[SV-2]

Cold Shock Response of an Antarctic *Streptomyces* Strain Showing Demulsifying Ability

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Abstract

The hydrophobic spores of *Streptomyces* sp. AA8321 isolated from the Antarctic coast displayed demulsification ability. The aerial spores demulsified an emulsion of kerosene/0.2% Triton X-100 (2:1 v/v) to 50% and 95% within 1 min contact at the concentrations of 5.0×10^7 and 1.0×10^8 spores/ml, respectively. A cold shock protein (*csp*) gene was cloned from the hydrophobic spore- producing *Streptomyces* sp. AA8321 using PCR. It encoded a low molecular protein with 68 amino acids showing very low homology with previously reported *csp* genes. Only the sequence of the first six amino acids was just the same and yet others were different. RNA blot analysis indicated that the csp gene was induced by cold shock, i.e., transferring from 30°C to 10°C, and this cold shock response proposed that the isolated gene be a new type of *csp* gene.

Introduction

The range of temperatures in which growth of organisms has been detected reaches from –12 °C to approximately 112 °C. Within this frame, the velocity of chemical reactions and the physical properties of biomolecules change dramatically, such that microorganisms are only able to grow within a limited thermal range. Among them, the microorganisms that can grow at near 0°C with an optimal growth temperature above 20 °C are classified into psychrophiles (cold loving). At low temperature, the rate of enzymatic reactions, the fluidity of cellular membrane, and the affinity of uptake and transport systems decrease (Phadtare et al. 2000). To overcome problems at low temperature, psychrophiles change biochemical composition and gene expression. Therefore, biomolecules of psychrophiles may show distinctive physical properties.

One of the properties is hydrophobic extracellular material showing demulsification ability. Demulsification is the process of destroying or breaking unwanted emulsion especially water-in-oil types. Oil-water emulsions are inhibitory in oil recovery process and can pollute environments. Demulsification using microorganisms has been examined in various petroleum emulsions, and the microorganisms-induced demulsification is associated with in the cell surface of the organisms.

Biosynthesis of cold shock proteins (CSPs) that are induced by low temperature is one of the cold responses of psychrophiles (Berger et al. 1997). CSPs are homologous small (~ 7.5 kDa) proteins reported from microorganisms, some of CSPs are essential for cellular growth and for efficient protein synthesis at both optimal and low temperatures (Graumann et al. 1997). Csp homolog has been also found in *Streptomyces* (Kormanec and Sevcikova 2000). In this study, demulsification ability toward

various oil-in-water emulsions was examined, and a *csp* gene was characterized from a soil microorganism *Streptomyces* sp. strain AA8321.

Results

1. Demulsification and hydrophobicity of spore surface

Demulsification of the standard emulsion was detected only with the spores obtained from solid culture, and the aerial spores displayed 50% and 95% of demulsification after 1 min contact to the emulsion at the concentrations of 5.0×10^7 and 1.0×10^8 spores/ml, respectively (Fig. 1). This result indicates that the spores from the solid culture were decisive in demulsification ability of the organism Correlation between demulsification and spore surface hydrophobicity was tested with the spores obtained from liquid and solid cultures. As shown in Table 1, hydrophobicity of the spores from the solid culture was correlated with both culture time and demulsification activity. However, de-emulsifying activity was not detected with neither mycelia nor spores from the liquid cultures, and the submerged spores displayed low hydrophobicity. Furthermore, the hydrophobicity of the submerged spores was independent from culture periods.

Demulsification ability of the spores from the solid culture toward hydrocarbons with low and high viscosity was studied with the emulsions consisting of 0.2% Triton X-100 aqueous solution (Fig. 2 and 3). Emulsions containing n-hexadecane, kerosene or diesel were demulsified more rapidly than emulsions of n-hexane and gasoline. However, demulsification rates were increased with decreasing ratio of paraffin or soybean oils to kerosene, and demulsification of the emulsions occurred completely within 10 min, when paraffin and soybean oils were diluted with kerosene in the ratio of 1:1. The demulsification of the emulsions prepared with kerosene and various emulsifiers by the spores from the strain was tested (Table 2). The emulsions with low concentration of the emulsifiers were separated more efficiently into two phases. The aerial spores of the strain AA8321 demulsified the emulsions containing Triton X-100 or Tween 80 less efficiently than the emulsions containing other emulsifiers.

