the process of characterizing this strain for its potential commercial usefulness, we observed the presence of low cellulose production under agitated growth conditions. We hypothesized that this phenomenon could be due to the growth of spontaneous mutants inca-

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pable of producing cellulose as reported by previous studies (Krystynowicz et al., 2002; Valla & Kjosbakken, 1982). We therefore investigated the potential influence of the presence of spontaneous mutations of the strain on cellulose production and report here on (i) the presence of a novel mutant phenotype and the effect of culture conditions on its selection; (ii) a comparison of the structural properties of cellulose produced by the mutant and the wild-type strains and (iii) used two-dimensional gel electrophoresis to compare the proteins produced by the wild-type and the mutant.

2. Materials and methods

2.1. Bacterial strains, culture medium and inoculum preparation

The *G. xylinus* K3 strain used in this study was isolated from commercially available Kombucha (Kombu Australia, Springwood, Queensland, Australia) and maintained at −80 °C on Protect Bacterial Preservers (Technical Service Consultants, Heywood, UK), a commercially available storage system for long-term preservation of bacteria. The working culture was prepared by resuscitation of the strain using Yeast Extract Peptone Mannitol agar (YPM; 25 g/l mannitol, 5 g/l yeast extract, 3 g/l peptone and 15 g/l agar) following incubation at 30 °C for 48 h. The culture media used in this study were (i) Hestrin and Schramm (HS) medium which consists of 20 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 2.7 g/l Na_2HPO_4, 1.15 g/l citric acid·H_2O and (ii) modified HS containing 20 g/l mannitol, 40 g/l corn steep liquor, 2.7 g/l Na_2HPO_4, 1.15 g/l citric acid-H_2O. Prior to sterilization at 121 °C the pH of the media was adjusted to 5.0 (Schramm & Hestrin, 1954).

Inoculum was prepared by transferring a single colony from the YPM working culture into 500 ml bottles containing 100 ml of HS medium and then incubated without agitation at 30 °C for 2 days. Following incubation, the broth culture was shaken vigorously to release the attached *G. xylinus* cells from the cellulose pellicle, with subsequent filtering of the broth culture through sterile gauzes. The resulting cell suspension was used as the seed culture for subsequent experiments. A volume of 1% (v/v) seed culture was inoculated into 75 ml specimen bottles containing 25 ml of HS or modified HS medium and incubated without agitation (static), or with shaking at 250 rpm (agitated), at 30 °C for 6 days, unless otherwise stated.

2.2. Experimental design

A wild-type colony (non-mucoid colony morphology) was propagated in both HS and modified HS media under both static and agitated conditions. Cultures were sampled every 24 h and the presence of different phenotypes was determined by different colony morphologies in order to investigate the effect of culture conditions on selection of spontaneous mutants.

In order to determine if the mutant phenotype was a stable heritable mutation or an unstable transient cell state, a single colony of the mutant phenotype was isolated and incubated in both HS and modified HS media under both static and shaken conditions at 30 °C for 48 h. A 100 μl aliquot from each medium and incubation type was subsequently transferred to fresh medium of the same type and incubated in the same way. This was repeated for four subsequent transfers. A volume of 100 μl of the cell suspension from each transfer was diluted, spread on YPM agar and incubated at 30 °C for 4 days after which colony morphologies were examined.

To test if there were any differences in cellulose production and cell growth among different phenotypes resulting from spontaneous mutations, a single colony of both the wild-type and the mutant phenotype were grown separately in both HS and modified HS media. Cultures were sampled every 24 h and the cell density and cellulose yield of the phenotypes were compared.

Cellulose pellicles produced by both the wild-type and the mutant phenotype were analyzed by solid state ^{13}C nuclear magnetic resonance (NMR) spectroscopy and scanning electron microscopy (SEM) to identify any differences in the supramolecular structure and microstructure of the cellulose samples. Proteins extracted from cells of the wild-type and the mutant phenotype obtained from the cultures were analyzed using two-dimensional gel electrophoresis.

