Characterization and spontaneous mutation of a novel gene, *polE*, involved in pellicle formation in *Acetobacter tropicalis* SKU1100

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Acetobacter tropicalis SKU1100 produces a pellicle polysaccharide, consisting of galactose, glucose and rhamnose, which attaches to the cell surface. This strain forms two types of colony on agar plates: a rough-surfaced colony (R strain) and a mucoid smooth-surfaced colony (S strain). The R strain forms a pellicle, allowing it to float on the medium surface in static culture, while the S strain does not. The pellicle is an assemblage of cells which are tightly associated with capsular polysaccharides (CPS) on the cell surface. In this study, a gene required for pellicle formation by the R strain was investigated by transposon mutagenesis using Tn10. The resulting mutant, designated Pel[−], has a smooth-surfaced colony and a defect in pellicle formation, as for the S strain. The mutant produced polysaccharide which was instead secreted into the culture medium as extracellular polysaccharide (EPS). An ORF was identified at the Tn10 insertion site, designated *polE*, upstream of which *polABCD* genes were also found. The deduced amino acid sequences of *polABCD* showed a high level of homology to those of *rfbABCD* which are involved in dTDP-rhamnose synthesis, whereas *polE* had a relatively low level of homology to glycosyltransferase. In this study a *polB* (*rfbA*) disruptant was also prepared, which lacked both CPS and EPS production. A plasmid harbouring the *polE* or *polB* genes could restore pellicle formation in the Pel[−] mutant and S strains, and in the Δ *polB* mutant, respectively. Thus both *polE* and *polB* are evidently involved in pellicle formation, most likely by anchoring polysaccharide to the cell surface and through the production of dTDP-rhamnose, respectively. The Pel[−] and Δ *polB* mutants were unable to grow in static culture and became more sensitive to acetic acid due to the loss of pellicle formation. Additionally, this study identified the mutation sites of several S strains which were spontaneously isolated from the original culture and found them to be concentrated in a sequence of 7 C residues in the coding sequence of *polE*, with the deletion or addition of a single C nucleotide.

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INTRODUCTION

Many bacterial species produce extracellular hetero- or homopolysaccharides, which are either tightly associated with the cell surface or secreted into the medium. The former are capsular polysaccharide (CPS) while the latter are extracellular polysaccharide (EPS). Bacterial CPS and EPS

are a focus of study, as they are involved in human and plant pathogenesis, and human- and plant-bacterium interactions; they are also involved in biofilm formation, which may be a part of the stress response to, for example, antibiotics (Sutherland, 1982; Roberts, 1996). Polysaccharide biosynthetic pathways involving glycosyltransferases have also been studied (Ishida *et al.*, 2002; Whitfield & Paiment, 2003).

Abbreviations: CPS, capsular polysaccharide; EPS, extracellular polysaccharide; Km, kanamycin; Tc, tetracycline; SEM, scanning electron microscopy; TFA, trifluoroacetic acid.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB195173.

Supplementary tables showing the bacterial strains and PCR primers used in this study are available with the online version of this paper.

Acetic acid bacteria are obligately aerobic α -*Proteobacteria*. They are oxidative bacteria that strongly oxidize ethanol to acetic acid and have thus been used for vinegar production. Such species maintain a high state of aeration by producing a pellicle which causes the cells to float on the medium surface in static culture. The pellicle is an assemblage of cells

that are tightly associated with CPS on the cell surface. Many species of acetic acid bacteria produce CPS, which seems to be related to pellicle formation. Specifically, *Acetobacter xylinum* (now called *Gluconacetobacter xylinus*) is widely known to produce a pellicle consisting of bacterial cellulose and thus it is the most famous model organism for the study of cellulose biosynthesis (Brown *et al.*, 1976). In addition to ultrastructural and biochemical investigations, the operons involved in cellulose biosynthesis, such as the *acs* operon (Saxena *et al.*, 1994) and the *bcs* operon (Wong *et al.*, 1990), have already been studied. Thus, the pellicle produced by other *Acetobacter* or *Gluconacetobacter* species has been considered to consist of cellulose or cellulose-like material.

We previously reported that *Acetobacter aceti* IFO 3284 (reclassified as *Acetobacter lovaniensis*) produces two different types of colony on agar medium, a rough-surfaced colony (R strain) and a smooth-surfaced colony (S strain). The R strain can produce a pellicle polysaccharide which allows it to float on the medium surface in static culture, while the S strain cannot (Matsushita *et al.*, 1992). The R and S strains are interconvertible by spontaneous mutation. The occurrence of such frequent mutation and the formation of two or more different types of colonies after serial transfer is well known in *Acetobacter* or *Gluconacetobacter* species, including *G. xylinus* (Shimwell & Carr, 1964; Valla & Kjosbakken, 1982). However, unlike *G. xylinus*, the R strain of *A. lovaniensis* produces a novel pellicle polysaccharide, which is a heteropolysaccharide composed of glucose and rhamnose (Moonmangmee *et al.*, 2002a). Furthermore, *Acetobacter tropicalis* SKU1100, a thermotolerant strain that can grow and form a thick pellicle in static conditions even at higher temperatures (37–40 °C), produces a pellicle polysaccharide consisting of galactose, glucose and rhamnose (Moonmangmee *et al.*, 2002b).