2. Cloning of the csp gene

We obtained the 200 bp amplified DNA fragment by PCR using a primer set which is conserved region of previously reported csp genes from *Streptomyces* species. The nucleotide sequence of the DNA fragment contained one ORF of 217 nucleotides encoding a low molecular protein with 68 amino acids (Fig. 4). The gene size of the ORF was similar to those of previously reported csp genes, but homology was very low (Fig. 5). Only the sequence of the first six amino acids was just the same and yet others were different. To identify cold response of the isolated gene, we checked whether cold shock induced the gene expression by RNA blot analysis (Fig. 6). *Streptomyces* sp. AA8321 was cultured to the mid-exponential phase at 30°C, after which it was subjected to a cold shock by

culturing at 10 ° C. The transcript of the isolated gene was monitored at various times after cold shock. They showed biphasic expression pattern. The transcript was induced at two minute after cold shock and the transcript level increased until 30 minute by approximately sixfold over the level at 30 ° C. The transcript level decreased at 60 minute but it reached maximal accumulation at 120 minute after cold shock again. This cold shock response proposed that the isolated gene be a new type of *csp* gene.

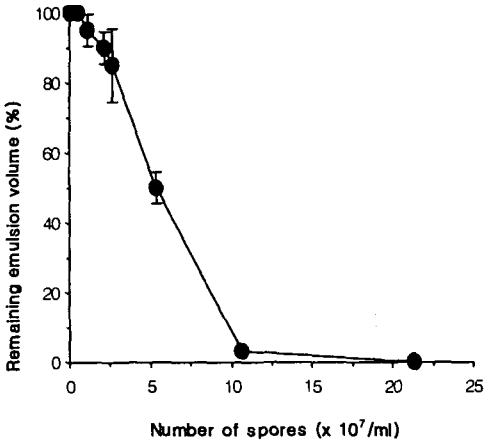

Fig. 1. Effect of spore concentration on demulsification ability toward the emulsion of kerosene/0.2% Triton X-100 (2/1, v/v). Relative demulsifying activity could be expressed as a percentage of decresed volume of remaining emusions.

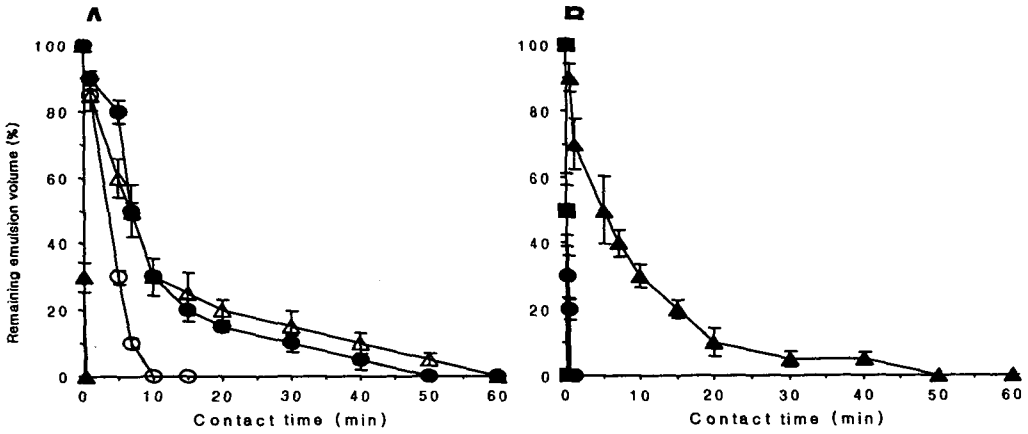

Fig. 2. Demulsification ability of the spores of the strain AA8321 toward emulsions of low viscosity hydrocarbons and 0.2% Triton X-100. Spores from the solid culture ($1X10^8$ spores/ml) were added into the emulsions. A: ●, n-hexane; ○, n-dodecane; ▲, n-hexadecane; △, cyclohexane. B: ●, kerosene, ▲, gasoline; ■, diesel. Relative demulsifying activity could be expressed as a percentage of decresed volume of remaining emusions.