2.3. Determination of cellulose yield and cell numbers

The cellulose yield was expressed as weight of cellulose per unit volume of culture medium (Son, Heo, Kim, & Lee, 2001). The cellulose from the cultures was purified by treatment with 0.5 M NaOH at 90 °C for 1 h to eliminate attached cells, followed by three washes with distilled water to remove medium components and other residues. The purified cellulose was subsequently dried at 105 °C to constant weight.

Cell numbers for each growth experiment were determined as total viable counts using 0.1% (w/v) peptone (pH 5.0) as the diluent and YPM agar as the plating medium. Colonies were counted after 4 days of incubation at 30 °C and numbers of cells was expressed as colony-forming units per ml (cfu/ml) (Kouda, Naritomi, Yano, & Yoshinaga, 1997).

2.4. Analysis of cellulose structure

The supramolecular structure of cellulose produced by the wild-type and the mutant cultivated in HS medium was investigated using solid state ^{13}C NMR spectroscopy. Prior to packing into the rotor, cellulose samples from *G. xylinus* cultures were rinsed with water and air-dried. ^{13}C NMR spectra were acquired at a frequency of 75.46 MHz and at 303 K on a Bruker MSL-300 spectrometer. The spectral width was 38 kHz with a contact time of 1 ms, the rotor was spun at 5–6 kHz at the magic angle of 54.7° and experimental recycle times were 3 s. At least 2400 scans were accumulated for each spectrum. The structural type, crystallinity and ratio of crystalline structures of both cellulose samples were determined on the basis of structures of native cellulose (VanderHart & Atalla, 1984; Yamamoto & Horii, 1993).

The microstructure of cellulose samples from the two phenotypes was observed using field emission scanning electron microscopy (FESEM). A total of five samples (5 mm discs) were randomly cut from freeze-dried cellulose material using a punch. The discs were mounted on carbon tabs and sputter coated (IB-5, EIKO, Tokyo, Japan) with platinum (6 mA for 180 s). Samples were viewed using a JEOL JSM-6300 FESE microscope (Japanese Electron Optics Limited, Tokyo, Japan) operated at an accelerating voltage of 6 kV and a working distance of 12–19 mm. Images were captured using ImageSlave image acquisition software (ImageSlave, Sydney, Australia).

2.5. Two-dimensional gel electrophoresis

In order to obtain bacterial cells for protein extraction, 100 ml of seed culture of the wild-type and the mutant were inoculated into 900 ml of HS medium and incubated without agitation for 6 days at 30 °C. The culture broths were then centrifuged at 13,131*g* for 20 min at 4 °C. The supernatants were discarded and the pellets were re-suspended in 40 mM Tris–HCl, pH 8.0. The suspensions were re-centrifuged and the bacterial cells were re-suspended in 2 ml of 40 mM Tris–HCl. Each suspension was sonicated on ice (20 cycles for 30 s each with 30 s intervals between cycles to allow for cooling; Minsonix XL2000 sonicator).

The cell extracts were transferred into new tubes and centrifuged at 12,100*g* at 4 °C for 20 min to remove whole cells and debris. The supernatant (total protein) was treated with DNase/RNase (Sigma, St. Louis, Mo.) to remove any of DNA or RNA which may cause streaking in the resulting gels. The supernatant was then left to incubate for 30 min at room temperature and centrifuged at 12,100*g* for 10 min. The protein concentration of the total protein extracts was determined using the Bradford protein assay dye reagent (Bio-Rad Laboratories, Hercules, USA. Cat. No. 500-0006) with IgG used as a standard in the assay. Total protein extracts were stored at −80 °C.

Two-dimensional (2D) gel electrophoresis was performed for the total protein extracts of the wild-type and the mutant cells as described by Rivas, Fegan, and Dykes (2008). Isoelectric focusing in the first dimension was performed in an isoelectric focusing (IEF) machine (Protean, Bio-Rad) and electrophoresis in the second dimension was performed in 1× TGS buffer at 200 V per gel for approximately 1 h. Each total protein extract was analysed in triplicate.

