Biosci. Biotechnol. Biochem., **67** (6), 1239–1244, 2003

Production and Characterization of Biosurfactants from *Bacillus licheniformis* F2.2

Jiraporn THANIYAVARN,[1,2,3,†] Niran ROONGSAWANG,[2] Takayuki KAMEYAMA,[2] Mitsuru HARUKI,[2] Tadayuki IMANAKA,[3] Masaaki MORIKAWA,[2] and Shigenori KANAYA[2]

[1]*Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Department of Material and Life Science, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan*
[3]*Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 686-8501, Japan*

Received October 23, 2002; Accepted February 22, 2003

A biosurfactant-producing strain, *Bacillus licheniformis* F2.2, was isolated from a fermented food in Thailand. The strain was capable of producing a new biosurfactant, BL1193, as well as two kinds of popular lipopeptide biosurfactants, plipastatin and surfactin. Mass spectrometry and FT-IR analysis indicated that BL1193 had a molecular mass of 1,193 Da with no peptide portion in the molecule. While plipastatin and surfactin were abundantly produced in a nutrient YPD medium, BL1193 was produced only in a synthetic DF medium containing no amino acids. According to an oil displacement activity test, the specific activity of BL1193 (6.53 kBS units/mg) is equivalent to that of surfactin (5.78–6.83 kBS units/mg).

Key words: *Bacillus licheniformis*; biosurfactant(s); lipopeptide(s); plipastatin; surfactin

Biosurfactants are biologically surface-active agents produced by several microorganisms.[1)] Their superior properties, such as low toxicity and high biodegradability, are undoubtedly environmentally acceptable.[2,3)] Being capable of lowering surface and interfacial tensions effectively, biosurfactants could be potential substitutes of widely used chemically synthesized surfactants. This means that biosurfactants are promising surfactants useful for the food industry, pharmaceuticals, cosmetics, specialty chemical industries, enhanced oil recovery (EOR), and cleaning oil spills by bioremediation.[4,5)] There are six classes of biosurfactants: glycolipids, lipopeptides or lipoproteins, neutral lipids, phospholipids, substituted fatty acids, and lipopolysaccharides.[1,6)] Production of an effective lipopeptide type biosurfactant, surfactin, was first reported for a strain of *Bacillus subtilis*.[7)] Since then, various kinds of lipopeptides with surface activity and/or antibiotic activity have been isolated from *Bacillus* strains. These are, for example, bacillomycin,[8)] iturin,[9,10)] mycosubtilin,[11)] plipastatin,[12)] surfactant BL86,[13)] halobacillin,[14)] and lichenysins A/B/C/G.[15–18)] As far as we know, production of biosurfactants other than lipopeptides has never been reported for *Bacillus* strains. The only exception is a bioemulsifier from *B. stearothermophilus* VR-8, which is a protein-polysaccharide-lipid complex with emulsifying activity.[19)] Here, we examined the culture conditions for the production of biosurfactants from the newly isolated *B. licheniformis* F2.2 and found that this strain produced a new non-lipopeptide type biosurfactant together with lipopeptides, plipastatin, and surfactin in an amino acid depleting medium.

Materials and Methods

Isolation and cultivation of strain F2.2. The strain was maintained in a nutrient YPD medium. YPD medium consisted of per liter; 10 g each of yeast extract (Difco, Detroit, MI), peptone (Difco), and glucose. Although this medium was useful for biosurfactant production by the strain, a synthetic DF medium was also used in the experiment. DF medium contained per liter; 20 g glucose, 4 g NH_4NO_3, 0.4 g $MgSO_47H_2O$, 0.2 g KCl, 0.1 g $CaCl_22H_2O$, 1.4 g $NaHPO_4 \cdot 12H_2O$ (pH 7.0) and 10 ml each of trace mineral solution and vitamin solutions.[16)] The strain F2.2 was grown aerobically at 30°C for 3 d in YPD medium for production of

