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Enhanced biosurfactant production by a mutant *Bacillus subtilis* strain*

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Summary. Ultraviolet mutation of *Bacillus subtilis* ATCC 21332 yielded a stable mutant that produced over three times more of the biosurfactant, surfactin, than the parent strain. By protoplast fusing the mutant (Suf-1) with the marker strain, *B. subtilis* BGSC strain IA28, the mutation was located between *arg*C4 and *his*A1 on the genetic map.

Introduction

Biosurfactants are produced as metabolic products or membrane components. These compounds are lipopeptides, glycolipids, lipopolysaccharides, neutral lipids and fatty acids or phospholipids (Cooper 1986; Cooper and Zajic 1980; Margaritis et al. 1979; Rosenberg 1982; Zajic and Steffens 1984). Surfactants are important as they are used in many multiphase processes (Layman 1985). Biosurfactants are biodegradable and potentially less toxic than the synthetic compounds currently used (Cooper and Zajic 1980). They can also be produced from a variety of substrates.

Surfactin, produced by *Bacillus subtilis* ATCC 21332 is one of the most effective biosurfactants known. It reduces the surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005% (Arima et al. 1968). This lipopeptide contains seven amino acids bonded to the carboxyl and hydroxyl groups of the 14-carbon acid (Kakinuma et al. 1969). It inhibits clot formation, lyses erythrocytes (Arima et al. 1968; Bernheimer and Avigad 1970), lyses bacterial spheroplasts and protoplasts and inhibits cyclic 3′,5′-monophosphate diesterase (Hosono and Suzuki 1983). It is produced from glucose instead of hydrocarbons as is the case of most biosurfactants (Cooper et al. 1979).

To increase commercial interest, yields of surfactin must be improved. Until now the only methods which have been utilized to enhance production are strain selection of the manipulation of environmental or nutritional factors (Cooper et al. 1981; Guerra-Santos et al. 1986). These methods, however, have their limitations as the levels of increase are marginal. Other approaches are therefore necessary. Our research has concentrated on the isolation of mutants which overproduce surfactin.

Materials and methods

Ultraviolet mutagenesis. The *B. subtilis* prototroph strain ATCC 21332 was grown to logarithmic phase and then approximately 3000 cells were plated on nutrient agar plates. The cells were then UV radiated for 35 s with short wave in a Chromato-Vue Cabinet Model CC-60 (UVP, San Gabriel, Calif, USA). This dosage of UV light had been previously determined to give 10%–20% survival of the colonies. The UV-irradiated cells were incubated at 37° C in the dark until colonies were visible.

Screening method for enhanced biosurfactant production. The *B. subtilis* mutants from UV mutagenesis were replica plated or individually spotted on to rich nutrient agar medium plates containing 5% sheep blood cells, 4% glucose, 0.1% nutrient broth, 0.1% yeast extract and mineral salts (Cooper et al. 1981). The cells were incubated at 37° C and the haemolytic zone surrounding the colonies was scored visually. The degree of lysis of red blood cells has been shown to be related to the level of surfactin production by *B. subtilis* (Mulligan et al. 1984).

Surfactin production in liquid media. From a sheep blood agar plate, *B. subtilis* was inoculated into 4% glucose and mineral salts medium, supplemented with 3.2×10^{-4} *M* $FeSO_4$ (100/500 ml flasks) (Cooper et al. 1981). After 3 days growth, 10 ml

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culture was transferred to another flask. After 6 h growth, 100 ml of this medium was used as an inoculum for a 3.7-l CHEMAP fermentor (Volketswil, Switzerland).

The continuously stirred tank fermentor was operated under the following conditions: a 2.0-l working volume, a temperature of 37° C, a 5.0 l/min aeration rate and pH control at 6.7. The surfactin was removed continuously in the foam into a flask on the air exhaust line (Cooper et al. 1981).

Growth was monitored by measuring the optical density (OD) of the fermentor broth at 600 nm; samples above OD 1.0 AUFS were diluted appropriately. For both strains, on OD of 1.0 AUFS represents 0.21 g/l biomass. Insignificant amounts of cells were found in the foam. Surface tension of the broth was measured by a Fisher Surface Tensiomat Model 21 (Montreal,Canada), a du Nouy tensiometer (Cooper et al. 1979).