Following electrophoresis, the gels were rinsed with distilled water and fixed using a fixing solution (7% (v/v) acidic acid and 10% (v/v) methanol) for 15 min. Gels were rinsed again with distilled water prior to staining with a colloidal coomassie stain (G250, Modified Neuhoff Stain; 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 34% (v/v) methanol, 0.1% (w/v) coomasie G-250). Following staining for 17 h, the gels were destained using a 1% acidic acid solution and washed with distilled water for 2–3 h and subsequently scanned using a densitometer (Molecular Imager GS-800; Bio-Rad). The raw images were processed using PDQuest software for 2D gels (Bio-Rad). Following background subtraction of spot detection, the gels were matched to each other to form a standard (synthetic) image that included all the proteins present in at least two of the three matched gels. Gels were stored in sealed bags containing 0.001% sodium azide solution.

Following analysis, five protein spots were selected based on a twofold or higher up regulation between the mutant and the wild-type and excised from coomassie blue stained gels for identification using matrix assisted laser desorption ionization (MALDI) mass spectrometry as described by Rivas et al. (2008).

2.6. Statistical analysis

All quantitative data, unless otherwise stated, are presented as the means of triplicates with errors represented by standard deviations. Significant differences of data sets were determined using means of paired *t*-tests on Minitab 14 for Windows® with the significance based on a level of 5% ($P < .05$).

3. Results and discussion

3.1. Effect of culture conditions on selection of spontaneous mutants by G. xylinus K3

It has been previously established that an oxygen-abundant culture achieved by shaking or agitation is an important factor in selecting spontaneous mutants incapable of producing cellulose by the populations of almost all the previously studied strains of *G. xylinus* (Krystynowicz et al., 2002; Valla & Kjosbakken, 1982). We therefore investigated the possibility of spontaneous mutation occurring in our strain under agitated conditions. We initially grew populations of the wild-type phenotype (non-mucoid colonies) in shaken broth cultures using both HS and modified HS media. After 3 days, colonies from the HS cultures plated on YPM agar showed phenotypic variation with two dominant phenotypes (non-mucoid and mucoid colonies) observed (Fig. 1). By contrast there was no phenotypic variation in the colonies from modified HS cultures i.e. they were all non-mucoid. Since HS and modified HS media are different with respect to carbon and nitrogen sources, this finding suggests that different nutrient conditions, rather than agitation conditions, may play a role in the selection of the spontaneous mutants by our strain. To confirm this possibility, we grew populations of the wild-type phenotype as before, but under static (without agitation) conditions. In line with our hypothesis, the colonies from the HS culture varied phenotypically, while there was no phenotypic variation in the modified HS culture.

These findings confirmed that different nutrient conditions rather than agitation conditions affect the growth of spontaneous mutants by our strain. This finding seems to be novel as spontaneous mutations of previously described strains of *Gluconacetobacter* spp. such as *G. xylinus* ATCC 10245 (Valla & Kjosbakken, 1982), *G. xylinus* E_{25} (Krystynowicz et al., 2002) and *G. hansenii* PJK (Park, Hyun, & Jung, 2004) have been reported to be due to oxygen abundance created as a result of stirring or shaking. Although the mechanisms of the spontaneous mutation of *G. xylinus* K3 were not investigated in our study, it appears that competition for both oxygen and nutrient levels are selective factors. In particular *G. xylinus* is a highly aerobic bacterium, and as a result cells at an air/liquid interface in static culture can access oxygen and utilize nutrients effectively through an increased metabolic rate (Ross et al., 1991). By contrast cells present in the oxygen-poor niche of a static broth culture are likely to have a restricted metabolic rate. Competition among the cells for oxygen and nutrients in this niche may favor the growth of spontaneous mutants which have a better ability to utilize nutrients. It is noted that the cells of *G. xylinus* K3 grew more slowly and produced less cellulose in HS medium than in modified HS medium as previously reported (Nguyen et al., 2008a). It is therefore possible that spontaneous mutants are selected in HS medium and not in modified HS medium.

The colonies grown from the mutant cultures were found to be phenotypically stable since the same phenotype was maintained after four growth passages. This suggests that the mutant phenotype is truly the result of a heritable mutation rather than a transient cell state. In addition, cellulose was found in the mutant cultures, indicating the mutant maintained cellulose-producing capability. Stability and maintenance of cellulose-producing capability distinguishes our mutant from the mutant phenotypes of strains previously reported in the literature which revert to cellulose-producing phenotypes and loose cellulose-producing capability (Krystynowicz et al., 2002; Valla & Kjosbakken, 1982).