† To whom correspondence should be addressed. Tel: +66-2-218-5070; Fax: +66-2-253-0337; E-mail: Jiraporn.Th@Chula.ac.th

Abbreviations: Ala, alanine; Asp, aspartic acid; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; Orn, ornithine; Pro, proline; Thr, threonine; Tyr, tyrosine; Val, valine; CMW, chloroform/methanol/water; HPLC, high performance liquid chromatography; LC/MS, HPLC/mass spectrometry; TLC, thin-layer chromatography; TFA, trifluoroacetic acid

plipastatin (RT-27G) and surfactin (RT-32G, RT-8I) or in DF medium (pH 7.0) for production of surfactin (RT-32G, RT-8I) and BL1193 (RT-11I).

Purification of biosurfactants from YPD and DF cultures. A cell-free culture supernatant containing biosurfactants was obtained by centrifugation at 10,000 × *g* for 20 min, 4°C. The supernatant was precipitated by adding conc. HCl to a final pH 2.0. The acid precipitates were collected by centrifugation at 20,000 × *g* for 20 min at 4°C and dissolved in 0.1 M Tris-Cl (pH 8.0). Then, a hydrophobic fraction containing biosurfactants was extracted five times with hexane. The hexane solution was dried up and the resulting pellet was dissolved in a solvent mixture, chloroform/methanol/water = 65:25:4 (CMW). This sample was further purified by Kieselgel 60 (Art. 5745, 2 mm thickness; Merck Co., Berlin, Germany) thin-layer chromatography (TLC) with CMW as a developing solvent mixture.[20] The gel bands containing a surface-active fraction were scratched off from the plate and extracted three times with CMW. Surface-active substances migrated at several positions in a range of R_f values from 0.56 to 0.94 and were further purified by high pressure liquid chromatography (HPLC). The HPLC was done with a Cosmosil 5C18-AR column (4.6 × 150 mm, Nacarai tesque, Kyoto, Japan) or Octadecyl-4PW column (4.6 × 50 mm, Tosoh, Tokyo, Japan) connected to a HP1100 system (Hewlett-Packard, Fort Collins, CO). The solvent conditions were as follows; 10% acetonitrile/0.1% trifluoroacetic acid (TFA) for 5 min, linear increase of acetonitrile concentration from 10 to 100% in 20 min, and 100% acetonitrile/0.1% TFA for 20 min. Flow rate was 0.7 ml/min at 30°C and the substances eluted were monitored by UV absorption at 210 nm. Because the substances that are migrated at R_f values larger than 0.8 on TLC were too hydrophobic to be soluble in the starting solvent (10% acetonitrile/0.1% TFA), they were dissolved in 100% acetonitrile. The HPLC was done by an isocratic elution mode, in this case with 100% acetonitrile/0.1% TFA.

Purification of plipastatin from YPD culture. Purification of plipastatin was optimized as follows. The YPD culture broth was precipitated by adding conc. HCl to a final pH 2.0. The acid precipitates were collected by centrifugation at 20,000 × *g* for 20 min at 4°C, and dissolved in 100 ml of 50 mM phosphate buffer (pH 7.5). Two volumes of cold acetone were added to the resultant solution at 4°C to generate insoluble precipitants containing most of the activity. These precipitants were dissolved in the phosphate buffer again, and active fractions were extracted by n-butanol and then put on a silica gel column Wakogel C-200 (Wako, Osaka, Japan) chromatography. After being dried up *in vacuo*, the sample was further purified by anionic exchange chromatography on a DEAE Sephadex A-50 column (Amersham Pharmacia, Buckinghamshire, UK) with linearly increasing NaCl concentrations from 0 to 1 M in 50 mM phosphate buffer (pH 7.5). Pure plipastatin was eventually obtained by gel filtration column chromatography on a Sephadex G-75 column (Amersham Pharmacia).

