Appearance of a stress-response protein, phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents

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A 28 kDa protein associated with the inner membrane was induced strongly in *Escherichia coli* K-12 cells grown in the presence of a hydrophobic organic solvent, n-hexane or cyclooctane. These organic solvents suppressed the growth (growth rate and yield) of *E. coli*. A partial amino acid sequence showed that this protein was the phage-shock protein PspA. PspA is known to be induced in *E. coli* cells under extreme stress conditions. The results suggest that *E. coli* cells are subject to strong stress in the presence of organic solvents. Introduction of a multi-copy plasmid vector carrying the *psp* operon into *E. coli* improved the survival frequency of cells exposed suddenly to n-hexane but not the growth rate of cells growing in the presence of n-hexane.

Keywords: *Escherichia coli*, organic solvent, phage shock protein A, *psp* operon

INTRODUCTION

In recent years, there has been increasing interest in culturing micro-organisms in two-phase liquid systems composed of an organic solvent and aqueous medium. This approach may provide a convenient means of bioconversion of hydrophobic organic compounds (de Smet *et al.*, 1983; Harbron *et al.*, 1986; Favre-Bulle *et al.*, 1991; Van Sonsbeck *et al.*, 1993; Aono *et al.*, 1994a; Doukyu & Aono, 1997).

In the case of micro-organisms, the toxicity of an organic solvent is correlated inversely with $\log P_{ow}$, when examined on the basis of microbial growth on an agar medium overlaid with organic solvent (Inoue & Horikoshi, 1989). Here, $\log P_{ow}$ is defined as the common logarithm of the partition coefficient (P_{ow}) of the organic solvent between n-octanol and water layers (Leo, 1993). Each bacterium has its own intrinsic tolerance level for organic solvents. This level is determined genetically although it is also affected by environmental factors (Aono *et al.*, 1991, 1994c, d; Nakajima *et al.*, 1995a, b; Asako *et al.*, 1997). The tolerance level of each micro-organism is represented by two terms: the index solvent and the index value. The index solvent is the most toxic organic solvent among those that can be tolerated by the organism. The index value is the $\log P_{ow}$ value of the

index solvent. Each bacterium can grow on agar medium overlaid with any one of the organic solvents having a $\log P_{ow}$ value greater than or equal to the index value (Inoue & Horikoshi, 1989; Aono *et al.*, 1994b).

This empirical rule derived from observations of microbial growth on agar media implies that each organism would also be able to grow in a two-phase liquid system consisting of a nutrient medium and an organic solvent with $\log P_{ow}$ greater than the index value. However, under such conditions growth of bacteria is suppressed by organic solvents having $\log P_{ow}$ values near the index value (Aono *et al.*, 1994b). We found that *Escherichia coli* grown in the presence of n-hexane ($\log P_{ow}$ 3.9) or cyclooctane ($\log P_{ow}$ 4.5) became increasingly susceptible to the solvent during the course of cultivation. Micro-organisms seem to be subject to strong stress in the presence of such harmful organic solvents.

In this paper we show that a 28 kDa protein appears in the inner membrane of *E. coli* cells growing in the presence of a harmful organic solvent. This protein was identified as phage shock protein A (PspA), a stress-response protein (Brissette *et al.*, 1991; Weiner *et al.*, 1991; Kleerebezem & Tommassen, 1993; Bergler *et al.*, 1994), which is induced in *E. coli* cells under various conditions including treatment with a water-miscible organic solvent, ethanol. This is the first report to show that hydrophobic water-immiscible organic solvents can trigger the induction of PspA.

Abbreviations: PSP, phage shock protein; P_{ow} , partition coefficient.

METHODS

Bacterial strains, phage and plasmids. The n-hexane-tolerant strain *E. coli* K-12 JA300 (F⁻ *thr leuB6 trpC1117 thi rpsL20* [Str^r] *hdsS*) (Kingsman *et al.*, 1979) was the main strain used in this study. *E. coli* K-12 LE392 (*supE44 supF58 galK2 metB1 trpR55 lacY1*) (Murray *et al.*, 1977) and *E. coli* K-12 JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ[lac proAB]/F'[traD36 proAB⁺ lacI^a lacZM15]*) (Yanisch-Perron & Messing, 1985) were used for phage stock and plasmid construction. Kohara's recombinant phage λ1C2 (Kohara *et al.*, 1987) was used as a donor of the *psp* operon. Plasmid pUC119 was used as a cloning vector. The recombinant plasmid (pPSP3) carrying the *psp* operon was constructed in this study.