In this study, we examined the polysaccharide production of *A. tropicalis* SKU1100 at the molecular level by searching for genes essential to pellicle formation. Since *A. tropicalis* also produces R and S strains, transposon mutagenesis was performed on an R strain isolated from its original culture. A mutant exhibiting a phenotype similar to the S strain was obtained and the genes involved in pellicle polysaccharide synthesis were isolated and characterized.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are shown in supplementary Table A (available with the online version of this paper). *A. tropicalis* SKU1100 and its derivatives were grown at 30 °C in potato medium consisting of 5 g glucose, 10 g yeast extract, 10 g polypeptone and 150 ml potato extract in 1 l tap water, or in YPG medium consisting of 5 g yeast extract, 5 g polypeptone and 10 g glycerol in 1 l tap water. *Escherichia coli* strains were all grown at 37 °C in LB medium consisting of 5 g yeast extract, 5 g NaCl and 10 g polypeptone in 1 l distilled water at pH 7.5. *A. tropicalis* transposon mutants were selected on YPG medium. Antibiotics were supplemented as

indicated at the following concentrations: tetracycline (Tc), 12.5 µg ml⁻¹; kanamycin (Km), 50 µg ml⁻¹; and ampicillin, 50 µg ml⁻¹.

Isolation of R and S strains of *A. tropicalis* SKU1100. *A. tropicalis* SKU1100 wild-type strain was cultured in potato medium with agitation. The culture was then diluted and spread onto potato agar, where almost all colonies were R-type, and an R-type colony was isolated as the R strain. To isolate the S-type colony, repeated shaking cultures were performed several times by transferring the culture to fresh potato medium every 24 h. The culture was then diluted and spread onto a plate from which an S-type colony was isolated as the S strain.

Comparison of growth behaviour. The *A. tropicalis* SKU1100 R strain, the Pel⁻ mutant (see below) and the Δ polB mutant (see below) were grown as seed cultures in potato medium for 24 h with agitation. For static culture, 25 µl of the seed culture was inoculated into 5 ml potato medium and cultivated statically at 30 °C. Cells were harvested from a single culture by centrifugation at 11 500 g in a microcentrifuge tube. Cell pellets were dried overnight at 120 °C and dry weight was measured. In the case of the shaking culture, 25 µl of the seed culture was inoculated into 5 ml potato medium and cultivated with agitation at 30 °C. Cell growth was measured as turbidity in Klett units.

DNA manipulation. The extraction of plasmid and genomic DNA from *A. tropicalis* strains was performed according to a standard protocol (Sambrook *et al.*, 1989). The extraction of plasmid DNA from *E. coli* and *A. tropicalis* was performed with a QIAprep Spin Miniprep Kit (Qiagen), where an incubation step at 37 °C for 30 min with 20 µg lysozyme ml⁻¹ was included in the case of *A. tropicalis*. PCR was carried out in a 25 µl reaction volume (puReTaq Ready-To-Go PCR beads; Amersham Biosciences) using a GeneAmp PCR System 2400 (Perkin-Elmer). Agarose gel electrophoresis was performed in a 0.8% agarose gel in TBE buffer (89 mM Tris/HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) and DNA excised from the gel was purified using a QIAquick Gel Extraction Kit (Qiagen). The PCR primers used in this study are listed in supplementary Table B (available with the online version of this paper).

Transposon mutagenesis. Random insertion of transposon Tn10 into the DNA of *E. coli* S17-1 harbouring pSUP2021 was carried out after infection with the transposon vehicle phage λ NK1323 (Reddy & Gowrishankar, 2000) as described by Sambrook *et al.* (1989). Tc^r colonies were then screened and their plasmids were extracted from Tc^r colonies. Mixtures of the plasmids were transformed into *E. coli* DH5 α . Then, a Tc^r clone was selected and pSUP2021 containing Tn10, pSUP2021Tn10, was isolated. pSUP2021Tn10 was transformed into an *E. coli* S17-1 donor strain for conjugal mating and transferred to the *A. tropicalis* SKU1100 R strain via conjugation. The recipient *A. tropicalis* strain and *E. coli* S17-1 harbouring pSUP2021Tn10 were grown to exponential phase ($\sim 10^8$ c.f.u. ml⁻¹) in potato medium and LB broth, respectively, and then mixed at a ratio of 3:2. The mixture was centrifuged for 5 min at 2460 g, and the pellets were resuspended in 100 µl potato medium and put onto a potato agar plate as a single spot. After incubation at 30 °C for 10 h, the cells were suspended in 1 ml distilled water and spread onto YPG agar containing 0.2% acetic acid and Tc. Tc^r colonies appeared after 3 days' incubation at 30 °C. A smooth-surfaced colony was obtained and subsequently used for further experiments.

