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Author(s)	Morikawa, Masaaki; Kagihiro, Shinji; Haruki, Mitsuru et al.
Citation	Microbiology, 152(9), 2801-2807 https://doi.org/10.1099/mic.0.29060-0
Issue Date	2006
Doc URL	https://hdl.handle.net/2115/14776
Type	journal article
File Information	M-S152.pdf



Biofilm formation by a *Bacillus subtilis* strain that produces γ -polyglutamate

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Received 12 April 2006
Revised 19 May 2006
Accepted 12 June 2006

The extracellular matrix produced by *Bacillus subtilis* B-1, an environmental strain that forms robust floating biofilms, was purified, and determined to be composed predominantly of γ -polyglutamate (γ -PGA), with a molecular mass of over 1000 kDa. Both biofilm formation and γ -PGA production by *B. subtilis* B-1 increased with increasing Mn^{2+} or glycerol concentration. γ -PGA was produced in a growth-associated manner in standing culture, and floating biofilms were formed. However, γ -PGA was produced in a non-growth-associated manner in shaking culture conditions. When *B. subtilis* B-1 was grown in a microaerated culture system, floating biofilm formation and γ -PGA production were significantly retarded, suggesting that oxygen depletion is involved in the initial steps of floating biofilm formation in standing culture. Proteomic analysis of membrane proteins demonstrated that flagellin, oligopeptide permease and Vpr protease precursor were the major proteins produced by cells in a floating biofilm and a colony.

INTRODUCTION

Biofilms are surface-attached communities of bacteria embedded in an extracellular matrix. Biofilm formation occurs in many settings, and in response to diverse environmental cues (O'Toole *et al.*, 2000; Parsek & Greenberg, 2005). For example, biofilms can form over solid surfaces, or at the surface of liquids. In the latter case, the floating biofilms are referred to as pellicles. The colonies that grow on semi-solid media can also be considered to be a form of biofilm. Macroscopic and microscopic observations of bacterial biofilms reveal highly ordered structural features that disappear when the components of the extracellular matrix are eliminated as a consequence of mutation (Branda *et al.*, 2005).

The extracellular matrix contributes to the mechanical stability of the biofilm, enabling it to withstand considerable shear forces, and the biofilm has been shown to contain

polysaccharides, proteins and nucleic acids (Branda *et al.*, 2005). Among the polysaccharides present in the biofilm matrix are: alginate, Pel and Psl in *Pseudomonas aeruginosa*; cellulose in *Escherichia coli* and *Salmonella typhimurium*; a tetrasaccharide repeat of D-glucose, L-fucose and D-glucuronic acid in *Klebsiella pneumoniae* and *Enterobacter aerogenes*; gellan in *Sphingomonas* spp.; and levan (β -D-fructan) in streptococci (Boyd & Chakrabarty, 1995; Danese *et al.*, 2000; Friedman & Kolter, 2004; Kiska & Macrina, 1994; O'Neill *et al.*, 1986; Yamazaki *et al.*, 1996). The matrix from *Vibrio cholerae* O1 biofilms has been shown to contain a polysaccharide composed of N-acetyl-D-glucosamine, D-mannose, 6-deoxy-D-galactose and D-galactose (Wai *et al.*, 1998).

In recent years, *Bacillus subtilis*, a spore-forming Gram-positive bacterium, has become a model organism for the study of biofilm formation (Branda *et al.*, 2001; Hamon & Lazazzera, 2001). It has been shown that wild strains of *B. subtilis* generally form more robust floating biofilms than strains derived from the domesticated laboratory strain *B. subtilis* 168. In the highly structured floating biofilms and colonies formed by the wild strain *B. subtilis* 3610, sporulation displays a high degree of spatio-temporal organization, occurring predominantly at the tips of aerial projections. This confirms that biofilm formation can be an integral part of the developmental processes of this organism. For *B. subtilis* 3610, a major component of the extracellular matrix in colonies and floating biofilms is the