Measurement of biosurfactant activity. Biosurfactant activity was measured by an oil displacement test.[20] This method is so sensitive that only a small amount of sample is required to measure the surfactant activity. A sample, solubilized or suspended in 10 μl of 10% acetonitrile or 0.1 M Tris-Cl (pH 8) buffer, was put on the center of an oil membrane which was formed on the surface of water in a large-size petri dish (15 cm diameter). The size of the resultant oil-displaced circle area reflects the activity of a surfactant. The larger this size is, the higher the activity of a surfactant is. One BS unit was defined as the amount of surfactants forming 1 cm^2 of oil-displaced area. The surface tensions were measured using a ring tensiometer (K6; Kruss, Germary).

Amino acid composition analysis. Peptide bonds in a lipopeptide biosurfactant were hydrolyzed by distilled 6 N HCl containing 0.2% phenol at 110°C for 24 h. Then the amino acid composition of the hydrolysate was analyzed with a type 835 amino acid analyzer (Hitachi, Tokyo, Japan) at The Institute for Protein Research, Osaka University.

Mass spectrometry. Mass spectra of the substances separated in HPLC (LC/MS) were obtained by an electrospray ionization mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA) under the conditions recommended by the manufacturer.

IR spectrometry. A purified biosurfactant sample was spotted onto a KRS-5 cell, and the Fourier transform-infrared spectrum (FT-IR) was measured by a JIR-AQS20M (JEOL, Tokyo, Japan) spectrometer at The Chemical Analysis Center in the Graduate School of Engineering, Osaka University.

Results

Isolation and characterization of a biosurfactant-producing strain F2.2

An effective biosurfactant-producing strain, F2.2, was isolated from a fermented food in Thailand. This strain was selected among the isolates based on the lowest surface tension of its culture supernatant.[21] Physiological characteristics of this strain are listed in Table 1. All of these features strongly indicate that strain F2.2 is *B. licheniformis*.[22] *B. licheniformis* is known to produce effective lipopeptide biosur-

Table 1. Characteristics of *B. lichemiformis* F2.2

Tests	Results	Tests	Results
Gram staining	+	Hydrolysis of	
Shape of cell	rod	casein	+
Spore formation	+	gelatin	+
Motility	+	starch	+
Catalase	+	tributyrin	+
VP test	+	Growth in NaCl	
Acid from		2%	+
D-glucose	+	5%	+
D-fructose	+	7%	+
D-mannitol	+	10%	+
D-xylose	+	Growth at	
L-arabinose	+	30°C	+
Anaerobic growth	+	40°C	+
Utilization of citrate	+	50°C	+
Gas from glucose	−	55°C	+
Production of indole	−	60°C	−

factants, surfactin, BL86, and the lichenysin family.[13,16,17] A new type from the lichenysin family, lichenysin G, has been recently isolated from *B. licheniformis* IM1307.[15] We further examined the structures of the biosurfactants produced by strain F2.2.

Identification of biosurfactants produced by strain F2.2—In YPD medium

The strain F2.2 grew well in YPD medium, and after cultivation for 3 days, the surface tension of the culture was decreased to 28 mN/m. Hexane extracts of the acid precipitates were directly analyzed by HPLC (Fig. 1a). It was found that two kinds of biosufactants, RT-27G and RT-32G, were produced under these culture conditions. Both RT-27G and RT-32G gave several peaks on HPLC. However, they were combined and purified for further characterizations, because a biosurfactant is usually produced in multiple forms which differ in the conformation and/or alkyl chain length.[23,24] Although the biosurfactant activity was slightly lower than that of RT-32G, RT-27G weakly inhibited the growth of Gram-positive bacteria (*B. subtilis*), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), and *Eumycetes* (*Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., and *Cladosporium* sp.). LC/MS analysis data demonstrated that the molecular weights of the compounds in RT-27G were 1,462 and 1,490 (figure not shown). It was also found that they contained amino acid residues at a ratio of Ala:Glu:Ile:Orn:Pro:Thr:Tyr = 1:3:1:1:1:1:2 and Glu:Ile:Orn:Pro:Thr:Tyr:Val = 3:1:1:1:1:2:1, respectively. These data indicate that these antibiotic and surface-active substances are plipastatins.[12] Structure of RT-32G was analyzed in the following experiments.

