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Expression of *sfp* gene and hydrocarbon degradation by *Bacillus subtilis*

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Abstract

Bacillus subtilis C9 produces a lipopeptide-type biosurfactant, surfactin, and rapidly degrades alkanes up to a chain length of C_{19}. The nucleotide sequence of the *sfp* gene cloned from *B. subtilis* C9 was determined and its deduced amino acid sequence showed 100% homology with the *sfp* gene reported before [Nakano *et al.* (1992) Mol. Gen. Genet. **232**: 313–321]. To transform a non-surfactin producer, *B. subtilis* 168, to a surfactin producer, the *sfp* gene cloned from *B. subtilis* C9 was expressed in *B. subtilis* 168. The transformed *B. subtilis* SB103 derivative of the strain 168 was shown to produce surfactin measured by its decrease in surface tension, emulsification activity, and TLC analysis of the surface active compound isolated from the culture broth. Like *B. subtilis* C9, *B. subtilis* SB103 containing *sfp* gene readily degraded aliphatic hydrocarbons (C_{10-19}), though its original strain did not. The addition of surfactin (0.5%, w/v) to the culture of *B. subtilis* 168 significantly stimulated the biodegradation of hydrocarbons of the chain lengths of 10–19; over 98% of the hydrocarbons tested were degraded within 24 h of incubation. These results indicate that the lipopeptide-type biosurfactant, surfactin produced from *B. subtilis* enhances the bioavailability of hydrophobic hydrocarbons.

Introduction

Various studies have been made on the microbial degradation of hydrocarbons having low water solubility, which limits their availability to biodegrading microorganisms. The biodegradation of hydrocarbons is often associated with the production of surface-active compounds from microorganisms (Koch *et al.* 1991). The surface-active molecules, biosurfactants contain both hydrophilic and hydrophobic components, a property that reduces the surface tension of aqueous media and thus emulsifies the hydrophobic compounds. A wide spectrum of microbial compounds, including lipopeptides, glycolipids, fatty acids, and polymeric biosurfactants, have been found to have surface activities (Georgiou *et al.* 1992, Desai 1987). We previously reported that *B. subtilis* C9, selected by the method of oil film-collapsing assay, produced a lipopeptide-type biosurfactant, which was determined to be surfactin (Kim *et al.* 1997a). *Bacillus subtilis* C9 readily degraded alkanes of chain length from C_{10} to C_{19} but hardly degraded hydrocarbons with the chain length of more than C_{21}.

A long-term goal of our research has been to develop a strain which degrades numerous hydrocarbons, including both aromatic and long-chain hydrocarbons. Our preliminary efforts to render competency to *B. subtilis* C9 were unsuccessful. Therefore, we initially decided to transform *B. subtilis* 168 to a surfactant producer. This approach was based on the fact that while strain 168 is neither a surfactant producer nor a degrader of hydrocarbons, it has been widely used as

a host strain to express the heterologous genes. It was expected that, once the *sfp* gene from *B. subtilis* C9 is successfully expressed in the strain 168, the transformant would degrade the hydrocarbons and thus it would be possible to, if necessary, construct an effective decomposer of mixed-hydrocarbons. As a first step toward this goal, we report here on the isolation of transformant to synthesis biosurfactant that enhances the bioavailability of hydrophobic compounds, and biodegradation of hydrophobic hydrocarbons by this transformant.

Materials and methods

Microorganisms, plasmids, and cultivation

Bacillus subtilis C9 KCTC (Korean Collection for Type Cultures) 8701P was used as a source of *sfp* gene. *Bacillus subtilis* strain 168 (Spizizen 1958) was a kind gift from Dr Frank Kunst in the Institute Pasteur and used as a host. Cells were grown in the basal salt medium (Kim *et al.* 1997b) containing 1% (w/v) glucose and 1% (v/v) *n*-alkane for the analysis of hydrocarbon biodegradation and in the biosurfactant production medium (Kim *et al.* 1997b) for surfactin production at 30 °C with shaking at 150 rpm. *Escherichia coli* strain DH5α used for propagation of plasmid DNA was cultured in Luria-Bertani (LB) medium (Miller 1972) at 37 °C. pGEM-T Easy vector (Promega, Madison, WI, USA) was used as a subcloning vector for DNA sequencing. pHPS9 shuttle vector was purchased from the BGSC (Bacillus Genetics Stock Center) at Ohio, USA and used as cloning and expression vector.