Culture media. *E. coli* JA300 was grown at 37 °C in LBGMg medium, consisting of 10 g Bacto Tryptone (Difco), 5 g Bacto yeast extract (Difco), 10 g NaCl, 1 g glucose and 10 mmol MgSO₄ l⁻¹ (Aono *et al.*, 1991). In some experiments, the organism was also grown in LBG medium (LBGMg medium without MgSO₄). When necessary, the media were solidified with 1.5% (w/v) agar. NZYM medium, consisting of 10 g NZ amine (Difco), 5 g yeast extract (Difco), 5 g NaCl and 2 g MgSO₄·7H₂O l⁻¹ (Luria & Burrous, 1957), was used for phage propagation. When necessary, ampicillin (50 μg ml⁻¹) was added to the media.

Preparation of inner-membrane protein fraction. The organism was grown in LBGMg medium. The cells were harvested by centrifugation (3500 g, 10 min, 4 °C), washed with cold 10 mM Na₂HPO₄/NaOH buffer (pH 7.0), then suspended in the buffer and broken by sonication in an ice-water bath. Unbroken cells were removed by centrifugation (3500 g, 10 min, 4 °C), the envelope fraction was precipitated by further centrifugation (100000 g, 45 min, 4 °C), and the pellet was washed once with the cold buffer. This envelope was incubated in the phosphate buffer containing 0.5% sodium N-lauroyl sarcosine (Sarkosyl) for 30 min at room temperature at a protein concentration of 3 mg ml⁻¹ (Camille *et al.*, 1973). The suspension was centrifuged at 100000 g for 45 min at 15 °C. The supernatant was used as the inner-membrane protein fraction. The pellet was washed twice with the Sarkosyl buffer and used as the outer-membrane protein fraction.

SDS-PAGE of proteins. Samples were dissolved in 1% SDS, 2.5% (v/v) β-mercaptoethanol, 20% (w/v) sucrose and 16 mM Tris/HCl buffer (pH 6.8), and heated for 5 min in a boiling bath. The samples were electrophoresed on a 0.1% SDS/15% (w/v) polyacrylamide gel by the Laemmli method (Laemmli, 1970). After electrophoresis, the protein in the gel was stained with Coomassie brilliant blue R-250.

Protein content. This was measured by the Lowry method.

N-terminal amino acid sequence. This was determined by Edman degradation using an Applied Biosystems protein sequencer 473A.

DNA manipulation. DNA manipulations, including preparation of plasmid DNA, restriction enzyme digestion, ligation, and transformation of *E. coli*, were carried out by standard methods. DNA from recombinant phage λ1C2 was isolated by using the QIAGEN Lambda kit with *E. coli* JM109 as a host.

Materials. The cyclooctane and n-hexane used were of the highest quality available; they were purchased from Wako Pure Chemical Industries. The log *P*_{ow} values of organic solvents were calculated by the addition rule (Leo, 1993) using the log *P*_{ow} calculation software, ClogP version 1.0.3 (Bio Byte Corporation).

RESULTS

Growth of *E. coli* JA300 in the presence of harmful organic solvents

We have previously examined the toxicity of organic solvents by observing microbial colony formation on agar medium overlaid with the organic solvent. For *E. coli* JA300, the index solvent is n-hexane (log *P*_{ow} 3.9) (Aono *et al.*, 1991). When an organic solvent with a log *P*_{ow} value much greater than the index value, such as dodecane (log *P*_{ow} 7.0), decane (log *P*_{ow} 6.0), or isooctane (log *P*_{ow} 4.8), was added suddenly to JA300 cells growing in LBGMg medium, growth was not inhibited for at least 20 h, as indicated by monitoring the turbidity and the number of viable cells. Fig. 1 shows the growth of JA300 after addition of isooctane. There was no evidence of a phase of adaptation to these organic solvents added suddenly.

In contrast, addition of n-hexane interrupted growth severely and caused a rapid decrease in the number of

Fig. 1. Effects of organic solvents with log *P*_{ow} near the index value on growth of *E. coli* JA300. The strain was grown in LBGMg medium aerobically at 37 °C. To the culture (OD₆₆₀ 0.1), a 10% volume of isooctane (log *P*_{ow} 4.8; ●), cyclooctane (log *P*_{ow} 4.5; ▲) or n-hexane (log *P*_{ow} 3.9; ■) was added at the time indicated by the arrow. Growth was followed by measuring culture turbidity at 660 nm (a) and viable cells (b). ○, No organic solvent added. Graphs show typical results obtained from three independent experiments.

