Importance of 3-Hydroxy Fatty Acid Composition of Lipopeptides for Biosurfactant Activity

Noha H. Youssef, Kathleen E. Duncan, and Michael J. McInerney*

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma

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Biosurfactant production may be an economic approach to improving oil recovery. To obtain candidates most suitable for oil recovery, 207 strains, mostly belonging to the genus *Bacillus*, were tested for growth and biosurfactant production in medium with 5% NaCl under aerobic and anaerobic conditions. All strains grew aerobically with 5% NaCl, and 147 strains produced a biosurfactant. Thirty-five strains grew anaerobically with 5% NaCl, and two produced a biosurfactant. In order to relate structural differences to activity, eight lipopeptide biosurfactants with different specific activities produced by various *Bacillus* species were purified by a new protocol. The amino acid compositions of the eight lipopeptides were the same (Glu/Gln:Asp/Asn:Val:Leu, 1:1:1:4), but the fatty acid compositions differed. Multiple regression analysis showed that the specific biosurfactant activity depended on the ratios of both *iso* to normal even-numbered fatty acids and *anteiso* to *iso* odd-numbered fatty acids. A multiple regression model accurately predicted the specific biosurfactant activities of four newly purified biosurfactants ($r^2 = 0.91$). The fatty acid composition of the biosurfactant produced by *Bacillus subtilis* subsp. *subtilis* strain T89-42 was altered by the addition of branched-chain amino acids to the growth medium. The specific activities of biosurfactants produced in cultures with different amino acid additions were accurately predicted by the multiple regression model derived from the fatty acid compositions ($r^2 = 0.95$). Our work shows that many strains of *Bacillus mojavensis* and *Bacillus subtilis* produce biosurfactants and that the fatty acid composition is important for biosurfactant activity.

Biosurfactants are compounds produced by a variety of microorganisms (3) that are capable of lowering surface and/or interfacial tension (3, 4, 13, 30) by partitioning at the water-air and water-oil interfaces (34, 39). They can have a variety of structures, including fatty acids, neutral lipids, phospholipids, glycolipids, and lipopeptides (13). Biosurfactants aid in the tertiary stage of oil recovery from low-production oil reservoirs by releasing oil trapped by capillary pressure (34).

The activity of biosurfactants depends on their structural components, e.g., the types of hydrophilic and hydrophobic groups and their spatial orientation (9). Most lipopeptide biosurfactants have been shown to have a structure similar to that of surfactin, the biosurfactant produced by *Bacillus subtilis* (2, 14, 20, 32). Surfactin is a cyclic lipopeptide with β -hydroxy fatty acids linked to a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) (2, 14). The solubility and surface activity of surfactin depend on the arrangement of the amino acid residues to produce two domains, a minor hydrophilic domain and a major hydrophobic domain (9). Changes in the amino acids at positions 2, 4, and/or 7 of surfactin to more hydrophobic residues increased the surface activity and decreased the critical micelle concentration (8, 31, 32, 35, 36). In contrast, Yakimov et al. (40) changed the fatty acid composition of lichenysin A, a lipopeptide produced by *Bacillus licheniformis* BAS50, by the addition of branched-chain amino acids to the growth medium. The increase in the percentage of

branched-chain fatty acids in lichenysin A decreased the activity of the biosurfactant.

Candidate microorganisms for enhanced oil recovery should produce biosurfactants at low oxygen tensions, slightly elevated temperatures, and high salt concentrations since these are the conditions encountered in many domestic oil reservoirs. The lipopeptide produced by *Bacillus mojavensis* strain JF-2 generates the low interfacial tension (<0.01 mN/m) needed for substantial oil recovery (24, 26). This strain grows and produces the lipopeptide anaerobically at salt concentrations up to 8% and temperatures up to 45°C (19, 24). However, most of the activity is lost after extended incubations (N. Youssef and M. J. McInerney, unpublished data), and complex nutrients are required for its anaerobic growth (25).

In an attempt to find better candidates for microbially enhanced oil recovery, a number of bacterial strains, mostly *Bacillus* strains, were screened for anaerobic growth and stable biosurfactant production (28, 42) in the presence of 5% NaCl. Biosurfactant activities varied markedly among the strains. To understand the factors that influence biosurfactant activity, the biosurfactant concentration and amino acid and fatty acid compositions of a number of lipopeptide biosurfactants produced by strains of *Bacillus subtilis* and *Bacillus mojavensis* were determined.