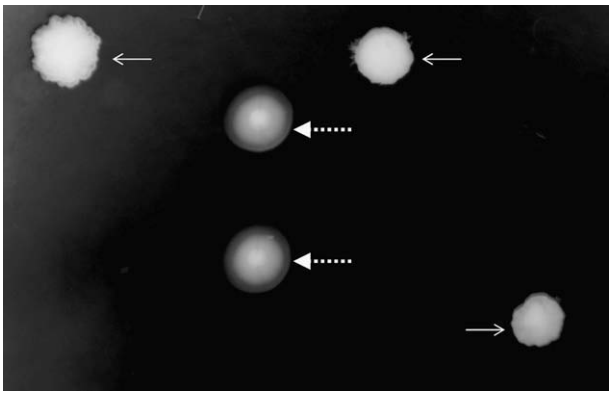

Fig. 1. Morphology of *G. xylinus* K3 colonies formed on YPM agar. The broken arrows indicate the non-mucoid colonies formed by the wild-type and the solid arrows indicate the mucoid colonies formed by the mutant.

3.2. Cellulose production and cell growth

The cellulose yields and cell density of the wild-type and the mutant were compared after six days in both HS and modified HS static cultures. In HS static culture, the mutant produced 0.153 ± 0.012 g/l cellulose, which was significantly lower ($P < .05$) than the cellulose yield of the wild-type (0.217 ± 0.027 g/l) (Fig. 2a). In contrast to reduced cellulose production, the mutant showed a significantly higher ($P < .05$) cell density than the wild-type after six days of growth (Fig. 2a). However, when subjected to modified HS static culture, both the mutant and the wild-type produced similar ($P > .05$) amounts of cellulose after six days and showed a similar cell density ($P > .05$) (Fig. 2b). These data suggest that the mutant appeared to reduce cellulose production to gain a comparative growth advantage as compared to the wild-type when grown in the environment (HS static culture) where it was selected for. This indicates that the adaptation of our strain to conditions of nutrient limitation may select for mutants that improve some aspects of nutrient catabolism by reducing unessential functions such as cellulose production that are costly to fitness. An adaption of microorganisms to conditions of nutrient limitation resulting in mutation has been reported in other bacteria such as *Escherichia coli* (Cooper & Lenski, 2000).

We also observed that the cellulose pellicle produced by the mutant grown in HS medium under static conditions gradually sank to the bottom of the culture after 2 days of floating on the culture surface (Fig. 3b) while the cellulose pellicle produced by the wild-type consistently floated on the culture surface (Fig. 3a). This observation suggested that the mutant may produce cellulose of a different kind from the wild-type. To test this hypothesis the structure of bacterial cellulose produced by two phenotypes in HS medium was compared using solid-state CP/MAS ^{13}C NMR spectroscopy and SEM.

3.3. Structural properties of bacterial cellulose

Comparison of NMR spectra of both cellulose samples indicated that the cellulose synthesized by the mutant had the same structural type of cellulose I (Nguyen et al., 2008a; VanderHart & Atalla, 1984) produced by the wild-type strain. This result eliminated our