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Abbreviations: CSF, competence-stimulating factor; CV, crystal violet; EPS, exopolysaccharide; HPTLC, high-performance silica gel thin layer chromatography; MACS, microaerated culture system; γ -PGA, γ -polyglutamate; SEM, scanning electron microscopy.

putative exopolysaccharide (EPS) produced by the *eps* locus (Branda *et al.*, 2001, 2005; Kearns *et al.*, 2005). However, the macroscopic features of colonies and floating biofilms formed by different wild strains of *B. subtilis* can be dramatically different. Such was the case when we compared *B. subtilis* 3610 with *B. subtilis* B-1, which is an environmental strain isolated from an oilfield (Morikawa *et al.*, 1992). Here, we report that γ -polyglutamate (γ -PGA) is a major extracellular polymeric substance, and that oxygen depletion is an important signal for floating biofilm formation in *B. subtilis* B-1. We also demonstrate that flagellin, oligopeptide permease and Vpr protease precursor are the major membrane proteins produced in floating biofilm cells.

METHODS

Culture media. Luria (L-) broth, containing (l^{-1}) 10 g Bacto tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl, was used for general cultivation of bacteria. L-agar is a semi-solid medium containing 1.2% agar in L-broth. E-medium is a medium used for effective production of γ -PGA (Cromwick & Gross, 1995), and it contains (l^{-1}): 20 g L-glutamic acid, 12 g citric acid, 80 g glycerol, 7 g NH_4Cl , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4 , 0.15 g $CaCl_2 \cdot 2H_2O$, 40 mg $FeCl_3 \cdot 6H_2O$ and 148 mg $MnSO_4 \cdot H_2O$. The pH of E-medium was adjusted to 7.4 by 2 M NaOH. The amounts of glycerol and $MnSO_4$ were adjusted as noted.

Scanning electron microscopy (SEM). Pellicles were gently placed on a glass plate that had been coated with poly-L-lysine. The specimen was fixed with glutaraldehyde and OsO_4 , dehydrated in ethanol, isoamyl acetate and critical CO_2 , and sputter coated with platinum (Glauert, 1975). Observations were performed with a Hitachi S800 scanning electron microscope.

Preparation and analyses of extracellular matrix. *B. subtilis* B-1 (Morikawa *et al.*, 1992) cells were grown overnight on L-agar, and the colonies were scraped off, and suspended in 0.9% NaCl. This enabled us to obtain almost pure extracellular polymeric substances by a simple two-step process: removal of cells by centrifugation (30 000 g at 4 °C for 30 min), and ethanol precipitation by addition of 3 vols cold ethanol to the supernatant. The ethanol precipitation step was repeated twice, and the resulting material was dried in a vacuum.

A portion of the purified material was hydrolysed in 5 M HCl at 100 °C for 24 h, and the products were separated on a silica gel 60 high-performance silica gel thin layer chromatography (HPTLC) plate (no. 05631; Merck), with a developing solvent mixture of ethanol/water (63:37). The remaining material was dissolved in D_2O (4%, w/v), and analysed by 1H -NMR and ^{13}C -NMR (UNITY-INIVA 600; Bruker). These analyses revealed that the recovered material consisted almost exclusively of γ -PGA. The DL configuration of the constituent glutamic acids was determined by reverse-phase HPLC analysis, after derivatization with Marfey's reagent (1%, w/v, in acetone) at 40 °C for 90 min. The HPLC column was a COSMOSIL 5C18-AR (i.d. 4.6 mm, length 20 cm; Nacalai Tesque), and the eluent was methanol/20 mM sodium acetate buffer (pH 4)/acetonitrile (2:7:1). Fractions were monitored by absorbance at 340 nm. Elution positions and the areas of the peaks were compared with standard D-glutamic acid and L-glutamic acid.

