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 PsI in Pseudomonas aeruginosa; cellulose in Escherichia coli and Salmonella typhimurium; a tetrasaccharide repeat of D-glucose, L-fucose and D-glucono-1,5-lactone 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 & Lazazera, 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
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 NH4Cl, 0-5 g MgSO4.7H2O, 0-5 g KH2PO4, 0-15 g CaCl2.2H2O, 40 mg FeCl3.6H2O and 148 mg MnSO4.H2O. The pH of E-medium was adjusted to 7-4 by 2 M NaOH. The amounts of glycerol and MnSO4 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 OsO4, dehydrated in ethanol, isoomyl acetate and critical CO2, and sputter coated with platinum (Claubert, 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 D2O (4 %, w/v), and 0-5 M NaH2PO4, 0-2 M Na2SO4, 0-05 M HCl, 0-01 g NaAc and 0-01 g NaN3. The pH of E-medium was adjusted to 7-4 by 2 M NaOH. The amounts of glycerol and MnSO4 were adjusted as noted.

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/DDBJ 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 & Chakraborty, 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,
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.

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).

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
the concentration of MnSO₄ 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**

<table>
<thead>
<tr>
<th>Peak</th>
<th>¹H-NMR (δ=p.p.m.)</th>
<th>¹³C-NMR (δ=p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-CH</td>
<td>4·19</td>
<td>55·44</td>
</tr>
<tr>
<td>β-CH₂</td>
<td>2·08, 1·93</td>
<td>28·34</td>
</tr>
<tr>
<td>γ-CH₂</td>
<td>2·34</td>
<td>32·89</td>
</tr>
<tr>
<td>CO</td>
<td>–</td>
<td>175·53</td>
</tr>
<tr>
<td>COOH</td>
<td>–</td>
<td>178·90</td>
</tr>
</tbody>
</table>

**Fig. 2.** SEM of pellicles formed by *B. subtilis* B-1.
extracellular protease precursor (Vpr; P29141), an oligo-
peptide permease (OppA; CAA39787/Spo0K, AAA62687),
and a mature flagellin (Hag; P02968), respectively. The
ComX–ComP–ComA signalling pathway is a major quo-
rum response pathway in \( B. \) subtilis, and it regulates the
production of \( \gamma \)-PGA (Comella & Grossman, 2005; Stanley &
Lazazzera, 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 (compe-
tence-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 \( \text{comA} \). CSF is produced
from pro-CSF, after being processed by extracellular serine
proteases such as Vpr, Epr and Apr (subtilisin E) (Lazzazerra,
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 micro-
array 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. 3.](http://mic.sgmjournals.org) Effects of MnSO\(_4\) and glycerol on \( \gamma \)-PGA production and biofilm formation. Bar and line graphs indicate \( \gamma \)-PGA produc-
tion (g l\(^{-1}\)) and amounts of biofilm (OD\(_{570}\)), respectively. Strain B-1 was grown in E-medium supplemented with MnSO\(_4\) (a) and glycerol (b) at 37 °C for 8 h. There was a direct corre-
lation between \( \gamma \)-PGA production and biofilm formation for var-
ious MnSO\(_4\) and glycerol concentrations (c).

![Fig. 4.](http://mic.sgmjournals.org) Comparison of standing and shaking culture systems in terms of cell growth (a) and \( \gamma \)-PGA production (b). ■, Shaking
culture; •, standing culture. Cells were grown in L-broth at
37 °C.

http://mic.sgmjournals.org
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).

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**REFERENCES**