MATERIALS AND METHODS

Bacterial strains and cultivation. The taxonomic affiliations and numbers of strains used for this study are shown in Table 1. All cultures were grown at 37°C in the presence and absence of O₂ in a mineral salt medium with 5% NaCl, with sucrose as the energy source, as previously described (42). For screening, duplicate 25-ml cultures were used, while duplicate or triplicate 1-liter cultures were used for biosurfactant extraction and purification. Each culture was grown until maximal activity was obtained (usually between 42 and 44 h of incubation). When

* Corresponding author. Mailing address: Department of Botany and Microbiology, University of Oklahoma, 770 Van Vleet Oval, Norman, OK 73019. Phone: (405) 325-6050. Fax: (405) 325-7619. E-mail: mcinerney@ou.edu.

TABLE 1. Numbers, taxonomic affiliations, growth properties, and biosurfactant production of bacterial strains used for this study

Species or isolates	No. of strains	No. of strains with growth ^a		No. of strains with biosurfactant production ^a		No. of strains with biosurfactant activity relative to JF-2 ranging from ^b :			Reference(s) or source ^c
		+O ₂	-O ₂	+O ₂	-O ₂	0.42–0.75	0.83–1.7	1.75–2.5	
<i>B. mojavensis</i>	23	23	1	11	1	4	5	2	33
<i>B. subtilis</i> subsp. <i>subtilis</i>	47	47	0	39	0	14	20	5	10, 12, 18
<i>B. subtilis</i> subsp. <i>spizizenii</i>	43	43	0	38	0	10	20	8	10, 12, 18
<i>B. megaterium</i>	15	15	0	7	0	4	3	0	18
<i>B. licheniformis</i>	12	12	12	8	1	7	1	0	12
<i>B. sonorensis</i>	9	9	9	4	0	4	0	0	29
<i>Bacillus</i> species	14	14	0	5	0	4	1	0	18
Oil well isolates	44	44	13	35	0	15	19	1	OU culture collection

^a All strains were grown with 5% salt. Duplicate cultures of each strain were analyzed for each condition.

^b Relative activity was measured by dividing the diameter of clearing of different strains by that of JF-2 (diameter of clearing = 1.2 ± 0.17 cm). The activity was measured in triplicate for each culture, and the coefficients of variation for duplicates of the same strain ranged from 0 to 17%.

^c The references provide the origins of the strains.

needed, amino acids (L-valine, L-alanine, L-leucine, and L-isoleucine) were added to the medium at 1 g/liter before autoclaving.

Screening for biosurfactant production. Biosurfactant activity was measured by the oil-spreading technique (28, 42). Fifty milliliters of distilled water was added to a large petri dish (25 cm in diameter), followed by the addition of 20 μ l of crude oil to the surface of the water. Ten microliters of a culture grown in mineral medium was added to the surface of the oil. The diameter of the clear zone on the oil surface was measured for triplicate samples from each replicate culture. Biosurfactant activity, defined as the diameter of clearing on the oil surface, in centimeters, ranged from 0 to 3 cm. The coefficients of variation ranged from 0 to 17% for replicates of the same strain. To compare biosurfactant stabilities, duplicate cultures of strains with the highest biosurfactant activities were sampled over a period of 14 days and tested for biosurfactant activity. The surface activity relative to that of the biosurfactant produced by *B. mojavensis* strain JF-2 was obtained by dividing the oil displacement diameter obtained with a given strain by the value obtained for *B. mojavensis* strain JF-2 (1.2 ± 0.17 cm).