Molecular mass was determined by TSKgel G4000SWxL gel filtration chromatography (i.d. 7.8 mm, length 30 cm; Tosoh), with 0.2 M sodium phosphate buffer (pH 6.9) and 0.2 M Na_2SO_4 . Elution of γ -PGA was monitored by absorbance at 220 nm. The level of production

of γ -PGA in the culture was estimated by comparing the peak area in gel filtration chromatography with those of the standards, or by weighing the dry material after purification.

Biofilm assays. Solid-surface-associated biofilm formation was estimated by the crystal violet (CV) staining method, with slight modifications (O'Toole *et al.*, 1999). Briefly, an overnight culture was diluted to an OD_{600} of 0.3, and 1 μ l was added to 99 μ l E-medium in a 96-well plastic titre plate. The plate was kept standing at 37 °C for 8 h. Then, the surface pellicles and the cultures were carefully removed from the wells. Each well was gently rinsed twice with distilled water, and the remaining cells and matrices were stained with 150 μ l of a 1% CV solution for 25 min at room temperature. After washing twice with distilled water, the CV attached to the biofilm was solubilized in 150 μ l DMSO, and quantified by measuring its absorbance at 570 nm.

Preparation and analyses of membrane proteins. Cells were harvested, and washed and suspended in 10 mM phosphate buffer (pH 7.0), after growing in standing, shaking or agar culture for 14 h at 37 °C. In the standing culture, cells formed floating biofilms on the surface (Fig. 1). Cell suspensions were subjected to lysozyme treatment (1 mg ml^{-1}) for 3 h at 37 °C, followed by sonication for 1 min on ice. After removing intact cells by centrifugation at 8000 g for 10 min, the supernatant was further fractionated by ultracentrifugation at 90 000 g for 1 h. The supernatant and precipitate were used as cytoplasmic and membrane protein samples, respectively. The membrane protein samples were separated on 12% polyacrylamide gel containing 1% SDS. Protein bands were visualized by Coomassie brilliant blue R-250 staining. The gel containing a protein band was subjected to trypsin digestion, followed by amino acid sequence analysis with LC-MS/MS (Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, MA, USA) and Sequest (Thermo Electron).

RESULTS AND DISCUSSION

Chemical structure of the extracellular polymeric substance produced by *B. subtilis* B-1

B. subtilis B-1, which was isolated from an oilfield, was found to form robust pellicles (floating biofilms) and solid-surface-associated biofilms in standing culture. It also formed highly structured colonies on L-agar medium that were strikingly mucoid in their centre (Fig. 1). In contrast, *B. subtilis* 168 formed flat colonies, and did not form significant pellicles or solid-surface-associated biofilms in L-broth. SEM observation of the pellicle produced by *B. subtilis* B-1 showed that the cells were densely packed side by side, and embedded in a matrix (Fig. 2). The 16S rRNA gene sequence of *B. subtilis* B-1 (EMBL/GenBank/DDJB accession number AB213262) is 99.6% identical to that of *B. subtilis* 168.

The extracellular polymeric substances produced by the colonies of *B. subtilis* B-1 were purified by ethanol precipitation, and found to yield negative results for both uronic acid and anthrone assays, suggesting that the material did not contain significant amounts of polysaccharide (May & Chakrabarty, 1994). In addition, the purified material did not contain significant amounts of protein or nucleic acid, as evidenced by the lack of absorbance at 280 and 254 nm. When the sample was hydrolysed, separated using HPTLC,