Chemical isolation of surfactin. Using concentrated hydrochloric acid, the pH of the collapsed foam after cell removal was adjusted to 2 ± 0.5 (Cooper et al. 1981). This precipitated the proteins and lipids. After decanting the supernatant, the residue was resuspended in dichloromethane in a separatory funnel and shaken vigorously. Surfactin was recovered in the organic (top) layer. Two more extractions were performed and the organic layers were pooled and evaporated. The residue was redissolved in slightly basic water (pH 8.0) and filtered through Whatman no. 1 paper to remove undissolved impurities. The filtrate was again adjusted to pH 2 ± 0.5 and extracted with dichloromethane three times and evaporated as described above.

Surfactin assay. The biosurfactant yield of the collapsed foam was determined by both critical micelle concentration (CMC) and amino acid analysis. The CMC values were determined by measuring the surface tension at various dilutions (Cooper et al. 1979). The logarithm of the dilution was plotted as a function of surface tension. The CMC is the point of abrupt surface tension increase. The surfactant concentration is thus a function of the CMC.

An aliquot of the collapsed foam was trichloracetic acid-treated to precipitate peptides and proteins. The supernatant was assayed to determine the free amino acids in the medium. The residue (pellet) was redissolved in 25% trifluoroacetic acid and an aliquot was removed, dried and acid hydrolysed for 2.5 h at at 150° C in a Waters PICO-TAG Amino Acid Analysis System (Mass, USA). This enabled us to determine the amino acids in the peptide portion of surfactin. According to the molecular formula (Kakinuma et al. 1969), the peptidyl portion of surfactin is 76.5% by weight of the intact molecule. All amino acid analyses were done on a Beckman System 6300 High Performance Analyser equipped with a Beckman Model 7000 Data Station (Palo Alto, Calif, USA).

Mass spectrometry. Mass spectra were obtained in the positive ion mode on a VG Analytical ZAB-HS double focussing mass spectrometer (Manchester, UK). The accelerating voltage was 10 kV and the fast xenon atom beam was operated with an emission current of 1 mA at 8 kV. Mass spectra were recorded with an integrated data acquisition sytem and calibration was performed with CsI. Spectra for samples are an average of ten scans.

Media for protoplast fusion. For the isolation of auxotrophs, transformants or transductants, Spizizen minimal medium (SMM) (Spizizen 1958) was used. For protoplast regeneration, HCP-1.5 medium containing glucose (5 g/l), casamino acids (5 g/l), K_2HPO_4 (3.5 g/l), KH_2PO_4 (1.5 g/l), L-tryptophan (0.1 g/l), polyvinylpyrrolidone (PVP) (mean molecular weight 700000, 15 g/l), $MgCl_2$ (1.9 g/l) and 25% (v/v) 2 *M* sodium succinate (pH 7.3); HCP-1.5 agar contained the same components supplemented with agar (8 g/l). The HCP-3 medium was the same as HCP-1.5 with the exception of 30 g/l PVP.