Biosurfactant extraction and purification. The method used for biosurfactant extraction and purification was modified from the work of Kim et al. (23). When the maximum oil displacement diameter was obtained, cells from duplicate or triplicate 1-liter cultures were removed by centrifugation at $14,300 \times g$ for 15 min at 4°C. The pellet was dried at 110°C overnight, and the dry weight was determined. Biosurfactant in the supernatant was precipitated with 40% (wt/vol) ammonium sulfate and incubated overnight at room temperature. The precipitate, containing the biosurfactant along with other compounds, was then collected by centrifugation at $14,300 \times g$ for 30 min at 4°C. The precipitate was extracted with 250 μ l of chilled acetone to remove most of the proteins. Instead of the column chromatography steps used by Kim et al. (23), further purification was achieved by preparative thin-layer chromatography (TLC) of the acetone extract. The whole acetone extract (250 μ l) was spotted onto preparative silica gel TLC plates (Whatman, Clifton, NJ) with a solvent system of isopropanol-water–28% (wt/vol) ammonium hydroxide (80:11:9). The TLC plates were developed with iodine vapor. Each fraction was scraped off the plate, dissolved in 250 μ l water, and tested for surface activity by the oil-spreading technique. Surface-active fractions were lyophilized. The weight of the lyophilized biosurfactant was determined and used to calculate the biosurfactant yield (biosurfactant weight/dry weight of cells). Biosurfactant yields of different strains varied from 0.9 to 3.1 mg g⁻¹ dry weight of cells. The coefficients of variation of biosurfactant yields between different batches of the same strain ranged from 4.9 to 27%.

To compare surface activities of different biosurfactants, 1- μ g μ l⁻¹ solutions of purified biosurfactants were prepared and tested by the oil-spreading technique. The specific activity of each purified biosurfactant was expressed as the diameter of the clear zone, in millimeters per microgram of purified biosurfactant. The biosurfactant specific activities of different strains varied from 0.7 to 4.5 mm μ g⁻¹. Triplicate samples were done for each culture. The coefficients of variation of specific activities between different batches of the same strain ranged from 4 to 26%, while those for the same batch of the same strain were <5%.

Amino acid analysis. The amino acid composition of each purified biosurfactant was determined by the molecular biology research facility of the William K. Warren Research Institute (Oklahoma City, OK). Purified biosurfactants were

acid hydrolyzed under a vacuum in sealed tubes with 6 N HCl at 110°C for 18 to 24 h. Each hydrolyzed sample was vacuum dried, dissolved in 0.01 N HCl, and filtered through a 0.45- μ m nylon filter before analysis. Amino acid analysis was performed by cation-exchange chromatography. Amino acid elution was accomplished with a two-buffer system. The sample was injected onto the column equilibrated with 0.2 N sodium citrate, pH 3.28. This buffer eluted the first nine amino acids. The remaining amino acids were eluted by 1 N NaCl in 0.2 N sodium acetate, pH 7.4. Amino acids were detected by an online postcolumn reaction with ninhydrin (Tritone; Pickering Laboratory, Inc.). Derivatized amino acids were quantified by their absorption at 570 nm, except for glutamic acid and proline, which were detected at 440 nm. The procedure was performed with a totally automated Beckman Gold model 126 HPLC amino acid analyzer.

Fatty acid analysis. A methanolysis procedure, modified from the method of Yakimov et al. (40), was used to analyze fatty acids. Two hundred micrograms of the purified biosurfactant was hydrolyzed under a vacuum for 16 h at 90°C with 4 ml of 25% 12 N HCl in methanol in sealed tubes. The hydrolyzed fatty acid methyl esters (FAME) were then extracted with 7 ml of 1:1 (vol/vol) ethyl acetate:hexane. The organic phase was concentrated under a stream of N₂ to 0.6 ml. The concentrated fractions were neutralized with 0.5 ml of 0.4 M phosphate buffer (pH 12) and incubated at room temperature for 10 min. The FAME in the organic layer were derivatized with BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] (Pierce, Rockford, IL) and analyzed by gas chromatography-mass spectrometry (6890N network GC system/5973 network mass selective detector; Agilent Technologies, Wilmington, DE). One microliter of each sample was used for injection; triplicate injections were made for each biosurfactant preparation. The oven temperature was set at 60°C for 5 min and then increased to 250°C over a 15-min interval. The column was a capillary column (0.25 mm by 30 m by 0.25 μ m). The carrier gas was helium, and the flow rate was 1.2 ml/min. The mass spectrometer was operated at 400 Hz. Peak areas obtained on the gas chromatogram were used to calculate the percentage of FAME isomers compared to the area of all FAME. The electron ionization mass spectra were dominated by fragment ions specific for trimethylsilyl (TMS) derivatives. The fragment ion at 175, which is specific for TMS-derivatized hydroxyl groups in the beta position, was used to extract the chromatogram to detect peaks corresponding to 3-hydroxy fatty acids. The M-15 fragments (loss of a methyl group) on the mass spectra were used to identify the carbon chain lengths of the fatty acid isomers. These corresponded to 301 for 3-OH-C₁₃, 315 for 3-OH-C₁₄, 329 for 3-OH-C₁₅, 343 for 3-OH-C₁₆, and 357 for 3-OH-C₁₇. M-31 fragments, characteristic of fatty acids and corresponding to the loss of methanol, were also detected. *Iso* isomers of fatty acids were identified by the presence of an M-43 fragment (loss of an isopropyl group) and the absence of an M-29 fragment (loss of an ethyl group) from the mass spectra. *Anteiso* isomers were identified by the presence of an M-57 fragment (loss of a secondary butyl group) and the absence of an M-43 fragment (loss of an isopropyl group) from the mass spectra. Fragments M-29, M-43, and M-57 were very small in comparison to others specific for TMS derivatives but were discernible by magnifying the mass spectra. Retention times and mass spectra were compared to authentic standard methyl 3-hydroxy tetradecanoate (Larodan Fine Chemicals, Malamö, Sweden).