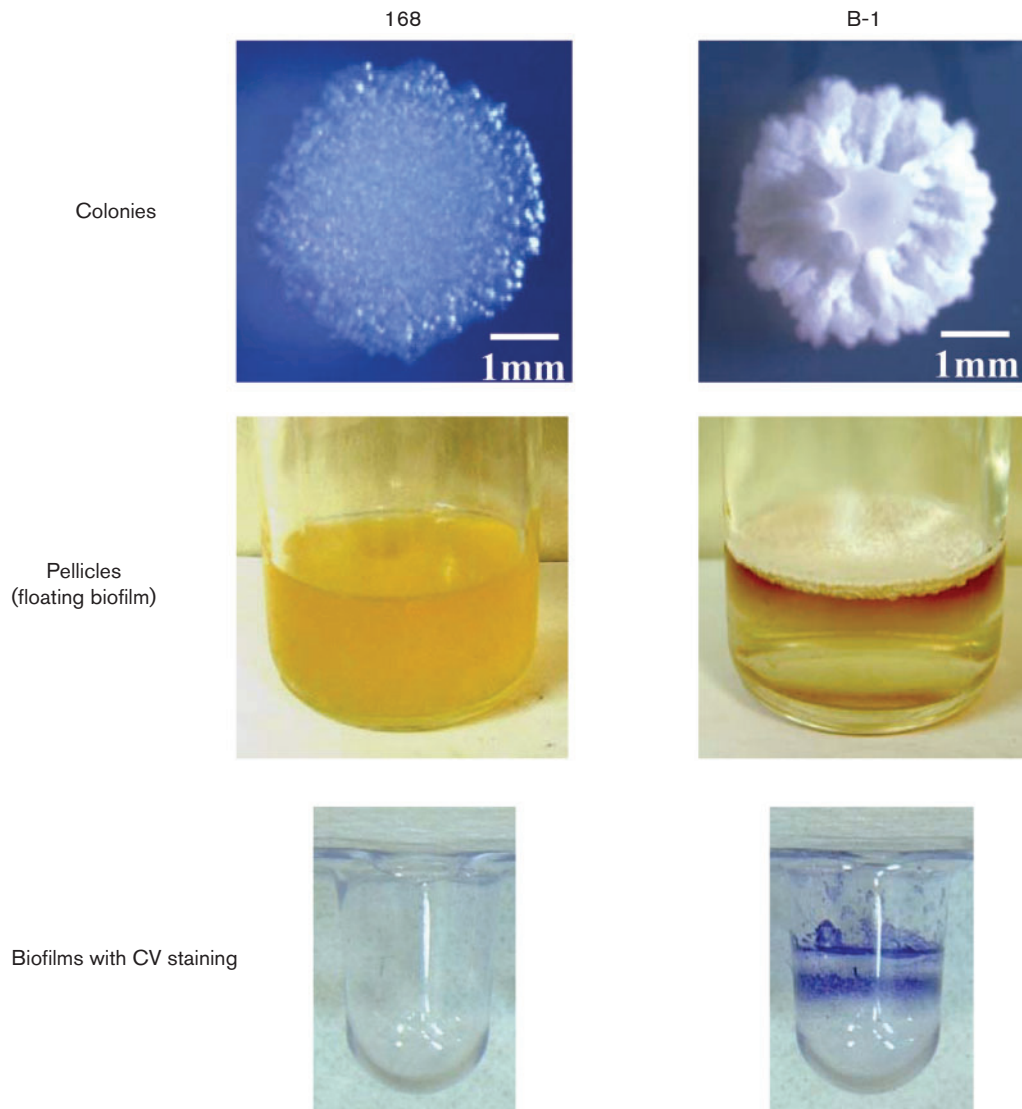


Fig. 1. Comparison of *B. subtilis* 168 (laboratory strain) and *B. subtilis* B-1 (environmental strain). Cells were grown on L-agar (colonies) and L-broth (pellicles, biofilms) at 37 °C for 14 h. Cells were taken by toothpick, and stabbed on L-agar to form a colony. Overnight liquid culture was added (1%) to L-broth for pellicle formation, which was visualized by CV staining (see Methods).

and stained by spraying with 0.1% ninhydrin ethanol solution, followed by baking at 100 °C, a single red-coloured spot was observed at a position corresponding to that of glutamic acid. The material was then analysed by ¹H- and ¹³C-NMR. Chemical shifts of the peaks are shown in Table 1. All of these results indicate that the material we isolated was composed mostly of γ -PGA. Relative glutamic acid peak areas, after separation using HPLC, indicated that the ratio of D- and L-glutamic acid, constituting the γ -PGA, ranged from 9:1 to 4:1 after 12 h cultivation at 37 °C in L-broth. The molecular mass of the isolated γ -PGA was determined to be over 1000 kDa by gel filtration chromatography. It should be noted that *B. subtilis* 3610 did not produce γ -PGA.