viable cells, as described previously (Aono *et al.*, 1994b). Immediately after the addition, the colony-forming ability of most cells was lost (Fig. 1). The cell survival frequency was 10–20% at 0.5 h after the addition of n-hexane. The survivors grew for 2 h in LBGMg medium overlaid with n-hexane at a growth rate (doubling time 25 min) similar to that found in the absence of n-hexane. Thereafter, the growth rate was retarded (doubling time 3 h). The culture turbidity increased constantly although the rate of increase was suppressed by n-hexane. The frequency of survivors found after the addition of n-hexane was sufficiently high to conclude that the survivors were not n-hexane-tolerant mutants which might appear at a low frequency. It is obvious that JA300 cells grew via physiological adaptation to an environment 'polluted' with n-hexane.

JA300 continued to grow for a while after the addition of cyclooctane ($\log P_{ow}$ 4.5) or diphenyl ether ($\log P_{ow}$ 4.2), although the growth rate was lowered slightly by each organic solvent. Fig. 1 shows the growth profile after the addition of cyclooctane. The number of viable cells began to decrease gradually 3 h after the addition of cyclooctane although the turbidity continued to increase at a rate lowered slightly by isooctane. These results indicated that an organic solvent having a $\log P_{ow}$ value which was 0.6 greater than the index value was sufficiently harmful to suppress the growth of JA300 in the late exponential phase of growth.

Appearance of a 28 kDa inner-membrane-associated protein in *E. coli* cells growing in the presence of organic solvents

We assumed that some alteration adapted to the environment containing organic solvents was caused in the membrane. Envelope fractions were prepared from *E. coli* JA300 grown in the presence of the organic solvents for 2 h. The envelope proteins were fractionated by solubilization with Sarkosyl and examined by SDS-PAGE (Fig. 2). A protein band corresponding to a molecular mass of 28 kDa was found in the Sarkosyl-soluble protein fraction prepared from the cells grown in the presence of n-hexane. This protein band was not found in the samples prepared from the cells growing without n-hexane. The Sarkosyl-soluble protein fraction is mainly inner-membrane protein (Camille *et al.*, 1973). There was no difference in Sarkosyl-insoluble envelope protein fractions, mainly containing outer-membrane proteins. Thus, the 28 kDa protein was assumed to be derived from the inner membrane, although the cytoplasmic protein was not examined.

E. coli JA300 was grown in medium overlaid with cyclooctane to observe whether the 28 kDa protein would appear after prolonged incubation in the presence of this solvent. Growth in the presence of organic solvent has been shown to be dependent on the culture conditions, such as nutrients, concentrations of alkaline earth ions and air supply (Aono *et al.*, 1994b; Doukyu & Aono, 1997; Noguchi *et al.*, 1997). Fig. 3(a) shows a

Fig. 2. Appearance of a 28 kDa protein in *E. coli* JA300 cells exposed to organic solvent. JA300 was grown in the presence of organic solvent as shown in Fig. 1. The envelope fraction was prepared from the cells recovered 2 h after the addition of organic solvent. The envelope fraction, containing 30 μ g protein, was extracted with 0.5% Sarkosyl at room temperature. Sarkosyl-insoluble (a) or -soluble (b) protein was electrophoresed on an SDS-15% (w/v) polyacrylamide gel. Protein was stained with Coomassie brilliant blue R-250. Lanes: M, molecular mass markers; 1, without organic solvent; 2, cyclooctane; 3, n-hexane. The arrow indicates the 28 kDa protein.

typical growth curve. The cells grew to 2×10^9 ml⁻¹ at 7 h. Thereafter, the number of viable cells decreased gradually and had fallen to 5×10^8 ml⁻¹ at 16 h. Inner-membrane protein fractions prepared from the cells were analysed by SDS-PAGE (Fig. 3b). A significant amount of the 28 kDa protein appeared in the inner membrane of the cells grown in the presence of cyclooctane after 6 h. At this time, the growth was retarded by cyclooctane. This protein did not appear within 16 h when the organism was grown without organic solvent (Fig. 3c).

The 28 kDa protein was induced in the cells when growth was retarded by cyclooctane (Fig. 3a, b). This was the case also for its induction in the presence of n-hexane. It is likely that this protein appeared in the inner membrane upon disturbance of membrane structure by

Fig. 3. Appearance of PspA in *E. coli* JA300 growing in the presence of cyclooctane. JA300 was grown in LBGMg medium overlaid with 10% (v/v) cyclooctane (●) or without added solvent (○). The number of viable cells in the medium layer was periodically examined (a). The cells were harvested from the culture with cyclooctane (b) or without added solvent (c) at the times indicated at the top of each gel. Sarkosyl-soluble protein obtained from the whole-envelope fraction, containing 30 µg protein, was electrophoresed on an SDS-polyacrylamide gel. Protein was stained with Coomassie brilliant blue R-250. The 28 kDa protein is indicated by the arrow. M, molecular mass markers.

the organic solvents. The protein remained in the inner membrane stably for a long period.