TABLE 2. Comparison of biosurfactant activities and fatty acid ratios of different biosurfactants

Species	Strain	Sp act (mm μg^{-1}) ^a	Fatty acid composition (%) ^b			
			C ₁₃	C ₁₄	C ₁₅	C ₁₆
<i>B. mojavensis</i>	JF-2	3 $\pm$ 2	1.3 $\pm$ 2.5	49.6 $\pm$ 85.4	11.3 $\pm$ 14.9	24.4 $\pm$ 45
<i>B. subtilis</i> subsp. <i>spizizenii</i>	TG6-19	2.4 $\pm$ 0.8	3.6 $\pm$ 7.1	58.4 $\pm$ 54.8	30.3 $\pm$ 54.8	3.5 $\pm$ 0.2
<i>B. subtilis</i> subsp. <i>subtilis</i>	T89-15	3 $\pm$ 2	2.4 $\pm$ 4.8	67.7 $\pm$ 47.2	22.7 $\pm$ 29.4	4.3 $\pm$ 3.8
<i>B. subtilis</i> subsp. <i>subtilis</i>	T89-42	1.63 $\pm$ 0.3	4.7 $\pm$ 9.4	67.1 $\pm$ 29.4	24.6 $\pm$ 47.2	4.3 $\pm$ 3.8
<i>B. subtilis</i> subsp. <i>spizizenii</i>	T88-8	3 $\pm$ 2.95	2.4 $\pm$ 4.8	61 $\pm$ 52.8	27.3 $\pm$ 52.8	6.3 $\pm$ 88
<i>B. subtilis</i> subsp. <i>spizizenii</i>	CL1-14	2.55 $\pm$ 2.1	4.2 $\pm$ 8.4	67.3 $\pm$ 56.9	25.8 $\pm$ 44.4	4.4 $\pm$ 4.9
<i>B. mojavensis</i>	T89-14	2 $\pm$ 2	6.4 $\pm$ 12.8	57 $\pm$ 69.5	32.8 $\pm$ 55.7	2.8 $\pm$ 1.2
<i>B. subtilis</i> subsp. <i>spizizenii</i>	T89-3	1.35 $\pm$ 1.3	6.8 $\pm$ 13.6	49.7 $\pm$ 44.3	35.5 $\pm$ 29	6.2 $\pm$ 0.7

^a Data are means $\pm$ standard deviations of triplicate cultures. The specific activity of each purified biosurfactant is expressed as the diameter of spreading in mm per μg of purified biosurfactant.

^b Data are means $\pm$ ranges of duplicate cultures. The numbers are percentages of different fatty acids (% mass values) in the lipid portion of the purified biosurfactant. The percentages were calculated by dividing the peak areas of individual fatty acids by the total peak area of all FAME.

Statistical analyses. SPSS for Windows (release 11.5.0; SPSS Inc., Chicago, IL) and Microsoft Excel for Mac (version 11.1.1) were used to calculate Pearson's correlation coefficients and test regression models.