Analyses

The residual hydrocarbons in the culture medium were extracted with two volume of *n*-hexane. The amount of *n*-alkanes was measured by gas chromatography equipped with a flame ionization detector under the following conditions: column, fused silica capillary column PTE-5 (QTM, 15 m, 0.53 mm ID, 0.5 μm; Supelco, PA, USA); carrier gas, nitrogen (30 ml min^{-1}); column temperature, 50–270 °C (10 °C min^{-1}), injection temperature, 200 °C; detector temperature, 250 °C.

The cell growth was determined by measuring the optical density of culture broth at 660 nm and expressed as gram dry cell l^{-1} by multiplying the O.D. values by 0.3 which was determined from calibration curves.

The surface tension of the culture broth was determined at 25 °C with a ring tensiometer (K10ST; Krüss, Hamburg, Germany).

TLC analysis was carried out on silica gel F_{254} plates (Merck) in chloroform/methanol/water (65:25:4, by vol.). Components on the plates were located by spraying them with 30% H_2SO_4 solution or the irradiation of short wavelength UV.

The concentration of surfactin was determined by measuring the CMD (critical micelle dilution) and the concentration of peptide moiety of surfactin (Kim *et al.* 1997b).

Emulsification activity was determined by the modified standard method of Rosenberg (1979) and Kim *et al.* (1997b). One unit of emulsification activity per ml was defined as the amount of biosurfactant that increased OD_{620} of 0.1 in the assay reaction. Emulsion turbidity was directly proportional to the concentration of the isolated surface active compound from *B. subtilis* C9 ranging from 0.1 to 3 mg l^{-1}.

DNA isolation and transformation

The chromosomal DNA of *B. subtilis* C9 was isolated by the method of Doi (1983). Plasmid DNAs were isolated by QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Preparation of competent *E. coli* and their transformation with plasmid DNA was carried out according to the procedure of Inoue *et al.* (1990). Transformation of *B. subtilis* was performed by the method of Anagnostopoulos and Spizizen (1961). The transformants producing surfactin were selected on the blood agar plate (Nakano *et al.* 1988) containing chloramphenicol (5 μg ml^{-1}).

PCR

PCR amplification of the *sfp* gene fragment from *B. subtilis* C9 was performed by using a PCR kit (Perkin Ellmer Co., Norwalk, CT, USA) with the following primers: 5′-CGCGGATCCGCTGTGGCAAGG CGGACA-3′ and 5′-CCGGAATTCCTGCCCGCCTCAAGAGTG-3′. The amplification was performed on GeneAmp PCR System 2400 (Perkin Elmer Co.), using program set to denaturation at 95 °C for 5 min, and then denature at 94 °C for 30 s, anneal at 55 °C for 30 s and extend at 72 °C for 1 min for a total of 25 cycles, with a final extension at 72 °C for 3 min. After the program was completed, the reaction mixture was electrophoresed on a 1% SeaKem

GTG agarose gel (FMC, Pockland, ME, USA), and the DNA fragment was extracted from the gel with Suprec-01 (Takara, Shiga, Japan).

DNA sequencing

The *sfp* gene was cloned into pGEM-T easy vector, and the nucleotide sequence was determined by the dideoxy-chain termination method (Sanger *et al.* 1977), using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA) with ABI 373A DNA sequencer (Applied Biosystems).

Isolation of lipopeptide-type biosurfactants

From the culture broth concentrated with ultrafiltration, lipopeptide-type biosurfactants were extracted by chloroform/methanol (2:1, v/v). The extracts were dissolved in chloroform and then placed on a column of silica gel equilibrated with chloroform. The surface-active compounds were eluted with methanol/chloroform (1:3, v/v). A crude lipopeptide-type biosurfactants were obtained by removing the solvent under reduced pressure.

Results and discussion

The microbial degradation of hydrocarbons by B. subtilis *C9*

The straight-chain hydrocarbons were readily degraded by *B. subtilis* C9. The cells of *B. subtilis* C9 were first cultured on the LB medium at 37 °C for 14 h. The cells were transferred into a basal salt medium containing 1% (w/v) glucose and 1% (v/v) of each *n*-alkane (C_{10-22}). Under these conditions, cells showed abundant growth within 24 h of incubation and rapidly degraded *n*-alkanes. The C_{10-19} alkanes tested were almost completely degraded within 24 h. The gas chromatography profiles of octadecane recovered from the culture of *B. subtilis* C9 showed that the residual amount of octadecane in the culture decreased linearly with time. During 15 h of incubation, the remaining octadecane in the culture decreased to 1% of the initial amount, though *n*-eicosane (C_{20}) was only degraded with a degradation rate of 50% for 48 h, and the long-chain hydrocarbons more than C_{21} were not degraded at all after 48-h incubation. It is supposed that the recalcitrant of long-chain hydrocarbons is due to their solid physical nature at 37 °C, below the melting point of *n*-eicosane. The aromatic hydrocarbons, benzene and naphthalene, were not completely degraded after 48-h incubation.