—In DF medium

For large-scale production of biosurfactants in

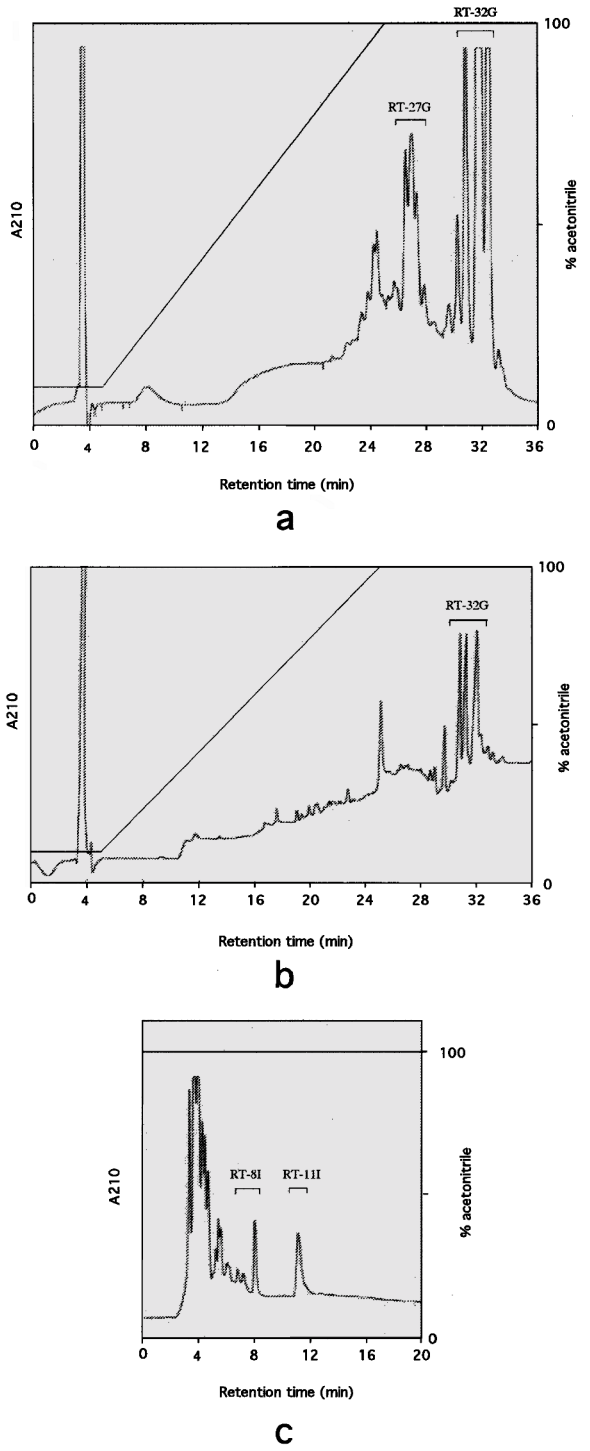

Fig. 1. Separation of Biosurfactants by HPLC.

a; A hexane extract of a YPD culture supernatant was separated on a Cosmosil 5C18-AR column. Peaks containing biosurfactant activity are labeled by a name in the chart. Multiple peaks under a bracket are isomers and conformers with different and the same alkyl chain length, respectively.

b, c; TLC purified samples from DF medium were put to a Cosmosil 5C18-AR column. Samples at R_f values smaller and larger than 0.8 on TLC plate were separately eluted from the HPLC column by a gradient mode (b) and an isocratic mode (c), respectively.