RESULTS

Screening of microorganisms. Thirty-five strains, mostly belonging to *Bacillus licheniformis* and *Bacillus sonorensis*, grew anaerobically with 5% NaCl (Table 1), and two of them produced a biosurfactant. One hundred forty-seven strains, mostly belonging to *Bacillus subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, produced a biosurfactant under aerobic conditions (Table 1). Sixteen strains produced biosurfactants with activities 1.75 to 2.5 times that of the JF-2 biosurfactant. Sixty-nine strains had biosurfactant activities comparable to that of JF-2 (0.83 to 1.7 times) (Table 1). Some *Bacillus mojavensis* strains (two of five tested) maintained their biosurfactant activity over a 14-day incubation period, in contrast to *B. mojavensis* strain JF-2, which lost 50% of its activity in 7 days.

Evaluation of a new protocol for biosurfactant purification. The lipopeptide produced by triplicate cultures of *Bacillus mojavensis* strain ROB-2 was used to compare the efficiencies of two purification methods. Method 1 involved acid precipitation (using 1 N HCl to adjust the pH of the cell-free culture fluid to 2) (41) followed by TLC. Method 2 used ammonium sulfate precipitation followed by acetone extraction and TLC. Seventy-five percent of the biosurfactant activity remained in the cell-free culture fluid after cell removal. The surface-active fraction obtained from the TLC plate by method 1 had 23% $\pm$ 7% of the activity originally present in the culture, while the surface-active fraction obtained from the TLC plate by method 2 had 63% $\pm$ 11% of the activity originally present in the culture. The specific biosurfactant activity of the surface-active fraction from the TLC plate for 12 different strains was 0.65 $\pm$ 0.07 mm μg^{-1} by method 1 and 1.9 $\pm$ 0.7 mm μg^{-1} by method 2. Method 2, being more efficient, was used to purify the biosurfactants.

Relationship between biosurfactant yield and activity. The biosurfactant yields of seven different *Bacillus* strains (duplicate cultures of each strain) with activities ranging from 0.5 to 4.25 times that of *B. mojavensis* strain JF-2 were determined. Biosurfactant activity was poorly correlated with the biosurfactant yield (linear correlation coefficient [r^2] = 0.09 and Pearson correlation coefficient [15] = -0.29). The biosurfactant activity did not always increase with an increase in biosurfactant yield,

i.e., some biosurfactants were produced with high yields but had relatively low activities, while others were produced with low yields but had high activities.

Biosurfactant structure-activity relationship. The lack of correlation between the biosurfactant yield and surface activity prompted us to study the effects of variations in structural components of different biosurfactants on activity. Amino acid analysis of eight purified biosurfactants showed that they contained the same amino acid composition (Glu/Gln:Asp/Asn:Val:Leu ratio, 0.99 $\pm$ 0.04:0.99 $\pm$ 0.04:1 $\pm$ 0.04:3.6 $\pm$ 0.12 [mean $\pm$ standard deviation of the mole ratio]). Since the acid hydrolysis method used for amino acid analysis does not differentiate between the acid and amide forms of the acidic amino acids (i.e., glutamate versus glutamine and aspartate versus asparagine), the peptide portions of these biosurfactants may differ in their Glu/Gln and/or Asp/Asn content.

The fatty acid portions of the biosurfactants contained 3-hydroxy tridecanoate (3-OH-C₁₃), tetradecanoate (3-OH-C₁₄), pentadecanoate (3-OH-C₁₅), and hexadecanoate (3-OH-C₁₆) (Table 2). The 3-OH-C₁₃ and 3-OH-C₁₅ fatty acids were present as mixtures of *iso* and *anteiso* isomers, while 3-OH-C₁₄ was comprised of normal and *iso* isomers. The 3-OH-C₁₆ fatty acid contained only the normal isomer. In some cases, the 3-OH-C₁₄ and 3-OH-C₁₅ fatty acids together constituted the majority of the fatty acids of the lipopeptide. However, in other cases, 3-OH-C₁₄ alone was the major fatty acid isomer. When the fatty acids of the biosurfactant purified from duplicate cultures of the same strain were analyzed, the fatty acid composition varied from one batch to another, along with the specific activity of the biosurfactant. Multiple regression analysis was used to determine the fatty acid isomers that contributed to activity (43). All fatty acid isomers, the sums of the tridecanoate, tetradecanoate, pentadecanoate, and hexadecanoate fatty acids, and the ratios of even *iso* to normal isomers and other combinations were used to construct multiple regression models. There was a significant positive correlation between the percent mass of the *iso*-3-OH-C₁₄ fatty acid and biosurfactant specific activity (Pearson's bivariate correlation coefficient; $r = 0.813$, $P < 0.001$) and a significant positive linear correlation between the ratio of *iso* to normal even-numbered fatty acids and biosurfactant specific activity (Pearson's bivariate correlation coefficient; $r = 0.953$, $P < 0.001$). No fatty acid other than the *iso*-3-OH-C₁₄ isomer showed a significant positive linear correlation with biosurfactant specific ac-