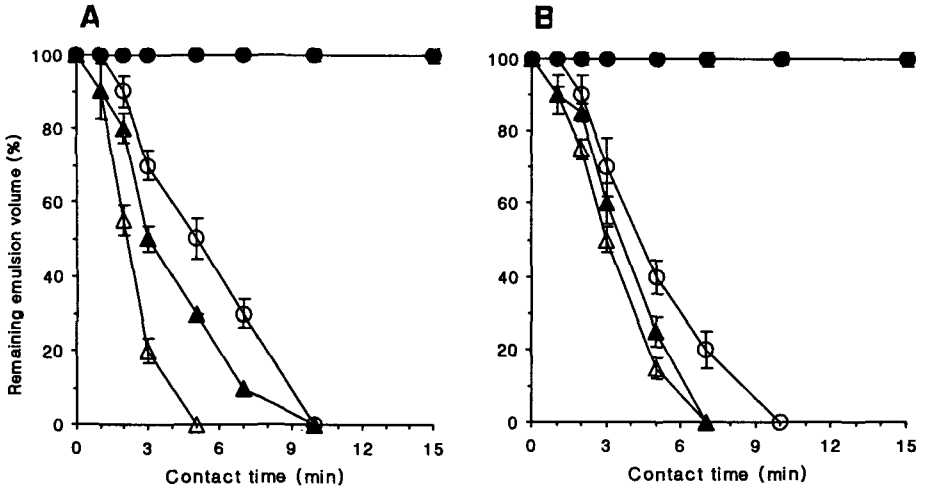

Fig. 3. Dimulsification ability of the spores toward emulsions of high viscosity. Spores from the solid culture ($1X10^8$ spores/ml) were added into the emulsions. A: paraffin oil (●) and paraffin oil diluted with kerosene (○, 50% paraffin oil; ▲, 30% paraffin oil; △, 10% paraffin oil). B: soybean oil (●) and soybean oil diluted with kerosene (○, 50% soybean oil; ▲, 30% soybean oil; △, 10% soybean oil). Relative demulsifying activity could be expressed as a percentage of decresed volume of remaining emusions.

Table 1. Demulsification activity and cell surface hydrophobicity of aerial and submerged spore solution of *Streptomyces* sp. AA8321 with different culture ages

Media	Culture age (d)	$T_{1/2}$	Hydrophobicity (%)
ISP4-ASW (solid)	4	10 h	20±1
	5	1 h	47±1
	6	25 min	60±2
	7	20 s	67±1
	8	20 s	70±1
	10	20 s	71±2
	15	20 s	72±1
ISP4-ASW (liquid)	4	> 24 h	29±5
	7	>24 h	33±2
Bennett's Medium (liquid)	4	> 24 h	4±2
	7	> 24 h	4±1
YED (liquid)	4	> 24 h	4±1
	7	> 24 h	4±1
MM(liquid)	4	> 24 h	36±12
	7	> 24 h	34±3

Spores were added into emulsions (kerosene/0.2% Triton X-100, 2:1) at the concentration of $1X10^8$ spores/ml.

Table 2. Demulsification activity of the spores of *Stretpmyces* sp. AA8321 toward oil-in-water emulsions of kerosene and water containing various emulsifiers

Emulsifier		$T_{1/2}$
Corexit 7664	0.2%	17 s
	0.5%	42 s
8667	0.2%	10 s
	0.5%	25 s
9550	0.2%	<5 s
	0.5%	<5 s
Finasol ORS2	0.2%	<5 s
	0.5%	<5 s
12	0.2%	<5 s
	0.5%	<5 s
51	0.2%	<5 s
	0.5%	<5 s
BP 700	0.2%	<5 s
	0.5%	<5 s
1100	0.2%	<5 s
	0.5%	<5 s
Triton X-100	0.2%[a]	>24 h
	0.2%	20 s
	0.5%	10 min
Tween 80	0.2%[a]	>24 h
	0.2%	<5 s
	0.5%	6 min

Spores from the solid culture were added into the emulsions at the concentration of $1X10^8$ spores/ml.

[a] Spores obtained from liquid cultures were added.

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1   ATG GCT CAG GGC ACC GTC AAT CCA CTG ATA GAA GCC AGC GCC TTT
      M   A   Q   G   T   V   N   P   L   I   E   A   S   A   F   15

46  ACC TCA AAA GGT AAG TGC TTA GCG AGA AAA GAT AAA TTC GAC CTT
      T   S   K   G   K   C   L   A   R   K   D   K   F   D   L   30

91  TTC TAT AAG AGG CAA CCG CAG TCT GTG CGC CAA CAG CGT CTG TTG
      F   Y   K   R   Q   P   Q   S   V   R   Q   Q   R   L   L   45

136 GCG GCG ATT CAT GCC AAT ATT ATC GAA TAC CCG GCG CAG GAC GCC
      A   A   I   H   A   N   I   I   E   Y   P   A   Q   D   A   60

181 AAT TCA ATC CCG ACA TGG TCC GCC TGA
      N   S   I   P   T   W   S   A   *                       68
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Fig. 4. Nucleotide and deduced amino acid sequences of a putative cold shock protein gene isolated from *Streptomyces* sp. Putative open reading frame (ORF) is shown below the nucleotide sequence in one letter symbol of amino acids.