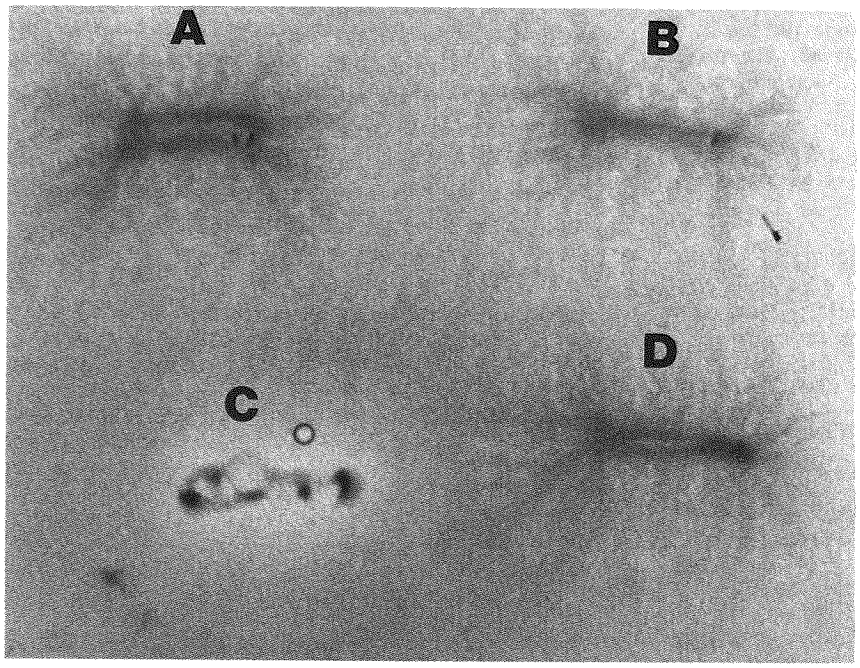

Fig. 1. Haemolysis by mutant and other strains on blood agar. Parent strain *B. subtilis* ATCC 21332 *(A)*, UV radiated colonies (*B* and *D*) and the mutant Suf-1 *(C)*. Growth was for 2 days at 37° C

Protoplast fusion. Protoplast fusion between the enhanced production mutant and BGSC strain 1A28 (*arg*C4, *his*A1 and *trp*C2) and regeneration was according to Akamatsu and Sekiguchi (1987). Cells were placed in SMM medium with 250 μg/ml lysozyme and incubated at 42° C for 45 min. The protoplasts were suspended in HCP-3 medium (30° C) for 3 h without shaking. The protoplast suspensions from the two strains were mixed and then centrifuged (4000 g, 10 min). The pellet was vortexed in SMM medium and 40% polyethylene glycol 4000 (average molecular weight 3000) was then added. The protoplast suspension was left to stand for one min followed by dilution with HCP-1.5 medium. The protoplasts were regenerated by placing 0.1 ml suspension on the surface of HCP-1.5 agar medium. Colonies were streaked on nutrient agar and single colonies were analysed for unselected markers.

Results and discussion

Selection of a mutant

To enhance biosurfactant production, mutation was chosen since any change in the regulatory system of biosurfactant synthesis and secretion could result in an altered level of production. After ultraviolet mutagenesis, approximately 1000 colonies were examined for enhanced haemolytic activity on sheep blood agar plates. One mutant, Suf-1, (C) produced a significantly larger haemolytic zone than the parent strain (Fig. 1). This mutant was not an auxotroph as it was able to grow in minimal medium. Without radiation, no colonies out of 1000 produced significantly higher levels of surfactant.

Biosurfactant production

To compare biosurfactant production by the mutant and parent strains, both were grown similarly in a fermentor. Suf-1 produced 3.4 times more biosurfactant than the parent strain over the same time period into the foam (Table 1). In both cases surfactin was totally (>99%) transferred from the fermentor medium into the foam. The growth of the two strains was approximately equal. Several single isolates of the same colony Suf-1 after many generations produced the same enhanced levels of surfactant in liquid medium. The *Suf*-1 mutation, therefore, was stable and did not revert in subculturing.

Table 1. Enhanced production of biosurfactant by *Bacillus subtilis* Suf-1

Strain	Growth after 40 h (optical density at 600 nm)	Surfactant yield (mg)[a]
ATCC 21332	8.2	328
Suf-1	8.3	1124

[a] Surfactant collected in the foam was determined by amino acid analysis and represents the amount produced in the fermentor

Biosurfactant characterization

The purified surfactant from the mutant and wild-type had similar amino acid profiles, which agreed with the compositon of surfactin (Kakinuma et al. 1969). Impure surfactant produced different peptidyl profiles. Furthermore, confirmation of the structure of the biosurfactants was obtained by FAB-MS (fast atom bombardment mass spectrometry). Based on the surfactin molecular formula ($C_{53}H_{93}N_7O_{13}$), the protonated molecular weight is 1036.69. The spectra of the compounds