Identification of the 28 kDa inner-membrane protein as PspA

E. coli JA300 was grown in medium overlaid with a 10% volume of cyclooctane for 20 h. The Sarkosyl-soluble membrane-protein fraction obtained from the cells was separated by SDS-PAGE. The 28 kDa protein was recovered from the gel, and the 19 N-terminal amino acid residues were sequenced. The sequence was

H₂N-A/GIFXXFADIVNANINALLE-. The N-terminal amino acid was Ala or Gly. The fourth and fifth residues were not clearly identified. This determined sequence was consistent with the sequence, H₂N-MGIFSRFADIVNANINALLE-, reported for PspA (Brissette *et al.*, 1991). The N-terminal Met residue was not found in the 28 kDa protein recovered from strain JA300. PspA is a 28 kDa peripheral inner-membrane protein of *E. coli*, inducible by various treatments that exert stress. A portion of PspA is present in the cytoplasmic protein fraction. PspA and the 28 kDa protein induced in the presence of organic solvents are identical to each other in location, molecular mass and N-terminal amino acid sequence. Thus, the 28 kDa protein was identified as PspA.

Construction of plasmid pPSP3 carrying the *psp* operon

Kohara's recombinant phage λ1C2 contains the *psp* operon, which includes the *pspA-E* genes (Kohara *et al.*, 1987; Brissette *et al.*, 1991). DNA from this phage was cleaved by *Eco*RI. A 4.5 kb *Eco*RI fragment containing the *psp* operon was inserted into an *Eco*RI site of vector plasmid pUC119. From the resulting recombinant plasmid (pPSP1), a 2.7 kb *Bgl*II-*Eco*RI fragment was subcloned into pUC119. This fragment carries *pspA-E* but not *pspF* (Bergler *et al.*, 1994). Genes *pspA-E* constitute one of the two divergent transcription units of the *psp* operon. The recombinant plasmid was designated pPSP3.

Expression of *pspA* in *E. coli* JA300(pPSP3)

PspA is one of the stress response proteins. Expression of the *psp* operon depends on σ^{54} and IHF (integration host factor), and is regulated positively and negatively by the operon genes (Weiner *et al.*, 1991). In recombinant plasmid pPSP3, the operon is inserted downstream of a *lac* promoter derived from the vector. The PspF-binding sites were deleted and the IHF-binding site was truncated in the cloned DNA. Therefore, the σ^{54} -dependent promoter is unlikely to be functional.

We examined whether the *psp* operon genes carried on the plasmid were expressed in *E. coli* JA300(pPSP3) under non-stress conditions (Fig. 4). A 28 kDa protein band corresponding to PspA was found in the inner-membrane protein fraction prepared from JA300(pPSP3), indicating that the plasmid-borne PspA was produced in JA300 cells not exposed to organic solvent. The expression of *pspA* in JA300 cells did not require addition of a *lac* inducer, such as IPTG. However, the amount of PspA expressed was less than that found in JA300 cells growing in the presence of n-hexane or cyclooctane (Figs 2 and 3). Although pPSP3 contains several *psp* genes, *pspA*, *B*, *C*, *D* and *E*, we did not detect any products of this operon other than PspA in the membrane fraction by protein staining. Nor were such products detected for JA300 cells treated with n-hexane or cyclooctane (Figs 2 and 3).

Fig. 4. Expression of PspA in *E. coli* JA300(pPSP3). JA300 transformed with pUC119 or pPSP3 was grown in LBGMg. Cells in the mid-exponential phase of growth were harvested from the culture. The whole-envelope fraction, containing 30 µg protein, was electrophoresed on an SDS-polyacrylamide gel. Protein was stained with Coomassie brilliant blue R-250. The arrow indicates the position of PspA. Lanes: 1, JA300(pUC119); 2, JA300(pPSP3); 3, JA300(pPSP3) treated with 10 mM IPTG for 4 h; M, molecular mass markers.