FIG. 1. Multiple regression analysis of fatty acid predictors of the specific activity of lipopeptide biosurfactants. Values on the x axis are the experimentally obtained specific activities of the different lipopeptide biosurfactants. Values on the y axis were obtained by using the multiple regression equation y (specific activity) = 0.39 (ratio of *iso* to normal even-numbered fatty acids) $- 0.09$ (ratio of *anteiso* to *iso* odd-numbered fatty acids) $+ 0.73$. The equation of the straight line was $y = 0.908x + 0.214$. The coefficient of linear regression (r^2) was 0.908 . The multiple regression equation shown above was used to predict the specific activities of four other strains. The coefficient of linear regression (r^2) between the predicted and actual specific activities for these four strains was 0.9134 ($y = 0.925x + 0.611$). Squares, values for experimentally obtained versus calculated specific activities for seven biosurfactants purified from duplicate cultures and one biosurfactant purified from triplicate cultures; diamonds, values for experimentally obtained versus calculated specific activities for four other purified biosurfactants.

tivity. We found that the best model of the specific activity of lipopeptide biosurfactants depended on both the ratio of *iso* to normal even-numbered fatty acids (positive dependence) and the ratio of *anteiso* to *iso* odd-numbered fatty acids (negative dependence). When the values expected for specific activity (obtained by using the multiple regression equation) were plotted against the values of specific activity obtained experimentally (Fig. 1, open squares) (43), the linear correlation coefficient (r^2) was 0.91 (15) and the Pearson correlation coefficient (r) was 0.94 (15). The variation not explained by the multiple regression model might be due to the probability of the presence of different amino acids in the peptide portions (Glu/Gln and/or Asp/Asn content) of the lipopeptides.

The multiple regression model accurately predicted the specific biosurfactant activity from the ratios of both *iso* to normal even-numbered fatty acids and *anteiso* to *iso* odd-numbered fatty acids for four other lipopeptide biosurfactants produced by *B. mojavensis* and *B. subtilis* subsp. *spizizenii* strains (Fig. 1, closed diamonds).

Effect of amino acid addition. Precursors of branched-chain fatty acids (21) were added to the growth medium of *B. subtilis* subsp. *subtilis* strain T89-42 to change the fatty acid composition and test the predictions of the multiple regression model (Table 3). When the strain was grown with 1 g/liter valine, the specific activity increased 3.2-fold, and the yield almost doubled compared to that in unamended cultures. The ratio of *iso* to normal 3-OH even-numbered fatty acids increased 2.8-fold (Table 3). The increase in both the specific activity and the ratio of *iso* to normal even-numbered fatty acids when valine

TABLE 3. Yields, surface activities, and fatty acid ratios of biosurfactants from *Bacillus subtilis* subsp. *subtilis* strain T89-42 grown in the presence and absence of exogenous amino acids in the medium

Amino acid added	Biosurfactant yield (mg/g dry wt of cells) ^a	Sp act ^b	Ratio of <i>iso</i> to normal even-numbered fatty acids ^c	Ratio of <i>anteiso</i> to <i>iso</i> odd-numbered fatty acids ^c
None	1.2 ± 0.25	2 ± 0.05	2.25 ± 0.5	0.55 ± 0.1
Alanine	0.28 ± 0.006	3.1 ± 0.4	2.7 ± 0.6	0.45 ± 0.3
Valine	2.3 ± 0.06	5.7 ± 0.6	6.33 ± 0.06	0.62 ± 1.05
Leucine	0.25 ± 0.04	4.1 ± 0.6	3.9 ± 0.38	0.037 ± 0.07
Isoleucine	0.37 ± 0.09	2.2 ± 0.4	3.9 ± 3.2	2.7 ± 0.6

^a Data are means $\pm$ standard deviations of triplicate cultures.