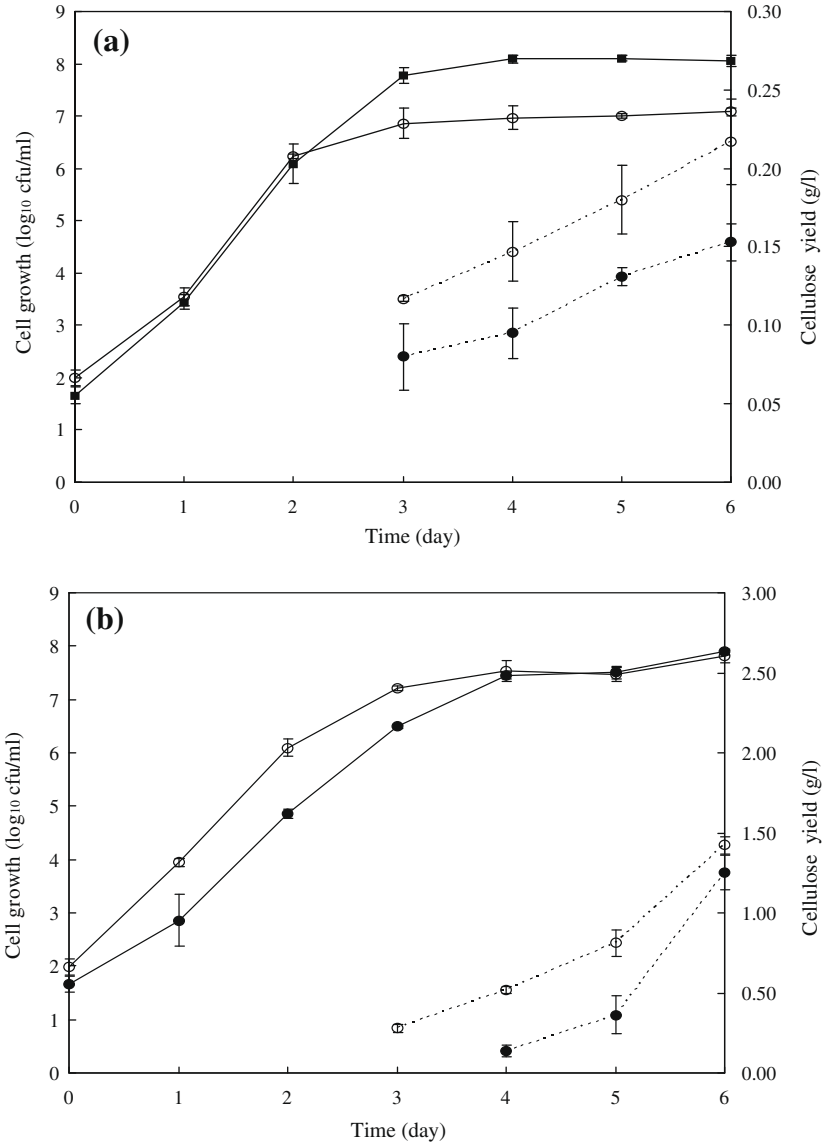

Fig. 2. The cell growth (solid lines) and cellulose yields (broken lines) of the wild-type (open circles) and the mutant (solid circles) in HS medium (a) and modified HS medium (b) under static culture for 6 days.

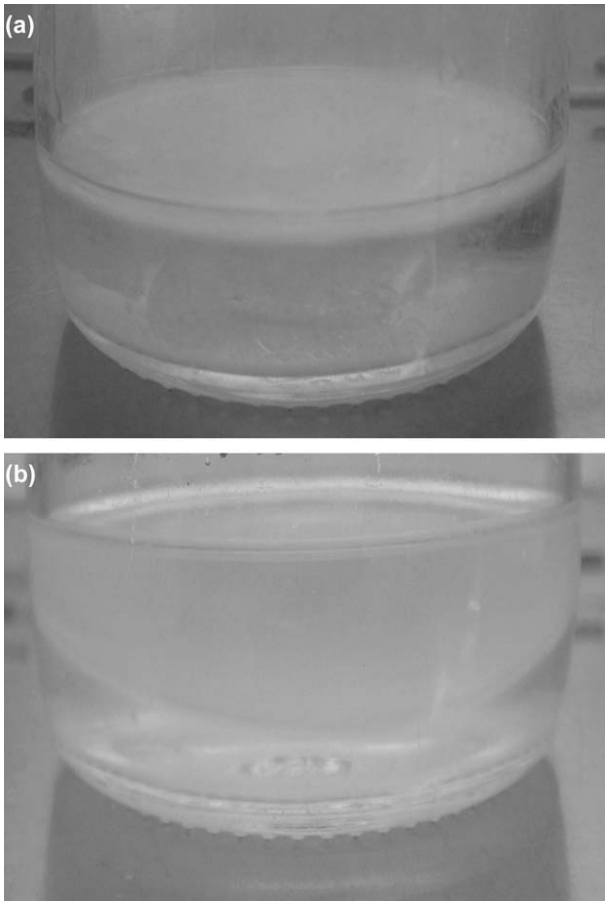

Fig. 3. Photographs of the floating type of cellulose produced by the wild-type (a) and the sinking cellulose mat produced by the mutant (b) in HS medium under static condition for 6 days.

