Journal of Industrial Microbiology (1996) 16, 257–260

Strain improvement of *Arthrobacter simplex* by protoplast fusion

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An L-tryptophan auxotroph and milky mutants were derived from an inducible cholesterol oxidase-producing bacterium, *Arthrobacter simplex* USA18, via UV-mutagenesis. Protoplasts of these mutants and a constitutive cholesterol oxidase producer, strain US3011, were prepared by growing cells in the presence of ampicillin (20 μg ml^{-1}) followed by digestion with lysozyme. Protoplast fusion between tested strains with complementary characteristics was achieved in the presence of 20–40% polyethylene glycol 6000. The fusion frequency was about 1.5–1.7 $\times$ 10^{-3}. The cholesterol oxidase activity of four fusants in a cholesterol-containing medium was 20–60% higher than that of parental strains. This study demonstrated that protoplast fusion is applicable to strain improvement of *Arthrobacter* strains for enzyme production.

Keywords: protoplast fusion; protoplasting; cholesterol oxidase; *Arthrobacter simplex*

Introduction

Coryneform bacteria (species of *Corynebacterium*, *Brevibacterium* and *Arthrobacter*) have been used extensively in the fermentation industry for production of amino acids [17], enzymes [9,16] and steroid drugs [12]. Traditionally, strain improvement of these industrial microorganisms aimed at increasing the yield of products has involved intensive programs of mutagenesis, followed by selection of strains with new characteristics [6]. With the advent of recombinant DNA and protoplast fusion techniques developed for species of *Bacillus* and *Streptomyces* [14], methods for the protoplasting of *Corynebacterium* [1,19] and *Brevibacterium* [4], have also been developed. It was reported that one of the major difficulties in using these methods for coryneform bacteria is that pre-treatment is necessary in order to facilitate the removal of cell wall materials, making the cells sensitive to lysozyme digestion.

To improve strains of *Arthrobacter* genetically, recombinant DNA techniques are considered to be useful, and a host-vector system has been developed for an *Arthrobacter* sp [16]. However, because the efficiency of the *Arthrobacter* host-vector system is not high and many *Arthrobacter* genes are not expressed in *E. coli*, cell fusion is still the appropriate method for strain improvement of *Arthrobacter* sp. In fact, the protoplast fusion technique has proved valuable for strain improvement of various industrial microorganisms for increased production of enzymes [13,15], organic acids [2,13], antibiotics [14] and amino acids [5].

To improve cholesterol oxidase productivity and strain stability, we have isolated inducible and constitutive mutants, USA18 and US3011, respectively, from *A. simplex* B7 using UV-mutagenesis [9]. The present paper is concerned with the strain improvement of these cholesterol oxidase-producing mutants by the protoplast fusion technique.

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Received 25 September 1995; accepted 23 January 1996

Materials and methods

Microorganisms

Inducible and constitutive cholesterol oxidase-producing mutants, USA18 [9] and US3011 [10], derived from *A. simplex* B7, were used in this study. Strain USA18 is resistant to 50 μg ml^{-1} of streptomycin, while strain US3011 is sensitive.

Materials

Yeast extract, tryptone, casamino acid, nutrient broth dehydrate and agar were obtained from Difco (Detroit, MI, USA). Streptomycin sulfate, ampicillin, lysozyme, L-tryptophan, sucrose, succinic acid (disodium salt) and maleic acid (disodium salt) were obtained from Sigma (St Louis, MO, USA). Cholesterol was purchased from Tokyo Kasei Chemical Company (Tokyo, Japan). Inorganic salts and other chemicals were all of reagent grade.

Media

Stock cultures were maintained on an enriched nutrient agar medium (10 g nutrient broth dehydrate, 10 g yeast extract, 10 g glucose and 20 g agar per liter of distilled water, pH 7.0). LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter of distilled water, pH 7.0) was used for routine bacterial growth. Minimal medium (10 g glucose, 2 g ammonium acetate, 0.5 g K_2HPO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ per liter of distilled water, pH 7.0) was used for the isolation of mutants auxotrophic for L-tryptophan. Protoplast buffer (SMB) was prepared by mixing equal volumes of SMM medium (1.0 M sucrose, 0.04 M sodium maleate and 0.04 M $MgCl_2 \cdot 6H_2O$, pH 6.5) and LBV medium (20 g tryptone, 1.0 g yeast extract and 10 g NaCl per liter of distilled water, pH 7.0). Protoplast regeneration agar medium

(DM3) was prepared by the following procedure. Firstly, 8 g agar, 5 g casamino acid and 5 g yeast extract in 350 ml distilled water was autoclaved. Then, the following items were autoclaved and added separately: 5 g glucose in 25 ml distilled water; 1.75 g K_2HPO_4 and 0.75 g KH_2PO_4 in 100 ml distilled water; 8.66 g $MgCl_2 \cdot 6H_2O$ in 20 ml distilled water; 0.1 g bovine serum albumin in 2 ml distilled water (filter sterilized); and 500 ml 1 M sodium succinate. Solid and soft agar media contained 20 g and 8 g agar per liter, respectively.