^b Data are means $\pm$ standard deviations of triplicate cultures. Specific activities of biosurfactants are expressed in mm of clearing μg^{-1} of biosurfactant.

^c Even-numbered fatty acids are *iso*-3-OH-C₁₄, normal 3-OH-C₁₄, and normal 3-OH-C₁₆. Odd-numbered fatty acids are *anteiso*-3-OH-C₁₃, 3-OH-C₁₅ (and 3-OH-C₁₇, but only in the case of isoleucine addition), *iso*-3-OH-C₁₃, and 3-OH-C₁₅ (and 3-OH-C₁₇, but only in the case of isoleucine and leucine additions). Data are Means $\pm$ ranges of the fatty acid ratios of the biosurfactants purified from duplicate cultures.

was added to the growth medium supports the finding that the specific activity is positively correlated to this ratio. When strain T89-42 was grown with alanine, the specific activity increased 1.7-fold and the ratio of *iso* to normal even-numbered fatty acids increased 1.2-fold, while the ratio of *anteiso* to *iso* odd-numbered fatty acids was about the same as that in the control without amino acid addition (Table 3). When leucine was present, the specific activity doubled (Table 3). The increase in specific activity with the addition of leucine could not be accounted for by an increase in the ratio of *iso* to normal even-numbered fatty acid isomers since the *iso* and normal isomers of even-numbered fatty acids with leucine addition comprised only 3.8% of the total fatty acids, compared to 48% of the total fatty acids in the control without amino acid addition. However, the decrease in the ratio of *anteiso* to *iso* odd-numbered fatty acids may explain the increase in specific activity since this ratio is negatively correlated with specific activity. When isoleucine was added to the growth medium, the specific activity was similar to that of the unamended control. An increase in the ratio of *iso* to normal even-numbered fatty acids (1.7-fold) might have counteracted the increase in the ratio of *anteiso* to *iso* odd-numbered fatty acids (2.7-fold) to keep the specific activity close to that of the control without amino acid addition.

Figure 2 shows that there was a strong linear correlation ($r^2 = 0.95$) (15) between the specific activities of biosurfactants produced in cultures with different amino acid additions and the specific activities predicted from the multiple regression equation based on fatty acid compositions. The Pearson correlation coefficient (r) was 0.98 (15).

DISCUSSION

The majority of strains examined in this study were members of *Bacillus subtilis* (subsp. *subtilis* and *spizizenii*), *Bacillus licheniformis*, or species closely related to them. Although many of these strains had originally been isolated for other studies (10, 12, 18, 33) and therefore not selected to be biosurfactant producers, 68% produced biosurfactants, compared to 80% of

FIG. 2. Correlation between experimentally obtained specific bio-surfactant activities and those predicted by the multiple regression equation based on known fatty acid compositions. The predicted specific activities were calculated from the fatty acid ratios given in Table 3 with the multiple regression equation given in the legend to Fig. 1. Data points represent each culture condition shown in Table 3. The error bars represent ranges for duplicate cultures for each growth condition. The equation of the straight line was $y = 0.323x + 1.12$. The coefficient of linear regression (r^2) was 0.902.

isolates from oil wells (Table 1). Approximately 14% of these strains that were examined with an oil-spreading technique had relative activities 1.75 to 2.5 times that of JF-2 (15 of 110; Table 1), while 1 in 44 strains isolated from oil wells (2%) had such a high relative activity. Therefore, in accordance with recent findings (7), we found that biosurfactant-producing microbes can be readily isolated from uncontaminated, undisturbed arid soils. However, our percentage of biosurfactant-producing isolates was quite high (e.g., 68% versus 3.4%), reflecting our focus on *Bacillus* species known to contain strains that produce biosurfactants rather than screening more broadly for novel biosurfactant producers. Strains varied greatly in biosurfactant yield (mg biosurfactant produced per gram dry weight of cells) and in the surface activity of active fractions collected from TLC plates. There was only a weak correlation between yield and surface activity.