Southern hybridization. Chromosomal DNA extracted from the *A. tropicalis* R strain and its transposon mutants was completely digested with *Sph*I, which does not cut the sequence of Tn10, and separated by agarose gel electrophoresis. Afterwards, the DNA was transferred to a nylon membrane by capillary blotting (Sambrook *et al.*, 1989) and fixed to the membrane by UV irradiation. The

1.1 kb PCR product for Tn10 that was amplified from pSUP2021Tn10 using primers TnL and TnR was used as a hybridization probe. Hybridization was carried out with ECL Direct Labelling and Detection Systems (Amersham Biosciences) according to the manufacturer's manual.

Cloning of the flanking sequence of the Tn10 insertion site.

Transposon flanking regions were cloned by the *in vitro* cloning method, where PCR is carried out between one primer that anneals to the known sequence (transposon DNA) and a second primer that anneals to the cassette DNA sequence (DNA cassette, LA PCR *in vitro* Cloning Kit; Takara) ligated to both sides of the DNA fragment and the intervening, unknown sequences are amplified. The chromosomal DNA of the Pel⁻ mutant (see below) was digested with *Bam*HI and ligated with a *Sau*3AI DNA cassette. Approximately 100 ng of the ligated DNA was used as template in each 25 µl PCR reaction with 10 pmol each PCR primer, C1/T1 or C1/T3. C1 and C2 were the primers for the cassette, whereas T1 and T2, and T3 and T4 were designed based on the transposon sequence at the 5' and 3' ends, respectively. The reactions (30 cycles) were carried out using PCR conditions of 94 °C for 30 s, 65 °C for 1 min and 72 °C for 3 min. After completion of the first PCR, each reaction mixture was diluted 100-fold and 1 µl of the diluted solution was then used as template for the second PCR, where primers C2/T2 or C2/T4 were used instead of C1/T1 and C1/T3 used in the first PCR, respectively. Furthermore, to determine the upstream sequence of the *Bam*HI site, a DNA fragment ligated to a *Sal*I DNA cassette was amplified with primers U1/U2 instead of T1/T2, respectively. Also, to determine the sequence downstream from the *Bam*HI site, a DNA fragment ligated to an *Sph*I DNA cassette was amplified with primers D1/D2 instead of T3/T4, respectively. D1 and D2 primers were designed based on the 5'-terminal sequence of the pTL1 insertion, whereas U1 and U2 were designed based on the 3'-terminal sequences of the pTR1 insertion. The PCR products were separated in 0.8% agarose gel, purified and ligated into pGEM-T Easy Vector (Promega) to create pTR2, pTR1, pTL1 and pTL2 with the primer sets of C2/U2, C2/T2, C2/T4, and C2/D2, respectively (see Fig. 4), and then subjected to sequence analysis.

Nucleotide sequence analysis. The DNA inserted into pGEM-T vector described above was subjected to sequence analysis using an ABI PRISM 310 (PE Biosystems) and the data were analysed using the GENETYX-MAC program (Software Development). Homology search analysis and alignment were performed using BLAST (Altschul *et al.*, 1990) and CLUSTAL W (www.ebi.ac.uk/clustalw), respectively.

Complementation analysis. The DNA fragment bearing the *polE* gene (see Fig. 4) was amplified from the *A. tropicalis* SKU1100 R strain with primers WFr and WRv using PCR conditions (30 cycles) of 94 °C for 30 s, 64 °C for 1 min and 72 °C for 2 min and were then ligated into the pGEM-T vector. A 1.5 kb *Eco*RI fragment was then cloned into the *Eco*RI site of a broad-host-range plasmid, pCM62 (Tc^r), resulting in pCMPolE. The plasmid was digested with *Bam*HI, blunt-ended and ligated with the 0.9 kb *Eco*RV fragment of the non-polar Km^r cassette from pTKm (Yoshida *et al.*, 2003) in the same transcriptional direction as *polE* to give pCMPolEkm. This plasmid was first transformed into *E. coli* S17-1 and then incorporated into the Pel⁻ mutant via conjugative transfer as described above. The transconjugants were selected on YPG agar containing 0.2% acetic acid and 75 µg Km ml⁻¹. pCMPolE was also transformed into the S strain by conjugation. The transconjugants were then directly selected on YPG agar containing 0.2% acetic acid and Tc.