Nucleotide sequencing analysis of sfp *gene on the genomic DNA of* B. subtilis *C9*

Three genes, *srfA*, *sfp*, and *comA*, required for the biosynthesis of the cyclic lipopeptide surfactin in *B. subtilis*, were identified and subsequently isolated (Nakano *et al.* 1988, 1989). The surfactin synthetases are encoded by the 27 kb *srfA* operon and bear a thioesterase domain (Cosmina *et al*. 1993). Surfactin is synthesized via the multiple-carrier thiotemplate mechanism from seven amino acids (Glu-Leu-Dleu-Val-Asp-Dleu-Leu) linked to a β-hydroxy fatty acid. The *sfp* gene which is located 4kb downstream of the *srfA* operon of *B. subtilis* encodes PPTase (4′-phosphopantethein transferase) required in addition to the *srfA* operon for surfactin production. The PPTase, which transfers the 4′-phosphopantetheinyl moiety of coenzyme A to the side chain hydroxyl of a serine residue in PCP (peptidyl carrier protein), is encoded by *sfp* in *B. subtilis*. The PPTase converts the PCP domain of surfactin synthetase from inactive apo- to phosphopantethein-containing holo-forms (Quadri *et al*. 1998, Reuter *et al*. 1999).

The nucleotide sequence of the *sfp* gene in *B. subtilis* C9 was determined and its deduced amino acid sequence was compared with those of *sfp* genes reported by Nakano *et al*. (1992) and that of the strain 168 as well. PCR amplification of the *sfp* gene fragment from *B. subtilis* C9 was performed with the primers designed from the determined *sfp* gene (Nakano *et al*. 1992). One fragment of 1 kb was obtained from the PCR. The nucleotide sequence of the fragment cloned in T7 vector was determined (data not shown). The sequence of this region revealed the existence of an open reading frame (ORF) consisting of 224 amino acids, and this ORF showed 100% homology with that of Nakano *et al*. (1992).

Expression of sfp *gene from* B. subtilis *C9 in* B. subtilis *168 and surface activities of the transformant*

It is known that in the *sfp* gene of *B. subtilis* 168, the frameshift mutation is occurred by a single-base (adenine) insertion on the ORF (Nakano *et al.* 1992). The adenine insertion on the ORF resulted in replacement of glutamic acid with glycine, and early termination of the translation of *sfp* gene resulting in

the defective PPTase in *B. subtilis* 168. Similar observations have been reported by Cosmina *et al*. (1993). They indicated that the frameshift mutation (a thymine insertion) is responsible for the sfp^0 phenotype in non-producing strain. Some *B. subtilis* strains unable to produce the lipopeptide surfactin were shown to have an intact *srfA* operon, but a defective *sfp* gene (sfp^0). It was also confirmed when the *sfp* gene was transferred to the surfactin-negative strain of *B. subtilis* by genetic transformation, the *sfp* was necessary and sufficient to make cells of a non-producing strain surfactin positive (Nakano *et al*. 1988).

We previously reported that *B. subtilis* C9 produced the lipopeptide-type biosurfactant, surfactin (Kim *et al*. 1997a). In this study, since *B. subtilis* C9 was difficult to be competent and was genetically uncharacterized, the *sfp* gene responsible for surfactin production was transferred from this strain to cells of the strain 168, which can be genetically manipulated. Nakano *et al*. (1992) reported that overexpression of *sfp* in *B. subtilis* did not cause production of an increased amount of surfactin and also resulted in the repression of the *srfA* operon. Therefore, the expression shuttle vector pHPS9 having relatively low copy number of 4 or 5 was used as an expression vector in *B. subtilis* 168. PCR amplification was carried out using the chromosomal DNA isolated from *B. subtilis* C9 as a template and the designed oligonucleotide primers (contained *Bam*HI and *Eco*RI site, respectively) from the sequenced *sfp* gene of *B. subtilis* C9. After the amplification was completed, the PCR product of 1 kb was digested by *Bam*HI and *Eco*RI. This fragment was ligated into pHPS9 vector, and was then introduced into *E. coli* DH5α cells. The recombinant plasmid, pHPS9-sfp (Figure 1) was recovered from transformant *E. coli* cells, and introduced into *B. subtilis* 168. Transformants producing surfactin (derivatives of the strain 168) were selected on the blood agar plate. This plate assay was based on the observation that the surfactin produced by *B. subtilis* 168 lyses red blood cells (Jain *et al.* 1991) resulted in the yellow-colored halo. Seven colonies showing a large halo on the blood agar plate were isolated. It was confirmed that the plasmids recovered from the isolated colonies were all the recombinant plasmid, pHPS9-sfp.