Isolation of auxotrophic mutants

Mutagenesis was performed by ultraviolet radiation [3]. Mid-log phase cells of strain USA18 freshly grown in LB medium at 30°C were harvested and suspended in saline solution at 10^8 cells ml^{-1}. After treatment with ultraviolet radiation for 6 min (99.99% killing), 0.1-ml samples, diluted appropriately with saline solution, were plated onto LB agar medium containing 50 μg ml^{-1} of streptomycin. After incubation at 30°C for 48 h, colonies were transferred to a minimal agar medium containing 50 μg ml^{-1} of streptomycin for the isolation of L-tryptophan auxotrophic mutants.

Protoplast formation and regeneration

Mid-log phase cells of strains freshly grown in LB medium at 30°C were harvested and resuspended in a fresh LB medium supplemented with 20 μg ml^{-1} of ampicillin. After incubation at 30°C for 12 h, cells were harvested by centrifugation at 1380 × *g* for 10 min. The pellet was resuspended in a suitable volume of SMB buffer (10^8 cells ml^{-1}), then 0.5–2.5 mg ml^{-1} of lysozyme solution was added. Suspensions were incubated at 30°C for 2 h with occasional mixing. The protoplasts were then harvested by centrifugation at 3000 × *g* for 5 min. Protoplast formation was monitored microscopically (phase contrast) and by plating onto hypertonic (DM3) and hypotonic (LB soft agar) media. Samples were withdrawn for viable counts before lysozyme addition and after the final incubation period. The pellet was resuspended, serially diluted in SMB buffer, and plated onto DM3 and LB soft agar plates. Colonies of regenerated protoplasts were counted after incubation at 30°C for 2–4 days. Viable cell counts were expressed as colony forming units ml^{-1} (CFU ml^{-1}).

Protoplast fusion

Equal volumes (0.5 ml) of the two protoplast suspensions were mixed in an Eppendorf tube and centrifuged at 3000 × *g* for 5 min. The pellet was resuspended in 0.5 ml SMM medium containing 20–60% polyethylene glycol 6000 (PEG). After incubation at 30°C for 15 min, the suspension was serially diluted in SMM medium. Samples (0.1 ml) were then plated onto DM3 plates and incubated at 30°C for 7 days. Colonies that appeared on DM3 plates were transferred to minimal agar medium supplemented with 50 μg ml^{-1} of streptomycin. Colonies that appeared in the above selection medium were isolated as fusants. These fusants were transferred again onto LB plates for observation of the color appearance of colonies.

Cultivation

The cultivation of parental strains and of fusants for the production of cholesterol oxidase was carried out in 500-ml flasks containing 100 ml cholesterol oxidase-producing medium (20 g cholesterol or 10 g glucose, 2 g ammonium acetate, 5 g yeast extract, 1 g KCl, 0.5 g K_2HPO_4, 5 g $MgSO_4 \cdot 7H_2O$ and 0.1 g Tween 80 per liter of distilled water, pH 7.5) which were shaken at 125 rpm for 48 h at 30°C. Culture filtrates were tested for the activity of cholesterol oxidase.

Estimation of cholesterol oxidase activity

Cholesterol oxidase activity was determined by a modification of Allain's method [8]. One unit of cholesterol oxidase activity is defined as the amount of enzyme oxidizing 1 μmol of cholesterol to cholest-4-en-3-one per min at 30°C.

Results

Isolation of auxotrophic and milky mutants

After UV-mutagenesis, an L-tryptophan auxotrophic mutant, UC3, and a milky mutant, UC4, were derived from an orange parental strain USA18. The deficient growth of mutant UC3 in a minimal medium containing 50 μg ml^{-1} of streptomycin could be reversed by addition of L-tryptophan. The minimal requirement of L-tryptophan was 0.5 μg ml^{-1}. The characteristics of milky mutant UC4 was similar to that of orange parental strain USA18 except that the colony was milky, not orange. The L-tryptophan requirement in strain UC3 and milky appearance in strain UC4 were used as markers in protoplast fusion experiments. The characteristics of parental strains and mutants such as antibiotic resistance, auxotrophic requirement and color appearance of the colony are summarized in Table 1.