Disruption and complementation of *polB* gene. To disrupt *polB* (see Fig. 4), the *polB* gene region was amplified by PCR from SKU1100 R strain chromosomal DNA using primers PolBFR and PolBRv. PCR cycling was done with the following conditions: 94 °C for 5 min, then 25 cycles of 94 °C for 30 s, 60 °C for 1 min and

72 °C for 3 min, followed by 7 min at 72 °C. The resultant 2.5 kb PCR product was ligated into pGEM-T vector to give pTPolB. The plasmid was digested with *Bam*HI, blunt-ended and ligated with the 0.9 kb *Eco*RV fragment of the non-polar Km^r cassette, as described above. At this step, it was confirmed that the transcriptional direction of both the cassette and the target *polB* gene was the same. The resultant plasmid, pTΔPolB, was digested with *Eco*RI and the 3.4 kb fragment was used for the disruption of *polB*. The SKU1100 R strain was electroporated with approximately 0.2 µg DNA fragment and Km^r transformants were selected on YPG medium containing Km. The disruption of the target gene was confirmed by PCR. The disruption mutant obtained was designated Δ*polB*. For the complementation of Δ*polB*, a 2.5 kb *Eco*RI fragment of pTPolB was inserted into the *Eco*RI site of pCM62, to place *polB* downstream of the *lac* promoter in the same transcriptional direction to give pCMPolB. The plasmid was then electroporated into the Δ*polB* mutant and the transformants were selected on YPG medium containing Km and Tc. The complemented strain was named Δ*polB*(pCMPolB) and the incorporation of plasmid was confirmed by agarose gel electrophoresis (data not shown).

Determination of sugar composition. *A. tropicalis* strains were cultured in potato medium by shaking (200 r.p.m.) with a rotary shaker at 30 °C for 24 h. The cells were then collected by centrifugation and washed twice with distilled water, whereas the culture supernatants were mixed with 2 vols 2-propanol to precipitate any polysaccharides. Approximately 50 mg of the cells and 20 mg of the crude precipitated polysaccharide were hydrolysed in an aqueous solution of 2 M trifluoroacetic acid (TFA) for 2 h at 120 °C in a glass screw-capped vial. The resultant solutions were subsequently taken to dryness at 40 °C in a vacuum-centrifuged evaporator. The dried products were then dissolved in 1 ml distilled water and evaporated to dryness again. Next the pellets were dissolved in 100 µl distilled water and 2 µl of the suspensions were applied onto a silica gel plate (silica gel60; Merck). After being developed with a solvent system of 1-propanol/distilled water (85:15, v/v), the plate was sprayed with 5% sulfuric acid in ethanol and baked for 10 min at 100 °C to visualize sugar spots.

Other analytical methods. Scanning electron microscopy (SEM) was performed by using a JEOL JSM6100 scanning electron microscope with the cells prepared as described previously (Moonmangmee *et al.*, 2002a). Sugar content was measured by the phenol/sulfuric acid method (Dubois *et al.*, 1956) using glucose as standard. Before analysis, *A. tropicalis* cells were washed twice with distilled water while the culture medium was dialysed against distilled water. Protein content was measured by the modified Lowry method (Dulley & Grieve, 1975) using bovine serum albumin as a standard.

RESULTS

Isolation and characterization of *A. tropicalis* SKU1100 R and S strains

The original culture of wild-type *A. tropicalis* SKU1100 included a small number of S-type strains which could be distinguished from the R strain on an agar plate as S and R colonies, respectively. The S colonies looked not only shiny but also slimy [Fig. 1A(b)], which is different from the shiny but not slimy S strain of *A. lovaniensis* (Matsushita *et al.*, 1992). The R and S strains were separated, maintained on agar plates and confirmed to be stable without any change after more than 10 successive cultivations. In static culture, the R strain grew on the medium surface by forming a thick pellicle, but the S strains did not produce pellicle, and thus barely grew

Fig. 1. Phenotypes of *A. tropicalis* SKU1100 strains. Colony morphology (A) and growth behaviour in static culture (B) of the R strain (a), the S strain (b), the Pel[−] mutant (c), Pel[−](pCMPolEKm) (d), the Δ polB mutant (e) and Δ polB(pCMPolB) (f). These strains were inoculated in 5 ml potato liquid medium and then cultivated under static conditions for 3 days.

[Fig. 1B(a, b)]. SEM showed that R cells, but not S cells, were covered by an amorphous material (Fig. 2a, b). As shown in Table 1, the sugar content in R strain cells was 3·6-fold higher than that of S strain cells, whereas the sugar content of the culture medium was higher in the S strain than in the R strain. The sugars detected in the culture medium of the S strain could be precipitated by isopropyl alcohol.

The sugar composition of the intact cells and the precipitated polysaccharides from the culture medium of the S strain was analysed by TLC as described in Methods. Similar to the original *A. tropicalis* cells (Moonmangmee *et al.*, 2002b),

Fig. 2. Surface morphology, observed by SEM, of *A. tropicalis* SKU1100 R strain (a), S strain (b), Δ polB mutant (c) and Δ polB(pCMPolB) (d). Bars, 1 μ m.

three sugars corresponding to galactose, glucose and rhamnose, based on R_F values and spot colour, were detected in the acid hydrolysate of the precipitated polysaccharides from the S strain culture medium (Fig. 3, lane 5) as well as the intact cells of the R strain (lane 1), but not in that of the intact cells of the S strain (lane 2). Thus, unlike the R strain, the S strain secreted polysaccharides into the culture medium instead of attaching them to the cell surface.