However, the hemolytic activity may have been associated with the presence of lytic enzymes instead of surfactin production by *B. subtilis*. Therefore, it was necessary to confirm the transformants really produce the lipopeptide biosurfactant, surfactin. The surface tension, emulsification activity, and surfactin concentration of the cell-free culture broth obtained from the cultures of the seven transformed colonies in the biosurfactant production medium were measured, and compared with those of *B. subtilis* C9 and non-surfactin producing *B. subtilis* 168. Among these, one clone showing the highest surface activities was selected as a potential producer of biosurfactant. This clone, a derivative of *B. subtilis* 168 containing pHPS9-sfp, was designated as the strain SB103.

As shown in Table 1, the transformed *B. subtilis* SB103 reduced the surface tension of the culture broth from 70 to 27.7 dyne cm^{-1}, showed the higher emulsification activity, and produced a larger amount of biosurfactant than *B. subtilis* C9. However, the *B. subtilis* strain 168 showed no significant levels of reduction of surface tension, emulsification activity against hydrocarbons, and of any surface-active compounds.

TLC analysis of surface-active compounds produced by *B. subtilis* SB103 was also carried out. The surface-active compound was isolated from the culture broth by ultrafiltration, solvent extraction, and silica gel column chromatography. The isolated compound showed a major spot having the same R_f value of 0.63 as the isolated surface-active compound from the culture broth of *B. subtilis* C9 and the authentic surfactin (Sigma) on TLC analysis. These results clearly confirmed that non-surfactin producer *B. subtilis* 168 was changed to the producer of lipopeptide biosurfactant, surfactin by the transformation of the *sfp* gene cloned from *B. subtilis* C9.

Biodegradation of hydrocarbons by the transformant, B. subtilis *SB103*

Given the results that the transformant, *B. subtilis* SB103, containing the *sfp* gene cloned from *B. subtilis* C9 became a lipopeptide-type biosurfactant producer, we subsequently investigated the relationships between the biosurfactant production and the biodegradation of hydrophobic hydrocarbons in *B. subtilis*. The patterns of hydrocarbons degradation by the *B. subtilis* SB103, C9, and 168 were examined with *n*-alkanes (C_{10-22}) (see Table 2). *Bacillus subtilis* SB103 degraded *n*-alkanes (C_{10-19}) almost completely within 24-h incubation at 37 °C. After 48 h of incubation, the remaining *n*-eicosane (C_{20}) in the culture was about 50% of the initial amount, and the long-chain hydrocarbons over C_{21} were, however, not degraded at all. These hydrocarbon degradation patterns were very similar to those of *B. subtilis* C9. But, the degradation rate of non-biosurfactant producer, *B. subtilis*