Protoplast formation

The optimal conditions for protoplast formation by lysozyme treatment were investigated. The appearance of spherical cells under the microscope and the disruption of these cells under hypotonic conditions were used as indices of protoplast formation.

Mid-log phase cells in LB medium were harvested by centrifugation. The pellets were suspended in SMB buffer, and 0.5–2.5 mg ml^{-1} of lysozyme solution was added. The level of osmotically sensitive cells under hypotonic conditions was parallel to the concentration of added lysozyme. However, spherical cells were not observed via light microscopy. The addition of EDTA or calcium chloride showed no apparent effect on the protoplast formation.

Mid-log phase cells in LB medium supplemented with 1% of glycine were harvested and treated with lysozyme. Most glycine-lysozyme-treated cells became osmotically sensitive. However, spherical cells were not observed via microscopy.

The growth of various *Arthrobacter* strains tested was completely inhibited by the addition of ampicillin at 40 μg ml^{-1}. Mid-log phase cells in LB medium supplemented with 20 μg ml^{-1} of ampicillin showed as swollen cells via microscopy. The osmotic sensitivity of these swollen cells in hypertonic medium was similar to that in hypotonic

Table 1 Comparison of the cholesterol oxidase activity of parental strains and fusants

Strain characteristics[a]	Cholesterol oxidase activity (U ml^{-1})[b]	
	(I)	(II)
(A) Parents		
US3011 (orange, Trp$^+$, Str^s)	0.23	0.75
UC3 (orange, Trp$^-$, Str^r)	0	0.47
Fusants (orange, Trp$^+$, Str^r)		
F12010	0.26	0.63
F1403	0.20	0.53
F1601	0.27	1.20
(B) Parents		
US3011 (orange, Trp$^+$, Str^s)	0.23	0.75
UC4 (milky, Trp$^+$, Str^r)	0	0.41
Fusants (orange, Trp$^+$, Str^r)		
F2208	0.25	0.87
F24010	0.27	0.96
F2605	0.23	0.88

[a]Trp$^+$: L-tryptophan non-requirement; Trp$^-$: L-tryptophan requirement; Str^r: Streptomycin resistant; Str^s: Streptomycin sensitive; Orange and milky: color appearance of colonies.
[b]Cells were cultivated in 500-ml flasks containing 100 ml basal medium (2 g ammonium acetate, 5 g yeast extract, 1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g Tween 80 per liter of distilled water, pH 7.5) supplemented with (I) 10 g glucose or (II) 20 g cholesterol at 30°C and shaken at 125 rpm for 48 h. Then, culture filtrates were tested for the activity of cholesterol oxidase.

medium (data not shown). As shown in Table 2, an apparent difference in viable cell number between the hypertonic and hypotonic media was found after these swollen cells were treated with lysozyme. The optimal lysozyme concentration for the formation of osmotically sensitive cells was 1.0–1.5 mg ml^{-1}. The appearance of spherical protoplast cells derived from rod-shaped parental strains via the ampicillin-lysozyme-treatment process was confirmed by microscopy.

Protoplast fusion

The protoplast fusions were performed between strain US3011 and strain UC3 or strain UC4. The parental strains were protoplasted, mixed, suspended in PEG solution for 15 min and plated on DM3 medium. Fusants with orange, Trp$^+$, Str^r characteristics were isolated on minimal agar medium supplemented with 50 μg ml^{-1} of streptomycin. The fusion frequency was expressed as the ratio of the number of fusant cells to the number of initial cells. The optimal PEG concentration for protoplast fusion in strain US3011 × strain UC3 and strain US3011 × strain UC4 was 20% and 40%, respectively (Table 3). The optimum fusion frequency was about 1.5–1.7 × 10^{-3} per initial cell. The fusion frequency decreased when the PEG concentration was increased up to 60%.