No peak corresponding to RT-11I was eluted even after keeping the concentration of acetonitrile at 100% for over half an hour in (a).

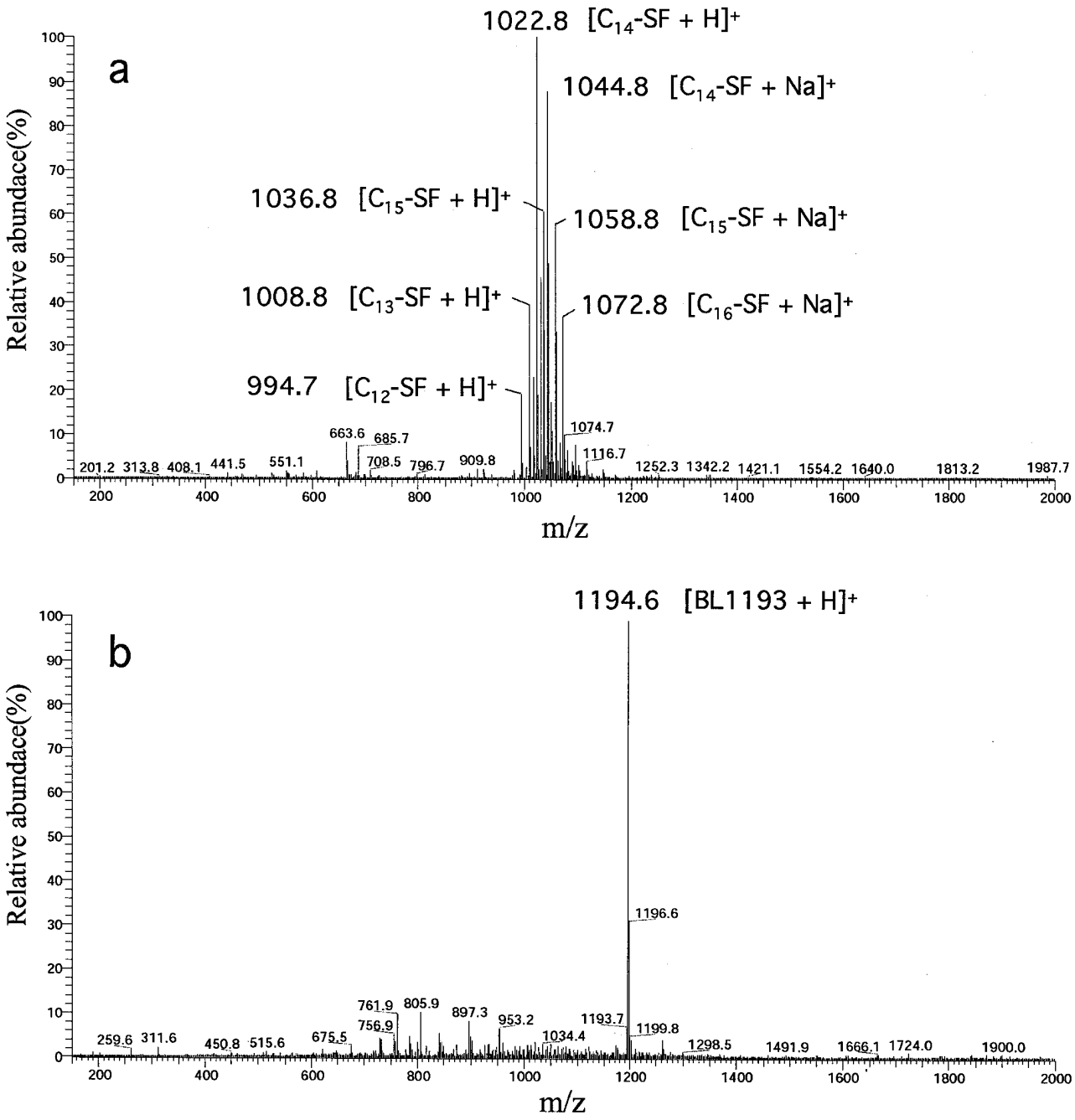

Fig. 2. Mass Spectrum Analyses of RT-32G, RT-8I, and RT-11I (BL1193).