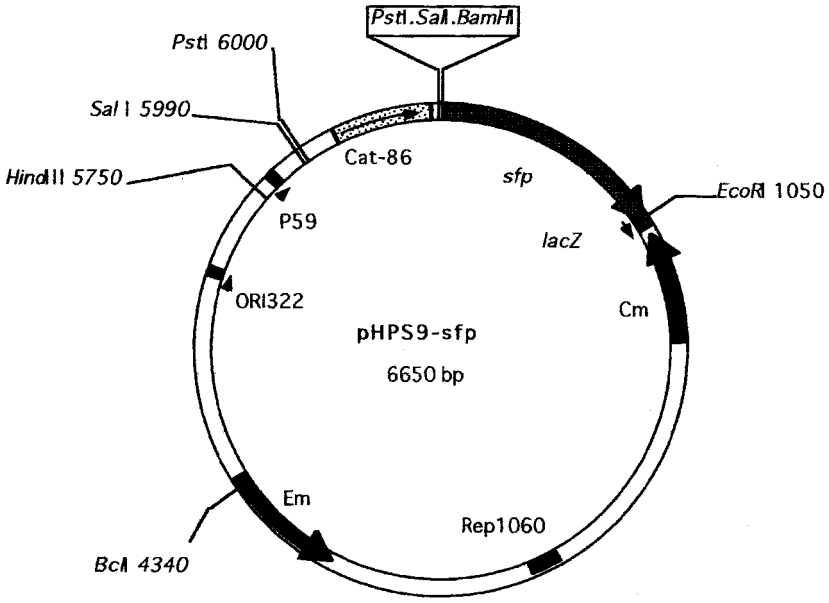

Fig. 1. Genetic and restriction map of the recombinant plasmid, pHPS9-sfp.

Table 1. The surface activities of the culture broth[1] of *B. subtilis* C9, 168, and SB103.

	Surface tension[2] (dyne cm^{-1})	Emulsification activity (unit ml^{-1})	CMD[3]	Conc. of surfactin (g l^{-1})
B. subtilis C9	28.5	138	40	4.2
B. subtilis 168	68.5	0	–	0
B. subtilis SB103	27.7	145	50	5.5

[1]Cells were cultured in 100 ml of the surfactin production medium in 500 ml-Erlenmyer flask at 30 °C for 3 days.
[2]Surface tension was measured with a 20-fold diluted cell-free culture broth with distilled water.
[3]CMD (critical micelle dilution) was determined as a dilution rate necessary to reach the CMC (critical micelle concentration), at which the surface tension starts to increase dramatically.

Table 2. Biodegradation of alkanes by *Bacillus* species and effects of surfactin addition on the hydrocarbons degradation.

Chain length	*B. subtilis* C9		*B. subtilis* 168		*B. subtilis* SB103		*B. subtilis* 168, surfactin addition	
	Growth (g l^{-1})*	Degradation (%)	Growth (g l^{-1})	Degradation (%)	Growth (g l^{-1})	Degradation (%)	Growth (g l^{-1})	Degradation (%)
C10	0.33	99	0.27	10	0.36	99	0.39	99
C12	0.48	99	0.45	18	0.54	99	0.54	99
C14	0.60	99	0.57	19	0.60	99	0.60	99
C16	0.66	99	0.57	22	0.60	99	0.60	100
C17	0.48	99	0.45	3	0.51	99	0.48	99
C18	0.54	100	0.51	3	0.57	99	0.57	99
C19	0.42	99	0.36	2	0.48	99	0.45	99
C20	0.42	52	0.21	0.3	0.42	49	0.45	62
C21	0.24	0.2	0.21	0.1	0.18	0.2	0.21	1.2
C22	0.21	0.2	0.21	0.1	0.21	0.2	0.24	1.5

The cells were grown aerobically in the biosurfactant production medium containing 1% of the individual hydrocarbon with varying carbon chain lengths, at 37 °C for 24 h.
*g dry cell per liter.

168 on *n*-alkanes of chain length from C_{10} to C_{16} reached only 10–20%, and this strain hardly degraded C_{17-22} alkanes in spite of the abundant cell growth. These results showed that the biosurfactant production is closely associated with the microbial degradation of hydrocarbons in *B. subtilis*. The effects of biosurfactant addition to the culture of non-surfactant producing *B. subtilis* 168 on the degradation of hydrocarbons were also examined (Table 2). The surfactin isolated from the culture of *B. subtilis* C9 was added to be a concentration of 0.5% by weight. The *n*-alkanes (C_{10-19}) examined were almost perfectly degraded within 24-h culture time by the non-biosurfactant producer, *B. subtilis* 168 in the presence of the lipopeptide biosurfactant, surfactin. These results showed that surfactin played a major role in hydrocarbons degradation by *B. subtilis*. As it has been demonstrated that the emulsification properties of biosurfactant can enhance the degradation of hydrocarbons (Andreas *et al.* 1991, Banat 1995), the biosurfactant used in this study served as an effective emulsifying agent, increasing the mineralization of hydrocarbons.

One of our interests is to develop a strain assimilating hydrophobic multi-compounds for the bioremediation. The development of a strain having genetic competency and producing biosurfactants might bring us a step closer to this goal. Further studies to examine the effects of surfactin on the rates of biodegradation of water insoluble compounds, such as long-chain hydrocarbons, aromatic hydrocarbons, bisphenols, and so on, and then transfer of the corresponding genes to *B. subtilis* SB103, are under continuation.

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