Construction of the Pel[−] mutant by Tn10 mutagenesis

To identify the genes involved in pellicle formation or pellicle polysaccharide synthesis in this strain, we attempted

Table 1. Sugar content of cells and culture medium of several *A. tropicalis* SKU 1100 strains

Each strain was cultivated in a potato liquid medium by shaking for 24 h at 30 °C. The cells were then harvested, washed and resuspended in distilled water. The sugar and protein contents of the intact cells were determined by the phenol/sulfuric acid method and the modified Lowry method, respectively. The culture media were dialysed against distilled water for 2 d before analysis.

<i>A. tropicalis</i> SKU1100 strain	Sugar content*	
	Cells [μmol (mg protein) ^{−1}]	Medium† [μmol (ml culture medium) ^{−1}]
R strain	3·73 ± 0·09 (3)	4·34 ± 0·16 (3)
S strain	1·40 ± 0·27 (3)	7·45 ± 0·37 (2)
Pel [−] mutant	0·99 ± 0·09 (3)	7·97 ± 0·02 (2)
Pel [−] mutant (pCMPolEKm)	3·91 ± 0·11 (2)	3·85 ± 0·20 (2)
S strain (pCMPolE)	3·16 ± 0·05 (2)	4·38 ± 0·16 (2)
Δ polB	1·28 ± 0·03 (2)	3·56 ± 0·10 (2)
Δ polB (pCMPolB)	3·46 ± 0·10 (2)	3·51 ± 0·04 (2)

*The sugar content is shown as a mean ± SD of two to three assays.
†The sugar content of the original potato medium was 4·75 ± 0·15 (2) after dialysis.

transposon mutagenesis. However, since *A. tropicalis* SKU1100 was found to be relatively resistant to Km, especially in the presence of acetic acid, Tn5 (Km^r) mutagenesis using suicide vector pSUP2021 could not be successfully applied. Since the Tn10 transposon, being resistant to Tc, has been known to randomly insert into the chromosome of Gram-negative bacteria at a single site (Alexeyev & Shokolenko, 1995), the pSUP2021 plasmid containing Tn10, pSUP2021Tn10, was constructed as described in Methods. When pSUP2021Tn10 was used for transconjugation into the R strain of *A. tropicalis* SKU1100, Tc^r transconjugant colonies were obtained at a frequency of 5×10^{-7} per recipient strain. Southern hybridization indicated that the mutants contained a single random insertion of Tn10 into their chromosome (data not shown). From approximately 2500 Tc^r colonies, only one colony exhibiting smooth-surfaced colony morphology similar to the S strain was isolated; this strain was designated Pel⁻ [Fig. 1A(c)]. This Pel⁻ strain could not form a pellicle in static culture [Fig. 1B(c)], lacked cell-attached polysaccharides and instead secreted polysaccharides into the culture medium (Table 1). As shown in Fig. 3, the constituents of the polysaccharide secreted by the Pel⁻ mutant (lane 6) were the same as those for the secreted polysaccharide of the S strain (lane 5) or the cell-attached polysaccharide of the R strain (lane 1).

Fig. 3. TLC profiles of the acid hydrolysis products of polysaccharides from *A. tropicalis* SKU1100 intact cells and from the culture medium. Each strain was cultured in 5 ml potato medium with shaking and then the cells and supernatant were separated. Lanes: 1, R strain cells; 2, S strain cells; 3, Pel⁻ mutant cells; 4, Pel⁻(pCMPoE) cells; 5, polysaccharides precipitated from the culture medium of the S strain; 6, precipitated polysaccharide from the Pel⁻ mutant; 7, Δ polB cells; 8, Δ polB(pCMPoB) cells; M, mixed authentic galactose (Gal), glucose (Glu) and rhamnose (Rha) in an equimolar ratio. Each sample was hydrolysed with 2 M TFA and developed on a silica gel 60/aluminium plate (Merck) with a solvent system of *n*-propyl alcohol/distilled water (85:15, v/v). Sugar spots were then detected with 5% sulfuric acid in ethanol. The rhamnose spot (yellow in colour) exhibits the same density as the other sugars, although it appears reduced compared to the other sugars in this black and white image.

Furthermore, to ascertain whether the Pel⁻ mutation affects both LPS synthesis and pellicle formation, we compared the LPS profiles between the R strain and the Pel⁻ mutant by 15 % SDS-PAGE. No difference in the mobility of LPS from both strains was observed (data not shown).