Biosurfactant produced in DF medium were analyzed by LC/MS. a, RT-32G. RT-32G is a mixture of the surfactin family with different alkyl chain lengths, Cn-SF. Cn abbreviates carbon number. RT-8I showed a similar chart to RT-32G. b, RT-11I.

industry, it is obviously beneficial to produce them by using a cheap synthetic medium. We tested DF medium for biosurfactant production with several kinds of nitrogen sources, such as ammonium chloride, ammonium nitrate, ammonium sulfate, and sodium nitrate. Because all the surface tensions of these cultures were lower than 30 mN/m, they were diluted by 100 times and examined for surface tensions. It was found that ammonium nitrate was the best nitrogen source among those tested for biosurfactant production. The surface tension (mN/m) of the diluted culture was 41.8 (ammonium nitrate), 47.2 (ammonium sulfate), 50.2 (ammonium chloride), and 58.5 (sodium nitrate). After cultivation for 3 d, surface-active substances were acidified to form precipitates, extracted with hexane, and further purified by TLC and HPLC described. The biosurfactant activities were eluted at a retention time of 32 min (RT-32G) in a gradient elution mode of HPLC (Fig. 1b), and at 8 min (RT-8I) and 11 min (RT-11I) in an isocratic elution mode (Fig. 1c). According to the mass spectrometry, RT-8I and RT-32G were

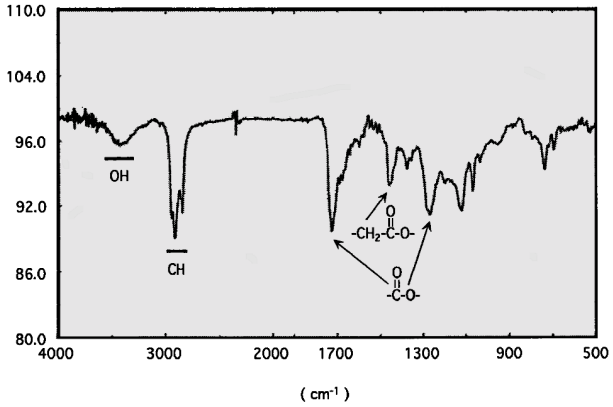

Fig. 3. FT-IR Specturm Analysis of RT-11I (BL1193). Major absorption valleys are assigned in the chart.

suggested to be surfactin, a most effective lipopeptide biosurfactant produced by *Bacillus* strains (Fig. 2a). The amino acid compositions of RT-8I and RT-32G were equally measured as Asp:Glu:Val:Leu = 1:1:1:4, which was identical to that of surfactin. As to RT-11I, the molecular weight was suggested to be 1,193 by mass spectrometry (Fig. 2b), namely BL1193. FT-IR spectrometry strongly suggested that BL1193 contained no amino acid residues (Fig. 3). Actually, after hydrolysis no amino acid was detected by amino acid composition analysis. A broad absorption valley at 3400 cm^{-1} indicated the presence of OH groups in the molecule. Strong absorption valleys observed in the range from 2850 to 2950 cm^{-1} demonstrate typical CH stretching vibration in the alkyl chain. Absorption valleys at 1740 and 1270 cm^{-1} are stretching vibration of C–O and C=O bonds in carboxyl esters, respectively. Scissoring vibration of a CH_2 group adjoining a carboxyl ester was also observed at 1370 cm^{-1}. As far as we know, the production of a biosurfactant with this molecular weight has never been reported for bacteria, including *Bacillus* strains. The surface activity of BL1193 was 6.53 kBS units/mg by the oil displacement test. Because the activity of RT-32G/RT-8I (surfactin) is 5.78–6.83 kBS units/mg, the surface activities of BL1193 and surfactin were found to be comparable. The production yields of surfactin and BL1193 in DF medium supplemented with 0.2% ammonium nitrate were roughly 0.58 mg/l and 0.03 mg/l, respectively.