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Streptomyces sp. AA8321      1 M A Q G T V N P L I E A S A F T S K G K
S. coelicolor                1 M A Q G T V K W F N A E K G Y G F I A V
S. hygroscopicus             1 M A Q G T V K W F N A E K G Y G F I A V
S. nodosus                   1 M A Q G T V K W F N A E K G Y G F I A V

Streptomyces sp. AA8321     21 C L A R K D K F D L F Y K R Q P Q S V R
S. coelicolor                  D G G A D V F V H Y S A I Q M D G Y R T
S. hygroscopicus               D G G A D V F V H Y S A I Q M D G Y R T
S. nodosus                     D G G A D V F V H Y S A I Q M D G Y R T

Streptomyces sp. AA8321     41 Q Q R L L A A I H A N I I E Y P A Q D A
S. coelicolor                  L E E G Q R V E F E I S Q G Q K G P Q A
S. hygroscopicus               L E E G Q R V E F E I S Q G Q K G P Q A
S. nodosus                     L E E G Q R V E F E I S Q G Q K G P Q A

Streptomyces sp. AA8321     61 N S I P T W S A *
S. coelicolor                  D M V R L S A *
S. hygroscopicus               D M V R V A V G *
S. nodosus                     D M V R V A G *
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Fig. 5. Comparison of the amino acid sequence of a putative cold shock protein of *Streptomyces* sp. with the partial sequences of *S. coelicolor, S. hygroscopicus* and *S. nodosus* cold shock protein.

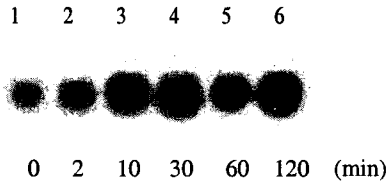

Fig. 6. RNA blot analysis for the transcript of a putative cold shock protein gene of *Streptomyces* sp. AA 8321.
Total RNA was extracted from each samples which was cultured at 30°C and transferred to 10 °C and cultured for 2 , 10, 30, 60, and 120 min.

Discussion

1. Demulsification and hydrophobicity of spore surface

Demulsification ability of *Streptomyces* sp. AA8321 increased with increasing concentration of the spores from the solid culture, and the hydrophobicity of spore surface increased with culture age and demulsifying activity. Similar results have been observed in the demulsification by *N. amarae* (Gray *et at.* 1984). Demulsification by the strain AA8321 seems to be associated with the spore surface as other de-emulsifying active bacteria, which activity is induced from hydrophobic cell surface (Stewart *et al.* 1983). Surface hydrophobicity of the spores appeared to facilitate adhesion of oil droplets in emulsions to spore surface, spreading of oil layer, and coalescence of the droplets, acting as chemical demulsifier. Demulsifiers, which are surface active and usually also emulsifiers, promote drop growth and emulsion disruption (Stewart *et al.* 1996).

The hydrophobicity of the aerial spores of *Streptomyces* is due to a sheath which encloses them, but the particular chemical components of the sheath responsible for its hydrophobic nature are

unknown (Rosenberg & Sar 1990). The hydrophobicity of an organism depends on the surface of lipophilic components, hydrophobins (Courtney *et al.* 1990). In the future work, the hydrophobin-like component of the aerial spores of the strain AA8321 has to be isolated to examine the correlation between the hydrophobin and demulsification ability of the organism. Lower de-emulsifying activities were observed in the emulsions of higher viscosity hydrocarbons and high concentration of emulsifiers. Surfactants and higher viscosity hydrocarbons stabilized interfacial tension and interfacial viscosity, and demulsification activity by the spores was lower in these emulsions, according to the suggestions of Kim *et al.* (1996). This paper has now demonstrated that *Streptomyces* sp. AA8321 has also the ability as microbial demulsifier. *Streptomyces* sp. has potential to be applied to de-emulsifying techniques by development of appropriate inexpensive culture conditions.

2. Cloning of the csp gene

The putative *csp* gene of *Streptomyces* sp. AA8321 was cloned using PCR. The deduced amino acid corresponds to a small protein of 68 amino acid residues that match CSPs, but this *csp* shared low level of homologies with *csp* genes previously reported from other *Streptomyces* species. Only the sequence of the first six amino acids was just the same and yet others were different. The sequence did not posses the previously defined RNP-1 and RNP-2 motifs involved in the binding to single-stranded nucleic acids, and no particular residue or domain that could be specific to *E. coli csp* genes emerged. Nevertheless, this *csp* was expressed very rapidly following cold shock, the transcript level was increased from two minutes after cold shock treatment, which reached maximum state at ten minutes after cold shock treatment. They showed biphasic expression pattern, which reported previously in *Bacullus subtilis cspB* (Willimsky et al. 1992). The cold shock response indicates that this *csp* gene of *Streptomyces* sp. AA8321 is supposed to be a new type of csp gene. The introduction of this *csp* gene is expected to increase survival rate after freezing or low-temperature treatment of useful bacteria. Further studies on this *csp* gene from psychrophilic *Streptomyces* may provide new insights on the molecular strategies adopted in response to cold stress.

Acknowledgement

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