Identification of the transposon-inserted gene and its flanking genes in the Pel⁻ mutant

To determine the gene in which the transposon insertion occurred in the chromosome of the Pel⁻ mutant, *in vitro* cloning was performed, as described in Methods. Conceptual translation of the nucleotide sequence revealed the presence of an ORF, designated *polE*, which encodes a protein consisting of 321 aa. Tn10 was inserted at a position 557 bp from the start codon. Upstream of *polE*, a promoter-like sequence (–35 TTTTCT and –10 TAGAAA) and four other ORFs, designated *polABCD*, were found; downstream an inverted repeat sequence (5'-AGAAGGAGGAGGCCTCCTTCT-3') which might work as a rho-independent terminator was found. Thus, *polABCDE* is expected to form an operon (Fig. 4). The deduced amino acid sequences of these genes showed a high level of homology to those of enzymes involved in polysaccharide synthesis, present in various micro-organisms (Table 2). *polABCD* showed significant homology to the *rfbBACD* genes involved in the dTDP-rhamnose synthesis pathway in Gram-negative bacteria (Boels *et al.*, 2004; Tsukioka *et al.*, 1997; Mitchison *et al.*, 1997; Marolda & Valvano, 1995), which encode the enzymes dTDP-glucose-4,6-dehydratase, glucose-1-phosphate thymidyltransferase, dTDP-4-dehydrorhamnose-3,5-epimerase and dTDP-4-dehydrorhamnose reductase, respectively. Conversely, *polE* showed relatively low homology to glycosyltransferases.

Restoration of pellicle formation to *A. tropicalis* SKU1100 Pel⁻ mutant and S strains

To confirm that the phenotype observed in the Pel⁻ mutant resulted from the transposon insertion, complementation analysis was carried out with the pCMPoEKm plasmid, which contains the *polE* gene and a non-polar Km^r cassette upstream of *polE*. The plasmid was incorporated into the Pel⁻ mutant via conjugation. After 3 days' incubation, transconjugants were obtained on selective plates. All colonies showed a rough surface and were able to grow and form pellicle in static culture (Fig. 1). The presence of pCMPoEKm in these colonies was confirmed by agarose gel electrophoresis (data not shown).

Since the Pel⁻ mutant exhibited a phenotype similar to the S strain (the formation of smooth and slimy colonies on agar plates, the absence of pellicle formation in static culture and the secretion of polysaccharides into the culture medium), the S strain may similarly have a mutation in the *polE* gene. To confirm this, the S strain was conjugated with pCMPoE and all the colonies that appeared on the selective plate displayed a rough surface and grew in static culture by producing a pellicle (data not shown).

Fig. 4. Genetic organization of *polABCDE* of *A. tropicalis* SKU1100. *Tn10* was inserted inside *polE* in the *PeI*[−] mutant. A physical map of the *polABCDE* region is shown. pTR1 and pTL1 were constructed by using the cassette primers and primers based on *Tn10*, whereas pTR2 and pTL2 were similarly constructed by using cassette primers and primers based on pTR1 and pTL1, respectively. White and black triangles indicate the promoter-like sequence site and the putative transcription terminator, respectively.

Δ*polB* mutant lacking both CPS and EPS productions

To identify the role of genes upstream of *polE*, the *polB* gene, which was expected to encode a glucose-1-phosphate

thymidyltransferase, was disrupted with a non-polar *Km* cassette; this cassette has been reported to create non-polar insertions during gene disruption (Yoshida *et al.*, 2003). Interestingly, the Δ*polB* mutant forms colonies which are flat and shiny, but not slimy and smooth on agar medium

Table 2. Sequence homology of Pol proteins of *A. tropicalis* SKU1100 to proteins from other organisms

<i>A. tropicalis</i> SKU1100 Pol proteins	Size (aa)	Organism	Identity/similarity (%)*	Related protein	Size (aa)	Accession no.
PolA (RfbB)	288	<i>Gluconobacter oxydans</i> 621H	71/79	dTDP-glucose 4,6-dehydratase	351	NC_006677
		<i>Gluconacetobacter xylinus</i>	62/72	dTDP-glucose 4,6-dehydratase	349	AJ250327
		<i>Vibrio fischeri</i> ES114	49/70	dTDP-glucose 4,6-dehydratase	364	NC_006840
PolB (RfbA)	293	<i>Gluconacetobacter xylinus</i>	69/88	Glucose-1-phosphate thymidyltransferase	306	AJ250327
		<i>Raoultella terrigena</i>	62/87	Glucose-1-phosphate thymidyltransferase	289	AY376146
		<i>Klebsiella pneumoniae</i>	59/83	Glucose-1-phosphate thymidyltransferase	301	AF097519
PolC (RfbC)	185	<i>Gluconobacter oxydans</i> 621H	80/91	dTDP-4-dehydrorhamnose-3,5 epimerase	187	NC_006677
		<i>Gluconacetobacter xylinus</i>	64/90	dTDP-4-dehydrorhamnose-3,5 epimerase	185	AJ250139
		<i>Rhodopseudomonas palustris</i>	51/74	dTDP-6-deoxy-D-glucose-3,5-epimerase	185	NC_005296
PolD (RfbD)	303	<i>Gluconobacter oxydans</i> 621H	71/90	dTDP-4-dehydrorhamnose reductase	297	NC_006677
		<i>Gluconacetobacter xylinus</i>	53/82	dTDP-4-dehydrorhamnose reductase	295	AJ250139
		<i>Mesorhizobium loti</i>	43/77	dTDP-6-deoxy-L-mannose-dehydrogenase	292	NC_002678
PolE	321	<i>Gluconobacter oxydans</i> 621H	46/78	Glycosyltransferase	312	NC_006677
		<i>Rhodobacter sphaeroides</i>	36/60	Glycosyltransferase	312	NZ_AA AE01000155
		<i>Nitrosomonas eutropha</i> C71	34/63	Glycosyltransferase	314	NZ_AA JE01000014
		<i>Lactobacillus plantarum</i> WCFS1	28/61	Rhamnosyltransferase	308	NC_004567
		<i>Streptococcus mutans</i>	27/56	Rhamnosyltransferase	311	T00087
		<i>Lactococcus lactis</i> Il1403	25/58	Rhamnosyltransferase	319	NC_002662