Discussion

B. licheniformis F2.2 was isolated from fermented foods in Thailand. It was found that the strain F2.2 has an ability to produce three kinds of surface-active compounds, plipastatin, surfactin, and BL1193. *Bacillus* strains have been reported to produce two lipopeptides simultaneouly.[10,25,26] Recently, we isolated a halotolerant *B. subtilis* BBK-1 that produces three kinds of lipopeptides, bacillomycin L, plipastatin, and surfactin.[21] Several *Bacillus* strains seem to have an ability to produce multiple lipopeptides. In the strain F2.2, plipastatin and surfactin were abundantly produced in nutrient YPD medium. In addition, a new non-lipopeptide type biosurfactant BL1193 was produced in a synthetic DF medium, which was not produced in YPD medium. This fact suggests that gene expression for BL1193 production is repressed in a medium that contains peptides and amino acids. It seems that lipopeptides are vigorously produced in nutrient YPD medium. In fact, it has been reported that the addition of amino acids to the culture medium affects the amino acid composition of lipopeptide biosurfactants.[27]

Because nutrient broth such as Luria-broth has been used in most cases for biosurfactant production from *Bacillus* strains,[16,28] production of a non-lipopeptide biosurfactant BL1193 could have been overlooked. Although Jenny *et al.* reported that lichenisin C was produced in a mineral medium,[29] it would be interesting to re-examine biosurfactant production by *Bacillus* strains on synthetic media. Further chemical structural studies of BL1193 are underway.

References

1) Fiechter, A., Biosurfactant: moving towards industrial application. *Trends Biotechnol.*, **10**, 208–217 (1992).
2) Desai, J. D., and Banat, I. M., Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.*, **61**, 47–64 (1997).
3) Muller-Hurtig, R., Blaszczyk, R., Wagner, F., and Kosaric, N., Biosurfactants for environmental control. In "Biosurfactants: Production, Properties, Applications", ed. Kosaric, N., Marcel Decker, New York, pp. 447–470 (1993).
4) Fiedler, H. P., and Umbach, W., Cosmetics and toiletries. In "Surfactants in Consumer Products: Theory, Technology and Application", ed. Falbe, J., Springer-Verlag, Heidelberg, pp. 350–398 (1987).
5) Kosaric, N., Biosurfactants: production, property, application. In "Surfactant Science Series" vol. 48, Marcel Dekker, New York (1993).
6) Cooper, D. G., and Zajic, J. E., Surface-active compounds from microorganisms. *Adv. Appl. Microbiol.*, **26**, 229–256 (1980).
7) Arima, K., Kakinuma, A., and Tamura, G., Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Comm.*, **31**, 488–494 (1968).
8) Besson, F., Peypoux, F., Michel, G., and Delcambe, L., Structure of bacillomycin L, an antibiotic from *Bacillus subtilis*. *Eur. J. Biochem.*, **77**, 61–67 (1977).
9) Peypoux, F., Guinand, M., Michel, G., Delcambe, L., Das, B. C., and Lederer, E., Structure of iturin A, a peptidolipid antibiotic from *Bacillus subtilis*. *Biochemistry*, **17**, 3992–3996 (1978).