Effect of the *psp* operon on the organic solvent tolerance of *E. coli* JA300

To examine whether the *psp* operon was involved in determination of the organic solvent tolerance level of *E. coli*, n-hexane was added to growing JA300(pPSP3) cells (Fig. 5). Alkaline earth cations have been shown to enhance the stability of *E. coli* cells and to elevate organic solvent tolerance levels (Aono *et al.*, 1994b). Therefore, JA300(pPSP3) was grown in medium containing or not containing 10 mM Mg^{2+} . Growth of JA300 and JA300(pPSP3) was inhibited by the addition of n-hexane. Growth rates of the survivors were almost identical among the cultures. Therefore, it was concluded that overproduction of PspA did not enhance the growth rate of JA300 in the presence of n-hexane.

On the other hand, the cell survival frequency after the addition of n-hexane differed from that observed with Mg^{2+} supplementation and transformation with the *psp* operon. When JA300 cells carrying or not carrying pPSP3 were grown in LBGMg medium, the survival frequencies were similar. However, the survival frequency of JA300(pPSP3) was higher than that of JA300 when these cells were grown without a high concentration of Mg^{2+} . Overproduction of PspA probably

Fig. 5. Effect of the *psp* operon on growth of *E. coli* JA300 after addition of n-hexane. JA300 (○, ●) and JA300(pPSP3) (□, ■) were grown in LBG (open symbols) or LBGMg (solid symbols) medium. n-Hexane (10% volume) was added to the growing cells at 0 h (solid lines). JA300(pPSP3) was grown without n-hexane as a control (broken lines). The number of viable cells was examined periodically in each culture after the addition of n-hexane. The data shown are the mean values for two experiments.

contributes to improvement of the organic solvent tolerance level, as indicated by the enhanced frequency of cell survival after the addition of n-hexane. The *E. coli* inner membrane might be stabilized by PspA, although the effect was much less than that obtained with 10 mM Mg^{2+} . However, it is possible that this stabilization was due to products of genes other than *pspA*.

DISCUSSION

PspA was first discovered in *E. coli* cells infected with filamentous phage (Brissette *et al.*, 1990). *pspA* is known to be expressed in *E. coli* cells under various stress conditions, such as heat shock at 50 °C, hyper-osmotic shock at 0.75 M NaCl, 10% (v/v) ethanol treatment, inhibition of protein secretion with globomycin, inhibition of fatty acid synthesis with diazaborine, inhibition of ATP synthesis with carbonyl cyanide *m*-chlorophenylhydrazine, or prolonged incubation in the stationary phase of growth (Brissette *et al.*, 1990; Kleerebezem & Tommassen, 1993; Weiner & Model, 1994). It is not clear how these stimuli cause induction of the *psp* operon (Model *et al.*, 1997), but these treatments are all likely to injure the cell membrane.

Hydrophobic organic solvent molecules intercalate into biological membranes (Sikkema *et al.*, 1994). As a result, lipid interaction is weakened and the membrane struc-

ture is disturbed. Previously, we found that inner and outer membranes of *E. coli* JA300 were often detached from each other when the cells were grown in the presence of n-hexane. The periplasmic space was expanded and filled with ribosome-like particles (Aono *et al.*, 1994b). This observation suggests that the inner membrane is damaged structurally upon exposure to n-hexane. We surmised that some adaptive feature might be seen in such damaged membranes.

PspA appeared in the inner membrane of JA300 2 h after the addition of n-hexane, or after 6 h in the presence of cyclooctane. At these times, the organism was in the retardation phase of growth due to growth suppression by the organic solvent. It seems that JA300 can grow under stress conditions and the period until appearance of the PspA protein depends on the toxicity of the organic solvent, as estimated on the basis of $\log P_{ow}$ value. The inner membrane was probably already damaged by the organic solvent at the time when PspA appeared, although its appearance in the cytoplasm was not examined.

PspA overproduced before the shock addition of n-hexane failed to improve the growth rate of JA300 (*psp*⁺) in the presence of n-hexane (Fig. 5). However, overproduction of PspA led to improved survival of JA300 growing in LBG medium exposed to the bactericidal action of n-hexane. Recently, it was reported that PspA plays a role in maintenance of the proton-motive force under stress conditions (Kleerebezem *et al.*, 1996). The organic solvent tolerance is dependent on the respiratory activity (Noguchi *et al.*, 1997). The unusual expansion of the periplasm seems to lower the periplasmic concentration of protons. PspA might be involved in adaptation to an environment polluted with organic solvents by contributing to improvement of the energy production process when the inner membrane has been disturbed by an organic solvent. To confirm this possibility, we need to do further experiments using a *pspA*-defective mutant and a recombinant plasmid containing only *pspA*.

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