*Scores are based on the overall length-wise comparison.

[Fig. 1A(e)]. It could not form pellicle under static culture [Fig. 1B(e)] and there was no amorphous material on the cell surface (Fig. 2c), indicating that this strain had lost CPS production. Unlike the S or Pel⁻ strains, however, the $\Delta polB$ strain exhibited low sugar content in both cells and culture medium (Table 1), and no EPS, which can be precipitated by alcohol, was detected in the culture medium. In TLC analysis of the acid-hydrolysed sample from $\Delta polB$ cells, no rhamnose spot was detected, while only weak galactose and glucose spots were seen (Fig. 3). All the phenotypes resulting from *polB* gene disruption could be recovered with plasmid pCMPolB which contained *polB*, indicating that *polB* is involved in CPS production.

Physiological effect of Pel⁻ and $\Delta polB$ mutation

Since Pel⁻ and $\Delta polB$ mutants were defective in pellicle formation and CPS production, we compared the growth of these mutants with the R strain in static and shaking cultures (Fig. 5). The results showed that Pel⁻ and $\Delta polB$ mutants, unlike the R strain, could not grow well in static culture, while all strains grew well in shaking culture. The S strain is also unable to grow in static culture, but can grow in shaking culture. Since a pellicle is expected to have the ability to resist some stress, we also compared growth in the presence of acetic acid, a typical stress agent for acetic acid bacteria. As shown in Fig. 5(c), both Pel⁻ and $\Delta polB$ mutants, as well as the S strain, were more sensitive to acetic acid than the R strain. $\Delta polB$ could not grow well with 0.5 % acetic acid and failed to grow with 1 % acetic acid, while the Pel⁻ mutant, similar to the S strain, could grow slightly in the presence of 0.5 % acetic acid but only marginally with 1 % acetic acid.

A spontaneous mutation site in S strains of *A. tropicalis*

Since the S strain obtained from the R strain seems to have a mutation in its *polE* gene, we compared the nucleotide sequence of the R strain with the sequences of the *polE* gene in several different S strains separately obtained by spontaneous mutation. As shown in Fig. 6, the *polE* gene sequence obtained from these S strains had a single C addition or deletion in a sequence of 7 C residues at position 378–384, resulting in a frameshift mutation. Incidentally, this repetitive sequence was not found in the other ORFs, from *polA* to *polD*. The mutation creates a stop codon at position 420 in the case of the deletion of a C, or at position 591 in the case of the addition of a C, both leading to mistranslation of the protein.

DISCUSSION

Acetic acid bacteria produce relatively large amounts of CPS or EPS. In contrast to *G. xylinus*, which produces cellulose, other acetic acid bacteria have been shown to produce heteropolysaccharide as their pellicle. *A. lovaniensis* IFO3284 and *A. tropicalis* SKU1100 produce novel pellicle polysaccharides composed of glucose and rhamnose, or of galactose, glucose and rhamnose, respectively

(Moonmangmee *et al.*, 2002a, b). Like several other *Acetobacter* species, these strains form two different types of colonies, R and S, on agar medium. In static culture, the R strain can grow by producing a pellicle which permits it to float on the medium surface, but the S strain cannot grow well due to a defect in pellicle production. The R and S strains can be reversibly interchanged by repeated static or shaking culture conditions, for example ~20 % of R strains are converted to S strains after eight successive shaking cultures, each culture being long enough to allow five to six generations (data not shown). Finally, the R strain predominates in static culture, whereas the S strain predominates in shaking culture.