10) Sandrin, C., Peypoux, F., and Michel, G., Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. *Biotechnol. Appl. Biochem.*, **12**, 370–375 (1990).
11) Peypoux, F., Pommier, M. T., Marion, D., Ptak, M., Das, B. C., and Griffin, W. M., Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. *J. Antibiot.*, **39**, 636–641 (1986).
12) Nishikiori, T., Naganawa, H., Muraoka, Y., Aoyagi, T., and Umezawa, H., Plipastatins: new inhibitors of phospholipase A2 produced by *Bacillus cereus* BMG302-fF67, III. Structural elucidation of plipastatins. *J. Antibiot.*, **39**, 755–761 (1986).
13) Horowitz, S., and Griffin, W. M., Structural analysis of *Bacillus licheniformis* 86 surfactant. *J. Ind. Microbiol.*, **7**, 45–52 (1991).
14) Trischman, J. A., Jensen, P. R., and Fenical, W., Halobacillin: a cytotoxic cyclic acylpeptide of the iturin class produced by a marine *Bacillus*. *Tetrahedron Lett.*, **35**, 5571–5574 (1994).
15) Grangemard, I., Bonmatin, J. M., Bernillon, J., Das, B. C., and Peypoux, F., Lichenysin G, a novel family of lipopeptide biosurfactants from *Bacillus licheniformis* IM 1307: production, isolation and structural evaluation by NMR and mass spectrometry. *J. Antibiot.* (*Tokyo*), **52**, 363–373 (1999).
16) Jenny, K., Deltrieu, V., and Kappelli, O., Lipopeptide production by *Bacillus licheniformis*. In ''Biosurfactants: Production, Properties, Applications'', ed. Kosaric, N., Marcel Dekker, New York, pp. 135–156 (1993).
17) Lin, S. C., Milton, M. A., Sharma, M. M., and Georgiou, G., Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Appl. Environ. Microbiol.*, **60**, 31–38 (1994).
18) Yakimov, M. M., Abraham, W.-R., Meyer, H., Giuliana, I., and Golyshin, P. N., Structural charcterization of lichenysin A components by fast atom bombardment tandem mass spectrometry. *Biochim. Biophys. Acta*, **1438**, 273–280 (1999).
19) Gunjar, M., Khire, J. M., and Khan, M. I., Bioemulsifier production by *Bacillus stearothermophilus* VR8 isolate. *Lett. Appl. Microbiol.*, **21**, 83–86 (1995).
20) Morikawa, M., Diado, H., Takao, T., Murata, S., Shimonishi, Y., and Imanaka, T., A new lipopeptide biosurfactant produced by *Arthrobacter sp*. Strain MIS 38. *J. Bacteriol.*, **175**, 6459–6466 (1993).
21) Roongsawang, N., Thaniyavarn, J., Thaniyavarn, S., Kameyama, T., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S., Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. *Extremophiles*, **6**, 499–506 (2002).
22) Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G., Bergey's manual of systematic bacteriology, vol. 2, Williams & Wilkins, Baltimore/London (1986).
23) Morikawa, M., Hirata, Y., and Imanaka, T., A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta*, **1488**, 211–218 (2000).
24) Kakinuma, A., Ouchida, A., Shima, T., Sugino, H., Isono, M., Tamura, G., and Arima, K., Conformation of the structure of surfactin by mass spectrometry. *Agric. Biol. Chem.*, **33**, 1669–1671 (1969).
25) Tsuge, K., Ano, T., and Shoda, M., Isolation of a gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. *Arch. Microbiol.*, **165**, 243–251 (1996).
26) Duitman, E. H., Hamoen, L. W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., Ullrich, C., Stein, T., Leenders, F., and Vater, J., The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proc. Natl. Acad. Sci. USA*, **96**, 13294–13299 (1999).
27) Peypoux, F., and Michel, G., Controlled biosynthesis of Val7 and Leu7 surfactin. *Appl. Microbiol. Biotechnol.*, **36**, 515–517 (1992).
28) Morikawa, M., Ito, M., and Imanaka, T., Isolation of a new surfactin producer *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulator gene, *psf-1*. *J. Ferment. Bioeng.*, **74**, 255–261 (1992).
29) Jenny, K., Kappeli, O., and Fiechter, A., Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl. Microbiol. Biotechnol.*, **36**, 5–13 (1991).