Isolation of cholesterol oxidase-producing fusants

Among 50 cholesterol oxidase-producing fusants with orange, Trp$^+$, Str^r characteristics, the six most active in the culture fluids incubated by shaking at 30°C for 48 h were selected (Table 1). The cholesterol oxidase activities of these six fusants could be detected either in a cholesterol-free medium containing glucose as carbon source or in a cholesterol-containing medium. These characteristics were similar to that of the constitutive cholesterol oxidase-producing parental strain US3011. On the other hand, cholesterol oxidase activity was not found with the L-tryptophan-requiring strain UC3 and the milky strain UC4 in choles-

Table 3 Effect of PEG 6000 concentration on the fusion frequency

Fusion pairs	PEG 6000 concentration (%)	Fusion frequency[a]
US3011 × UC3	20	1.5 × 10^{-3}
	40	4.0 × 10^{-4}
	60	3.0 × 10^{-4}
US3011 × UC4	20	1.2 × 10^{-3}
	40	1.7 × 10^{-3}
	60	9.0 × 10^{-4}

[a]Fusion frequency was expressed as the ratio of the number of fusant cells to the number of initial cells.

Table 2 The optimal lysozyme concentration for formation of osmotically sensitive cells from ampicillin-pretreated cells

Strain	Initial cell concentration (CFU ml^{-1})	Lysozyme concentration[a] (mg ml^{-1})	Lysozyme-treated cells[b] (CFU ml^{-1})	
			SMB	H_2O
UC3	1.26 × 10^8	1.0	1.4 × 10^6	1.4 × 10^3
		1.5	4.5 × 10^5	1.6 × 10^3
		2.5	3.1 × 10^5	1.0 × 10^3
UC4	1.86 × 10^8	1.0	3.6 × 10^7	1.1 × 10^5
		1.5	1.3 × 10^7	4.1 × 10^4
		2.5	1.0 × 10^7	1.0 × 10^4
US3011	1.32 × 10^8	1.0	1.0 × 10^8	1.0 × 10^5
		1.5	3.0 × 10^7	2.5 × 10^4
		2.5	1.7 × 10^7	4.8 × 10^4

[a]Cells were grown in LB medium supplemented with 20 μg ml^{-1} of ampicillin at 30°C for 12 h, then harvested by centrifugation. The pellets were resuspended in SMB buffer supplemented with various concentrations of lysozyme and incubated at 30°C for 2 h.
[b]Lysozyme-treated cells in hypertonic buffer (SMB) and hypotonic buffer (H_2O).

terol-free medium containing glucose as carbon source. This characteristic was similar to that of the inducible cholesterol oxidase-producing parental strain USA18. However, in a cholesterol-containing medium, the enzyme productivity of the four fusants was 20–60% higher than that of parental strains. These fusants exhibited two favorable characteristics compared with the parental strains: resistance to streptomycin and the ability to produce cholesterol oxidase either in a cholesterol-free medium or in a cholesterol-containing medium.

Discussion

The genus *Arthrobacter* is industrially important for enzyme production [9,16], steroid transformation [12], and degradation of aromatic compounds [11]. They are Gram-negative rods in young culture and become Gram-positive cocci in older cultures [18]. The cell wall of *Arthrobacter* is composed of a basal peptidoglycan structure and a special sulfated polysaccharide structure [7]. Shaw [16] reported that *Arthrobacter* cell walls could be readily removed by lysozyme digestion and protoplast cells appeared in a hypertonic solution. In the present study, a large proportion of lysozyme-treated cells became osmotically sensitive cells. However, these osmotically sensitive cells did not appear as spherical protoplast cells in a hypertonic medium, indicating that pre-treatment of *Arthrobacter* cells is necessary for the formation of protoplast cells via lysozyme digestion.

It was reported that protoplasts of *Corynebacterium* [1,19] or *Brevibacterium* [4] could be prepared by growing cells in the presence of glycine or penicillin G, respectively, followed by digestion with lysozyme. In the present study, most of the glycine-lysozyme-treated cells became osmotically sensitive. However, no spherical protoplast cells were observed. On the other hand, ampicillin-lysozyme treatment not only made the cells osmotically sensitive (Table 2) but also produced spherical protoplast cells. It is apparent that the protoplasting system of *Arthrobacter* was similar to that of *Brevibacterium*.

Protoplast fusion is a useful technique for obtaining hybrids or recombinants of different microorganisms. It has proved to be a valuable tool for the improving industrial microorganisms with dominant characteristics such as increased product yield [2,5,13–15]. As shown in Table 1, the cholesterol oxidase activity of four fusants in a cholesterol-containing medium was 20–60% higher than that of the parental strains. These results indicate that protoplast fusion is applicable to strain improvement of *Arthrobacter* strains for enzyme production.

Acknowledgements

Financial support for this study from the National Science Council of Republic of China (NSC81-0418-B-002-03) was highly appreciated.

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