Production of γ -PGA, and biofilm formation

Recently, Stanley & Lazazzera (2005) have reported that following transfer of the genetic determinants controlling γ -PGA formation from a wild strain to the domestic *B. subtilis* 168, the latter is able to produce biofilms. However, deletion of *ywsC*, a gene encoding γ -PGA synthesis, in the wild *B. subtilis* RO-FF-1 does not lead to a marked decrease in surface-associated biofilm formation. This result demonstrates that the production of γ -PGA is not essential for biofilm formation by *B. subtilis*. As a result of this, we were interested in determining if there was a correlation between γ -PGA production and biofilm formation in *B. subtilis* B-1. Reports by others have shown that changes in

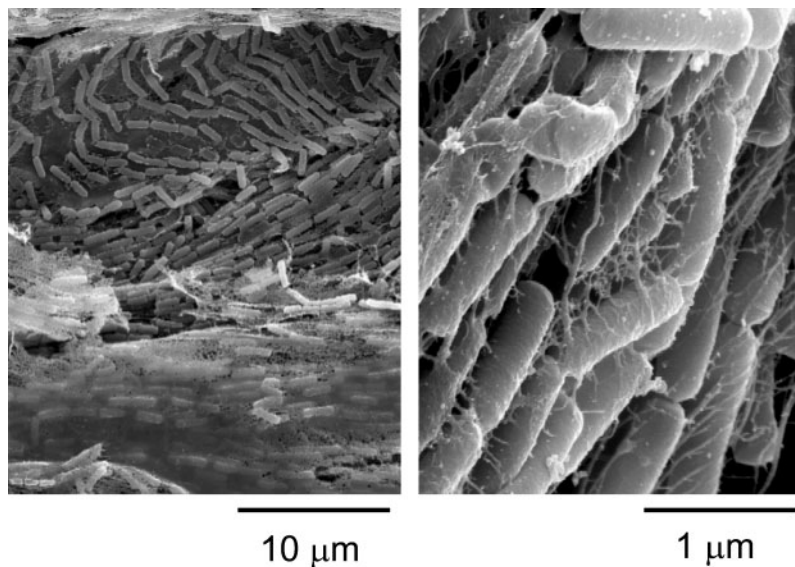


Fig. 2. SEM of pellicles formed by *B. subtilis* B-1.

the concentration of MnSO_4 and glycerol affect γ -PGA production by *Bacillus licheniformis* ATCC 9945A (Cromwick & Gross, 1995; Ko & Gross, 1998). We found similar results for *B. subtilis* B-1 (Fig. 3a, b). Importantly, this allowed us to show that there was a direct correlation between γ -PGA production and biofilm formation in *B. subtilis* B-1 (Fig. 3c). This correlation also held for another *B. subtilis* strain, *B. subtilis natto* BEST 195, which is a moderate γ -PGA producer (data not shown; Itaya & Matsui, 1999). We cloned the *ywsC* gene from *B. subtilis* B-1, and tried to generate a B-1-derived *ywsC* knockout mutant, but we were unable to do this.

In order to examine whether pellicle formation is a cause or a result of γ -PGA production, growth kinetics and γ -PGA production by *B. subtilis* B-1 were compared in standing and shaking culture. The cells did not form pellicles in the shaking culture because of the continuous shear stress. Production of γ -PGA in standing culture started 6 h after inoculation, when pellicle formation had not yet started. Moreover, γ -PGA was produced in the shaking culture, albeit after a significant lag, about 12 h after inoculation (Fig. 4). Eventually, after 24 h, the levels of γ -PGA produced were similar in standing and shaking culture. These results

indicate that pellicle formation is not a requirement for γ -PGA production in B-1.