The lack of correlation between yield and activity suggested that the variability in the surface activity of biosurfactants produced by the closely related strains used for this study was due to structure differences rather than a result of changed gene expression. The three-dimensional structure of surfactin, the biosurfactant produced by *B. subtilis* (9), showed that the carboxylic groups of both glutamate and aspartate form a minor hydrophilic domain and that the nonpolar residues in position 4, and to a lesser extent, positions 2 and 7 form major hydrophobic domains with the lipid tail. The presence of these two domains was found to be important for surface activity. Since then, structural variants of surfactin have been obtained via chemical modification (38), cultural modification, and genetic recombination (8, 14, 31, 32, 35, 36) to obtain biosurfactant molecules with more surface activity. A replacement of valine with isoleucine in position 4 decreased the critical micellar concentration twofold and increased the surface activity, possibly due to expansion of the major domain by the incorporation of the more hydrophobic isoleucine (8). Monoanionic biosurfactants (e.g., lichenysin A, with an asparagine in posi-

tion 5) had higher surface activities than dianionic biosurfactants (e.g., surfactin, with an aspartate in position 5) (14, 41). In this study, all of the biosurfactants tested had one valine and four leucines. Glutamine and/or asparagine could replace glutamate and aspartate in positions 1 and 5, respectively, since these amino acids could not be distinguished by the method used for amino acid analysis. The presence of glutamine or asparagine in the peptide chain would mean that the biosurfactant is monoanionic and hence should have more activity. A more detailed study of the presence of the amide forms of acidic amino acids is required to rule out their effect on biosurfactant activity.

The fatty acid composition of the lipopeptide also affects its activity. Yakimov et al. (40) found that an increase in the percentage of branched-chain fatty acids in lichenysin A of *Bacillus licheniformis* strain BAS50 decreased the surface activity and that an increase in the percentage of straight-chain 3-hydroxytetradecanoate (n-3OH-C₁₄) increased the surface activity. This is in contrast to our results showing that the percentage of 3-hydroxy *iso* even-numbered fatty acids (in our case, *iso*-3-OH-C₁₄ was the only even-numbered branched-chain fatty acid) was correlated with the surface activity. However, Yakimov et al. (40) studied only one lipopeptide, lichenysin A. Lichenysin A is a monoanionic lipopeptide with a heptapeptide (Glu:Asn:Val:Leu:Ile ratio, 1:1:1:3:1). The presence of the amide-form asparagine and the more hydrophobic isoleucine residue results in a lipopeptide with different properties from those of the lipopeptides compared in this study.

Kaneda (21, 22) showed that the biosynthesis of branched-chain fatty acids proceeds from the corresponding acyl coenzyme A esters derived from branched-chain amino acids (L-valine, L-isoleucine, and L-leucine). Since the fatty acid composition of the biosurfactant is controlled by the abundance of fatty acid precursors in the cell (1, 5, 6, 11, 16, 17), we added exogenous branched-chain amino acids to the growth medium to determine the effect of changes in fatty acid composition on biosurfactant activity. The results of exogenous amino acid additions to the growth medium (Table 3) suggest that altering the ratio of even-numbered fatty acids has a more pronounced effect on specific activity than does altering the ratio of odd-numbered fatty acids. Increases (2.8-fold and 1.2-fold) in the ratio of *iso* to normal even-numbered fatty acids (with valine and alanine additions, respectively) led to 3.2-fold and 1.7-fold increases in specific activity, respectively. However, a 15-fold decrease in the ratio of *anteiso* to *iso* odd-numbered fatty acids only led to a 2-fold increase in the specific activity when leucine was added to the medium. A 2.7-fold increase in the latter ratio with an isoleucine addition did not change the specific activity much compared to the control without amino acid addition. We hypothesize that branched even-numbered fatty acids (in this case, *iso*-C₁₄ was the only branched even-numbered fatty acid) might give the optimum hydrophilic-lipophilic balance required for optimum surface activity. A more definitive conclusion could be drawn if the lipopeptide with only the *iso*-C₁₄ fatty acid could be purified from the mixture of lipopeptides. A higher activity in this case would support this hypothesis.

This work shows that the fatty acid composition of lipopeptide biosurfactants is important for their activity and that manipulation of the medium composition to change the compo-

sition of the lipopeptide fatty acids may result in biosurfactants with higher specific activities. This may be a more useful approach than the molecular engineering of lipopeptides (27, 35, 37) since various regulatory policies make it difficult to use recombinant strains for in situ applications.

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