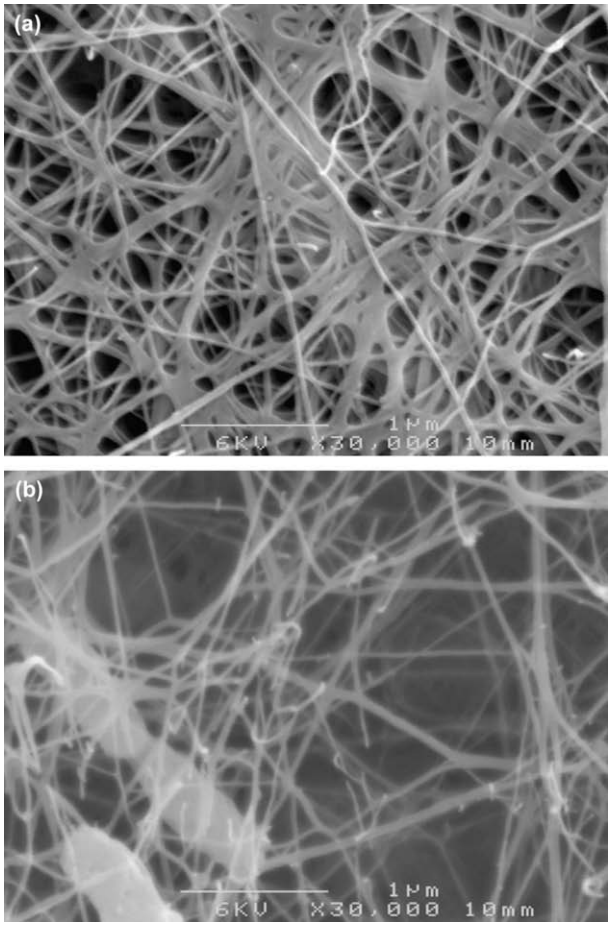

Fig. 4. Representative SEM images of bacterial cellulose produced in HS medium under static condition. (a) Bacterial cellulose produced by the wild-type shows the dense network of cellulose fibrils. (b) Bacterial cellulose produced by the mutant shows sparse density of cellulose fibrils.

speculation that the mutant may have been producing cellulose type II which has been reported to be produced by the mutant of *G. xylinus* ATTC 23769 (Kuga et al., 1993). Integration of the C4 signals revealed that the crystallinity of both cellulose samples was almost the same with approximately 80% crystalline structure (VanderHart & Atalla, 1984). Peak fitting analysis of the crystalline regions of the C4 signals (Yamamoto & Horii, 1993) indicated that both cellulose samples had a similar ratio of two crystalline structures, cellulose I_α and cellulose I_β which was approximately 50%.

The microstructure of the cellulose pellicles produced by two phenotypes in HS medium was compared using SEM. Although multiple SEM micrographs were taken of the pellicles produced by each phenotype, only one micrograph is presented here (Fig. 4). The micrograph presented is representative of all the micrographs for each pellicle type. Cellulose produced by the mutant (Fig. 4b) was observed to have fewer fibrils and larger gaps between fibrils compared to the cellulose produced by the wild-type, which was observed to have a denser network of cellulose fibrils with smaller gaps (Fig. 4a).

These results showed that although the mutant produces the same kind of cellulose as the wild-type, the cellulose mat had a lower fibril density and sank to the bottom of the growth medium. Cellulose has a higher molecular density than water and therefore the wild-type cellulose mat probably floats by trapping air to give it buoyancy. It is not so much the reduced cellulose production by the mutant that causes it to sink, but rather an increase in inter-fibril distances (Fig 4b) that allows displacement of air by liquid medium. Once the trapped air fraction within the mat is sufficiently small that the buoyancy provided by the air is outweighed by the density of cellulose, the mat sinks. This suggests not only a qualitative difference in cellulose production by the mutant and the wild-type but also disadvantages for the growth of the mutant. In particular, cellulose has been hypothesized to assist *G. xylinus* cells attached to the cellulose to reach the air/liquid interface for aerobic growth (Ross et al., 1991). In this case, static fermentation of the mutant results in a cellulose mat that sinks and therefore takes bacteria away from the air/liquid interface which would appear to be a major fitness disadvantage.