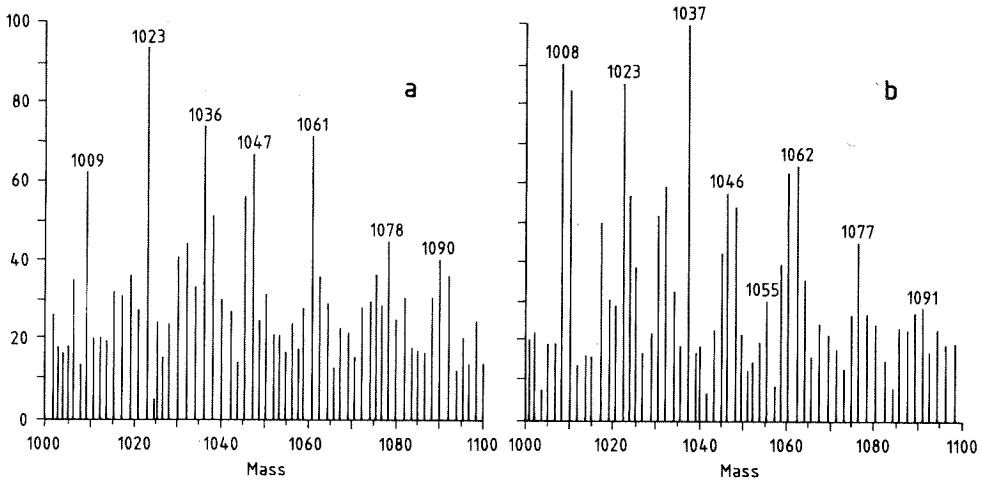

Fig. 2. Mass spectra of the purified biosurfactants isolated from the parent organism **(A)** and the hyperproductive mutant **(B)**

produced by the parent strain (Fig. 2A) and the mutant strain (Fig. 2B) were quite similar with molecular ions at 1036 and 1037, respectively. Their fragmentation pattern was also in harmony, with minor differences in intensities.

The screening method (Mulligan et al. 1984) for enhanced surfactin production was confirmed as these isolated compounds, solubilized in slightly basic water (pH 8.0), promoted haemolysis of the blood agar plates.

Location of the suf-1 mutation

Protoplast fusion was performed to locate the area on the chromosome of the mutation responsible for enhanced surfactin production. The *B. subtilis* BGSC strain IA28 was chosen for the three markers (*arg*C4, *his*A1 and *trp*C2), evenly spaced on the genetic map (Piggot and Hoch 1985). The marker strain produced very small amounts of biosurfactant on blood agar, no different from strain ATCC 21332. The linkage of the Suf-1 phenotype and the marker gene should give the approximate location of the *suf*-1 mutation with respect to the markers on the *B. subtilis* chromosome.

Loss of enhanced surfactant production by Suf-1 during fusion occurred upon specific recombinations of the mutated and marked areas (Table 2). This evidence suggested that the *suf*-1 mutation corresponded with the area between *arg*C4 and *his*A1 and furthest from *trp*C2. A single mutation in a specific region rather than multiple mutations throughout the genome must be responsible for increased surfactin production.

In summary, we increased biosurfactant production greater than threefold with *B. subtilis* Suf-1 by ultraviolet mutagenesis. Surfactin produced by this strain was identical to that produced by the wild-type. The mutation for increased production was mapped between *arg*C4 and *his*A1 on the *B. subtilis* chromosome, as determined by protoplast fusion. This information provides a target site for future genetic manipulation.

Table 2. Distribution of unselected marker classes among recombinants obtained by protoplast fusion between *B. subtilis* IA28 (*Arg*C4 *His*A1 *Trp*C2) and Suf-1

Number of colonies carrying indicated unselected markers			Numbers	% Frequency of Suf-1
Trp	His	Arg		
+	+	+	153	80 (121/153)[a]
+	+	−	88	49 (43/88)
+	−	−	32	0 (0/32)

[a] In parentheses are the numbers of Suf-1 recombinants per number of recombinants examined in each class. Presence of Suf-1 was indicated by haemolysis of blood agar plates

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