Depletion of dissolved oxygen, and biofilm formation

It is known that vigorous aeration is effective for high γ -PGA yields (Cromwick *et al.*, 1996). In order to test the effect of dissolved oxygen on γ -PGA production during pellicle formation, a microaerated culture system (MACS), obtained by utilizing the microporous Teflon tube Poreflon (Sumitomo Electric Fine Polymer), was used (Fig. 5b). MACS enabled us to aerate the culture without visible air bubbling. It was found that when the MACS was adopted, the cells grew in the liquid phase for as long as 9 h without forming pellicles. This result is in contrast with the observation that the cells started to form a pellicle at 6 h after inoculation in a standing culture system, and almost no cells grew in the liquid phase thereafter (Fig. 5a). These observations indicate that oxygen depletion in standing culture is involved in the initial steps of floating biofilm formation by *B. subtilis* B-1.

Analysis of membrane proteins in the biofilm and planktonic cells

In order to obtain further insight into the physiological difference between the biofilm and planktonic cells, membrane proteins prepared from the pellicles, planktonic cells and colonies were comparatively analysed by SDS-PAGE, followed by Coomassie brilliant blue staining (Fig. 6). It was obvious that the protein profiles of biofilm and colony cells were simpler than those of planktonic cells. Proteins with sizes of 77 (P77), 57 (P57) and 36 kDa (P36) were produced predominantly in the cells from pellicles and colonies when compared with planktonic cells. Analyses by Sequest, based on the partial amino acid sequences of these proteins, indicated that P77, P57 and P36 were a minor

Table 1. Chemical shift values of NMR analyses of EPS from *B. subtilis* B-1

Peak	$^1\text{H-NMR}$ (δ =p.p.m.)	$^{13}\text{C-NMR}$ (δ =p.p.m.)
α -CH	4.19	55.44
β -CH ₂	2.08, 1.93	28.34
γ -CH ₂	2.34	32.89
CO	—	175.53
COOH	—	178.90