3.4. Protein profiles

Total protein extracts obtained for the wild-type and the mutant following growth in HS medium without agitation were analyzed using 2D electrophoresis. The 2D electrophoresis protein gels of the wild-type and mutant had similar protein profiles, but analysis using PDQuest software detected some five spot variations between the two strains (Fig. 5). Spots 1, 2 and 3 (circles) were observed to be up-regulated (twofold or greater) in the mutant compared to the wild-type and were therefore selected for protein identification using MALDI-MS. Spots 4 and 5 (squares) found to be down-regulated in the mutant compared to the wild-type. Spots 1, 2 and 3, up-regulated in the mutant, were identified as chaperonin GroEL, short-chain dehydrogenase/reductase (SDR) and deoxythymidine diphosphate (dTDP)-4-dehydrorhamnose 3,5-epimerase, respectively. Chaperonin GroEL is a general stress-related protein which

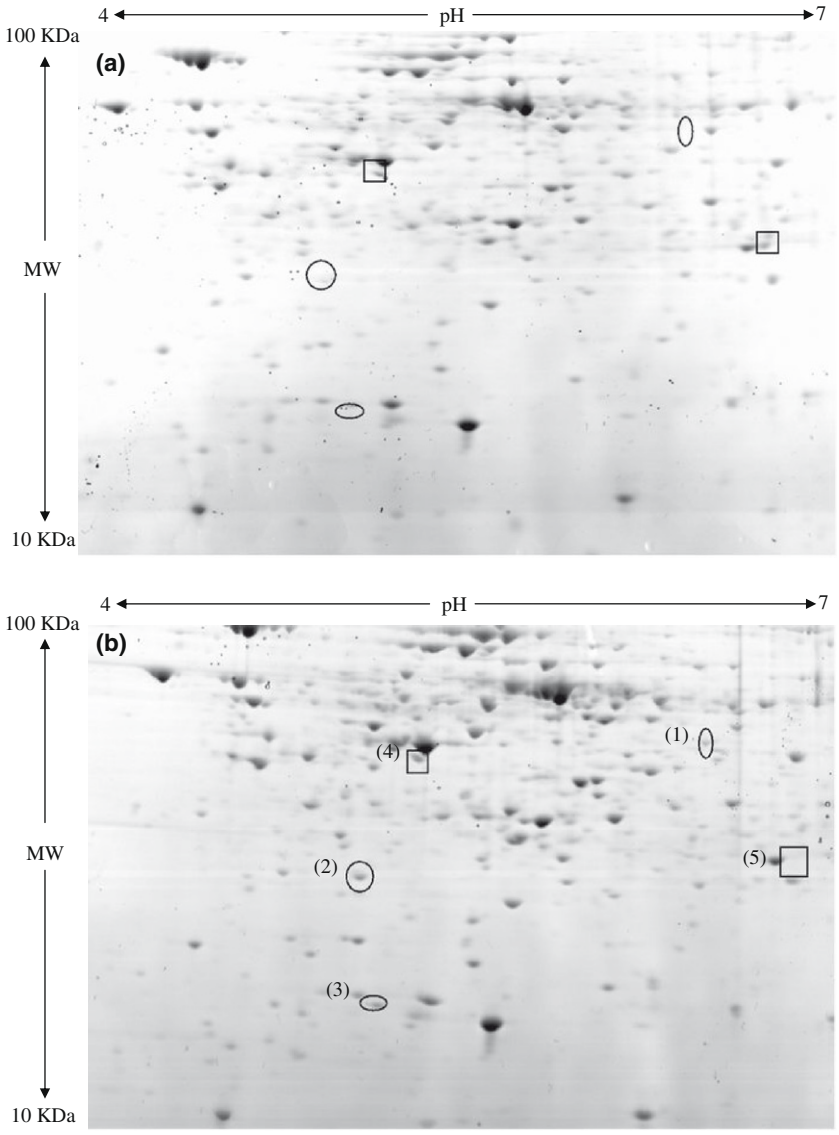

Fig. 5. Two-dimensional gel of total proteins extracted from the wild-type cells (a) and the mutant cells (b). The circles and squares indicate the differences between the protein profiles of the wild-type and the mutant. Molecular weight and isoelectric point are present on the *x* and *y* axes, respectively. The proteins marked in the circles were identified by MALDI-MS as chaperonin GroEL (spot 1), short-chain dehydrogenase/reductase (spot 2), and deoxythymidine diphosphate (dTDP)-4-dehydrorhamnose 3,5-epimerase (spot 3).