In this study, we investigated genes related to polysaccharide synthesis and pellicle formation in *A. tropicalis* SKU1100, and also the mechanism by which the R strain loses the ability to produce polysaccharide and is easily converted into the S strain. In *A. tropicalis* SKU1100, the S strain isolated from the original R strain did not contain CPS attached to the cell, but secreted polysaccharide which had an identical sugar composition as the CPS into the culture medium. This contrasts with the *A. lovaniensis* IFO3284 S strain (Moonmangmee *et al.*, 2002a) and $\Delta polB$ mutant of *A. tropicalis* which do not produce any CPS or EPS. Although no growth differences were seen among R strain, S strain, Pel⁻ mutant and $\Delta polB$ mutant cells under shaking conditions, the S strain, Pel⁻ mutant and $\Delta polB$ mutant demonstrated virtually no growth in static culture, suggesting that CPS production is essential not only for pellicle formation but also for growth of *A. tropicalis* SKU1100 in static culture. More interestingly, the S strain, Pel⁻ mutant and $\Delta polB$ mutant were more sensitive to acetic acid than the R strain, which is more critical for $\Delta polB$ as it produces neither CPS nor EPS. Thus CPS may serve as a barrier, protecting R strain cells from acetic acid stress, reminiscent of a biofilm in relation to antibiotic resistance.

To identify the genes involved in pellicle formation or pellicle polysaccharide synthesis in *A. tropicalis*, transposon mutagenesis was performed on the R strain. A transposon mutant (Pel⁻) exhibited the same smooth-surface colony morphology and phenotype as the S strain. An ORF, *polE*, was identified at the transposon insertion site of the Pel⁻ mutant. Four other ORFs, *polABCD*, were found upstream of *polE*, and the ORFs were tightly linked in the same orientation, implying that *polABCDE* has an operon structure. *polABCD* showed significant homology to the *rfbACD* genes involved in the dTDP-rhamnose synthesis pathway, which is expected to consist of the following four sequential reactions; glucose 1-phosphate + dTTP is converted to dTDP-glucose (RfbA), then to dTDP-4-keto-6-deoxy-D-glucose (RfbB), then to dTDP-6-deoxy-L-mannose (RfbC) and finally to dTDP-L-rhamnose (RfbD). We found that mutation of *polB* of *A. tropicalis* SKU1100 led to completely defective CPS and EPS synthesis. Since the structure of *A. tropicalis* CPS is expected to have a main chain consisting of rhamnose and many branches with

Fig. 5. Growth behaviour of wild and mutant strains of *A. tropicalis* SKU1100. (a, b) The growth of the R strain (●), the Pel⁻ mutant (□) and the Δ*polB* mutant (▲) in 5 ml potato liquid medium was monitored by measuring the dry weight of cells (static cultures, a) or by measuring the culture turbidity (shaking cultures, b), as described in Methods. (c) The effect of acetic acid on the growth of the R strain, the S strain, the Pel⁻ mutant and the Δ*polB* mutant was examined on potato agar plates containing 0, 0.5 and 1% acetic acid. Each strain was grown in a potato liquid medium under shaking conditions until the mid-exponential phase, then streaked onto the plates and incubated for 2 d at 30 °C.

glucose and galactose residues (S. Moonmangmee and others, unpublished), it is reasonable that a defect in dTDP-rhamnose synthesis would eliminate the production of CPS.

On the other hand, PolE exhibited a relatively low level of homology to other glycosyltransferase homologues, compared to PolABCD. However, there are PolE homologues (25–46% identity) among putative glycosyltransferases of the *α-Proteobacteria*, close to the genus *Acetobacter*, and also among putative rhamnosyltransferases from lactic acid bacteria (Table 2). Furthermore, similar to *polE* which is located directly downstream of *polABCD*, these homologous genes, except for the *Nitrosomonas* enzyme, are also located within the *rfbBACD* gene cluster. Thus, it is expected that *polE* encodes a rhamnosyltransferase, which transfers a rhamnosyl residue onto an the oligosaccharide unit being synthesized, working together with the *rfbBACD* gene products. However, this may not be the case, because the Pel⁻ mutant (Δ*polE*) still produces

EPS with the same sugar composition as the wild-type CPS, although the purified EPS was shown to have a different molecular mass (unpublished data). Despite a relatively low homology, these PolE homologues have four clear consensus motifs: DDGSxD and DQDDxW near the N-terminal region followed by HDWxx and xYRQH present downstream, as shown in Fig. 7. Although the former two motifs are similar to, but not the same as, domain A (putative catalytic sites) of ExoU and HasA families of *β*-glycosyltransferases, the latter two consensus motifs downstream of domain A are rather different from domain B (putative substrate-binding sites) of the same families (Keenleyside & Whitfield, 1996). Thus, it cannot be concluded that PolE homologues are a kind of glycosyltransferase. In addition, PolE has very low homology (14% identity/49% similarity) to a functionally identified rhamnosyltransferase (WbbL) of *Serratia marcescens* (Rubires *et al.*, 1997), which does not have any PolE consensus motifs. Thus, PolE seems not to work as a

Fig. 6. Mutation points of the *polE* gene in three different S strains. Asterisks indicate nucleotides identical in all sequences.

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