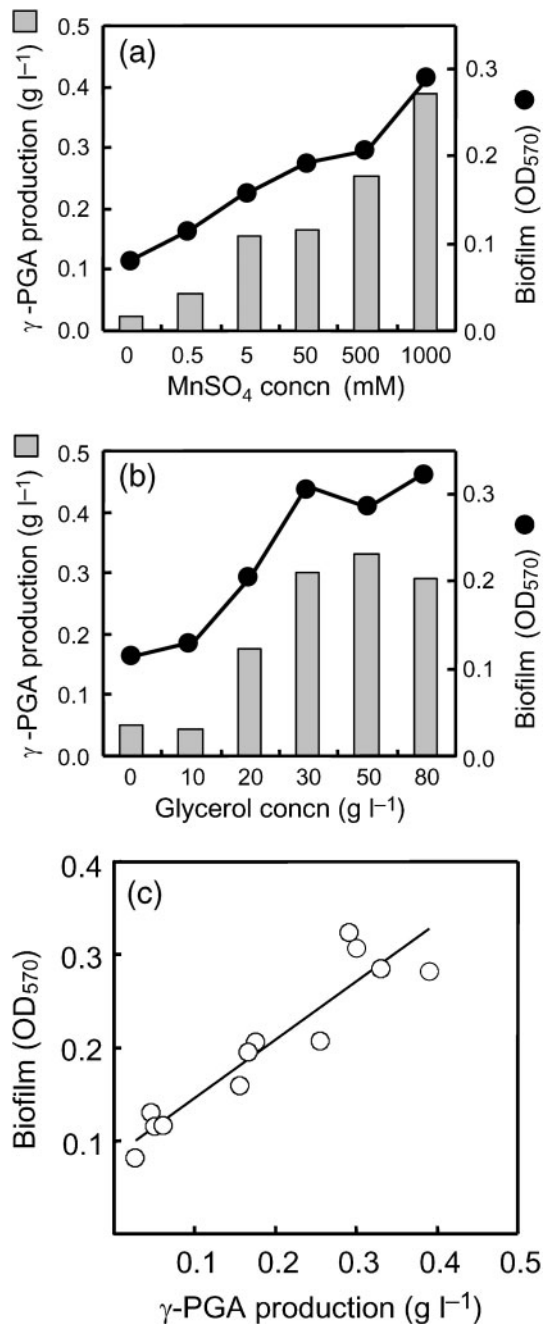


Fig. 3. Effects of MnSO₄ and glycerol on γ -PGA production and biofilm formation. Bar and line graphs indicate γ -PGA production (g l⁻¹) and amounts of biofilm (OD₅₇₀), respectively. Strain B-1 was grown in E-medium supplemented with MnSO₄ (a) and glycerol (b) at 37 °C for 8 h. There was a direct correlation between γ -PGA production and biofilm formation for various MnSO₄ and glycerol concentrations (c).

extracellular protease precursor (Vpr; P29141), an oligopeptide permease (OppA; CAA39787/Spo0K, AAA62687), and a mature flagellin (Hag; P02968), respectively. The ComX–ComP–ComA signalling pathway is a major quorum response pathway in *B. subtilis*, and it regulates the

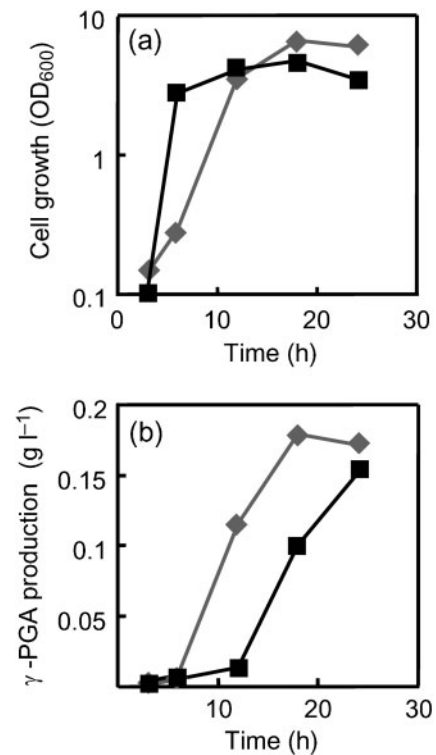


Fig. 4. Comparison of standing and shaking culture systems in terms of cell growth (a) and γ -PGA production (b). ■, Shaking culture; ◆, standing culture. Cells were grown in L-broth at 37 °C.

production of γ -PGA (Comella & Grossman, 2005; Stanley & Lazazzerra, 2005). Accumulation of ComX pheromone stimulates the membrane histidine kinase ComP, resulting in autophosphorylation. Phosphorylated ComP donates the phosphate to the response regulator ComA, leading to activation of ComA-dependent promoters. CSF (competence-stimulating factor; derived from the *phrC* gene product) is another pheromone that stimulates ComA activity (Lazazzera, 2000). CSF inhibits RapC, which negatively regulates transcription of *comA*. CSF is produced from pro-CSF, after being processed by extracellular serine proteases such as Vpr, Epr and Apr (subtilisin E) (Lazazzera, 2006). CSF is transported into the cell by an ATP-binding cassette (ABC) transporter system containing oligopeptide permease (OppA/Spo0K). The observation that OppA/Spo0K (P57) and Vpr precursor (P77) were predominantly expressed in the pellicle and colony samples suggests that a quorum-sensing system actively functions in these cells. It is interesting that P36 was found to be flagellin, and that it was significantly produced by the pellicle and the colony cells. Although Chagneau and Saier have demonstrated that flagella are required for biofilm formation, a DNA microarray analysis of *B. subtilis* biofilms has shown that the expression of genes encoding flagella is repressed (Chagneau & Saier, 2004; Stanley *et al.*, 2003). Another report has shown that there is no significant difference in the expression level of