functions as an unfolded polypeptide chain binding factor. As proteomic analysis were performed after the wild-type and the mutant were grown to stationary phase (six days), cells may face nutrient limitation and respond to starvation through the expression of stress-related proteins. It is therefore possible that chaperonin GroEL was present not only in the mutant but also in the wild-type, with the mutant expressing a different isoform of GroEL. The SDR protein is an enzyme with oxidoreductase activity and has not been reported to be involved in cellulose synthesis. The protein spot identified as dTDP-4-dehydrorhamnose 3,5-epimerase is an enzyme which may be associated with the cellulose synthesis-reducing effect of the mutation. This enzyme is involved in the dTDP-rhamnose synthesis pathway which consists of three steps: (i) the synthesis of dTDP-4-keto-6-deoxy-D-glucose from glucose 1-phosphate and dTDP, (ii) the conversion of dTDP-4-keto-6-deoxy-D-glucose to dTDP-4-keto-L-rhamnose by dTDP-4-dehydrorhamnose 3,5-epimerase and (iii) the reduction of dTDP-4-keto-L-rhamnose to dTDP-L-rhamnose (Christendat et al., 2000; Melo & Glaser, 1968). Rhamnose is known to be one of the components of acetan produced by some strains of *G. xylinus* which consists of glucose, mannose, glucuronic acid and rhamnose in the proportions of 4:1:1:1 (Ishida, Sugano, & Shoda, 2002; Jansson, Lindberg, Wimalasiri, & Dankert, 1993). It is therefore possible that the presence of dTDP-rhamnose may activate acetan synthesis, which in turn may reduce cellulose production of the mutant as both of these polysaccharides are synthesized from the same carbon substrates (Ross et al., 1991). The presence of acetan was not analysed for in the current study. Ideally this feature should have been investigated to confirm the results obtained from the 2D protein identification work. However, this hypothesis is supported by the lack of dTDP-4-dehydrorhamnose 3,5-epimerase in the wild-type and our previous findings showing an absence of acetan in the broth culture of the strain (Nguyen et al., 2008a). In addition, the mucoid nature of colonies formed by the mutant could be due to the presence of a water-soluble polysaccharide such as acetan. Also, the ability of our mutant phenotype to produce cellulose suggests a different molecular mechanism than

that suggested for cellulose deficiency in the non-cellulose producing mutants (Krystynowicz et al., 2005; Valla et al., 1989). Further identification of a mechanism for the novel mutation in *G. xylinus* K3 is required as it is not clear from the present study why the enzyme dTDP-4-dehydrorhamnose 3,5-epimerase was present in the mutant but not in the wild-type. However, it is possible that in the wild-type, transcription of the gene responsible for the expression of dTDP-4-dehydrorhamnose 3,5-epimerase is repressed by a transcription factor, and that a mutation in the gene encoding the transcription factor has rendered it non-functional.

4. Conclusion

In conclusion, our results describe a novel type of spontaneous cellulose production mutation associated with *G. xylinus* K3 from Kombucha which is generated under conditions of nutrient limitation. The mutant demonstrated phenotypic difference and produced less cellulose as compared to the wild-type in the environment where it was selected. However, the cellulose produced by the mutant had the same crystalline structure as that produced by the wild-type. The presence of enzyme dTDP-4-dehydrorhamnose 3,5-epimerase in the in the mutant but not in the wild-type may be involved in mutation. However, further work is required to determine whether the enzyme is associated with acetan synthesis and reduced cellulose synthesis by the mutant. In addition, although not performed in the present study, further confirmation of protein expression using quantitative real-time PCR or Western blotting, and investigation into the molecular nature of differences in the spontaneous mutant, are recommended.

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