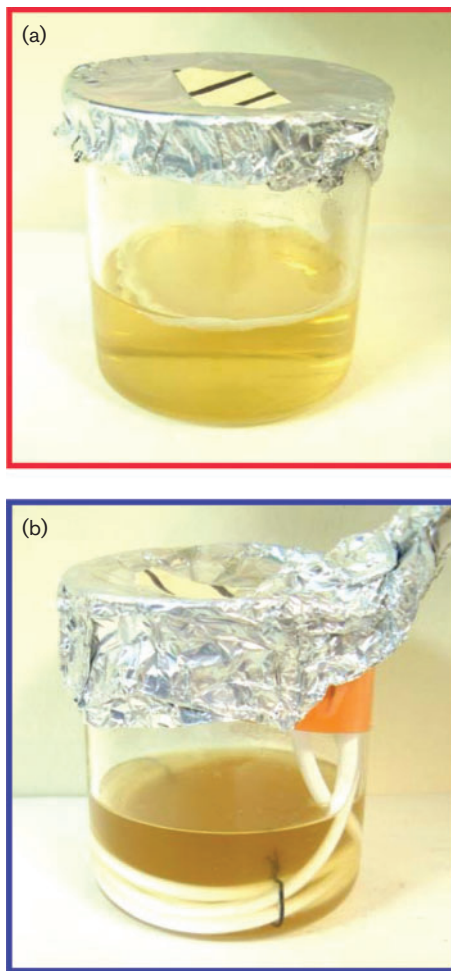


Fig. 5. Comparison of cell growth in standing culture (a) and MACS (b). Both culture vessels were placed at 37 °C for 9 h without shaking.

the gene encoding flagellin in biofilm cells, when compared with planktonic cells (Ren *et al.*, 2004). These contradictions remain to be explained. However, the existence of flagella in biofilm cells was also confirmed by electron microscopic observation after uranyl acetate staining (not shown). Our results may suggest that flagella in B-1 biofilm cells are produced for a purpose other than cell swimming. In the case of *Aeromonas hydrophila* AH-3, production of lateral flagella has been shown to be specifically increased on semi-solid media and surfaces, and to be important for adherence and biofilm formation (Canals *et al.*, 2006).

ACKNOWLEDGEMENTS

The authors are grateful to Professor M. Itaya (Keio University) for kindly providing *B. subtilis* natto BEST 195, and E. Oiki (Osaka University) for his excellent assistance in SEM observation. This work was supported by grants from the Institute for Fermentation, Osaka, and J-Power, to M. M., and the National Institutes of Health to R. K. (GM58213).

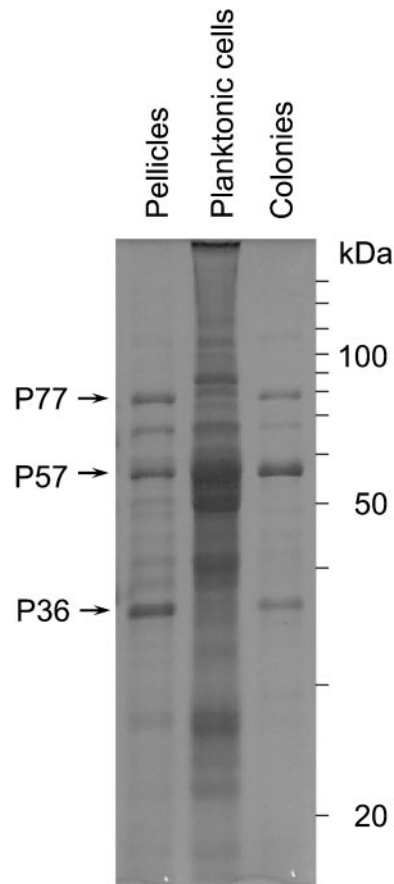


Fig. 6. Membrane protein analyses of B-1 cells from the floating biofilm (pellicles), shaking culture (planktonic cells) and colonies. Cells were grown at 37 °C for 24 h, with an inoculum size of 5%.

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