Release of penicillinase by *Escherichia coli* HB101 (pEAP31) accompanying the simultaneous release of outer-membrane components by Kil peptide

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The plasmid pEAP31 contains an alkaliphilic-*Bacillus* penicillinase gene and a colicin E1 *kil* gene. *Escherichia coli* HB101 carrying pEAP31 grown at high temperature released outer-membrane proteins, lipopolysaccharide and phosphatidylethanolamine into the culture medium. Concurrently, penicillinase that had accumulated in the periplasm of the organism was released from the cells. Phospholipase A₁–A₂ in the outer membrane was not activated in the organism. The results suggest that the release of accumulated periplasmic penicillinase from the producer cells was caused by partial disruption of the outer membrane mediated by the Kil peptide.

INTRODUCTION

The penicillinase of alkaliphilic *Bacillus cereus* no. 170 (F.E.R.M. 3221) was cloned into *Escherichia coli* HB101 with pMB9 as a vector. The enzyme produced by *E. coli* HB101 carrying the plasmid pEAP31 accumulates in the periplasm and then is released from the cells, according to the culture conditions. This extracellular production of the penicillinase is concluded to be caused by activation of a dormant *kil* gene on the plasmid pMB9 by a cloned DNA fragment (Kudo *et al.*, 1983; Kato *et al.*, 1983, 1986; Kobayashi *et al.*, 1986; Aono, 1988a, 1989). The *kil* gene is responsible for the release of colicin E1 from its producer cells (Sabik *et al.*, 1983; Suit *et al.*, 1983; Zhang *et al.*, 1985). In fact, a mature product of the *kil* gene is found in the envelope of *E. coli* HB101 cells carrying the plasmid pEAP 31 and releasing the penicillinase (Aono, 1988b).

Genes that encode peptides showing function similar to that of the Kil peptide are known for a number of colicin plasmids. It is proposed that dormant phospholipase A₁–A₂ in the outer membrane is activated by these peptides and hydrolyses phosphatidylethanolamine (PE) to produce lysophosphatidylethanolamine (LPE). LPE accumulates in the outer membrane and consequently makes the membrane permeable for proteins, including colicins (Hakkaart *et al.*, 1981; Watson & Vinsentin, 1982; Pugsley & Schwartz, 1983, 1984). However, there is no direct evidence as to whether the penicillinase permeates through an outer membrane that is enriched in LPE.

The present paper reports that *E. coli* HB101 (pEAP31) released several components of the outer membrane, i.e. proteins such as OmpA protein and the major lipoprotein, lipopolysaccharide (LPS) and PE, and that LPE does not accumulate in the organism. The outer membrane is altered in its composition owing to the release of the components induced by the Kil peptide without activation of the phospholipase A₁–A₂. The penicillinase

may be released from the cells because of this partial disruption of the outer membrane following the release of some of its components.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

E. coli HB101 [*hsdS20* (*hsdR hsdM*) *recA13 proA2 leuB leuB1 ara14 lacY1 galK2 xyl-5 mtl-1 supE44 endA rpsL20* (*str^r*) F[–]] was used. Plasmids used were pEAP4 and pEAP31, containing the penicillinase gene from alkaliphilic *Bacillus cereus* no. 170. The *kil* gene is active in pEAP31 but inactive in pEAP4 (Kato *et al.*, 1986; Kobayashi *et al.*, 1986).

The culture medium used was LG broth containing 10 g of Bacto tryptone (Difco Laboratories, Detroit, MI, U.S.A.), 5 g of Bacto yeast extract (Difco Laboratories), 2 g of glycerol, 1 g of glucose and 10 g of NaCl per litre of deionized water. The organism was cultured on a reciprocal shaker (Aono, 1988a).

Determination of LPS

Concentrations of LPS released into the culture broth were determined by a colorimetric Limulus test with the Pyrodict kit (Seikagaki Kogyo, Tokyo, Japan). The kit contains a *p*-nitroanilide derivative as a chromogenic substrate (Obayashi, 1984).

An envelope fraction was prepared from *E. coli* HB101 grown at 30 °C (Aono, 1988b). The fraction was extracted twice with methanol/chloroform/2% (w/v) NaCl (10:5:4, by vol.) and then with warm phenol/water (4:5, v/v) (Westphal & Jann, 1965). LPS was sedimented by centrifugation (120 000 *g* for 100 min at 4 °C) from the aqueous layer after it had been dialysed against running water. The LPS preparation was treated with ribonuclease A, washed several times by centrifugation and used as a reference for the LPS determination.

Abbreviations used: LPS, lipopolysaccharide; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine.

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Polyacrylamide-gel electrophoresis of LPS

A culture supernatant was mixed with 5 vol. of cold ethanol and held at -20°C overnight. The precipitate was collected by centrifugation at 12000 *g* for 15 min and then dissolved in 1% (w/v) SDS/1% (v/v) 2-mercaptoethanol/15% (w/v) sucrose/10 mM-sodium phosphate buffer, pH 7.2. Proteins in the sample were denatured by heating in a boiling-water bath for 2 min and thoroughly digested with proteinase K at 37°C overnight. These samples were electrophoresed on a 0.5% SDS/6 M-urea/12% (w/v) polyacrylamide gel (Aono, 1988b). LPS in the gel was oxidized with periodic acid and was detected by silver staining (Hitchcock & Brown, 1983).

Extraction and analysis of lipids

The culture was centrifuged at 7000 *g* for 10 min at 4°C . Lipids in the spent medium were extracted by the method of Bligh & Dyer (Rouser & Fleischer, 1967). The intact cells recovered from the culture were immediately suspended in 1% (w/v) NaCl. The suspension was extracted as described above.

The lipids were analysed by silica-gel t.l.c. in chloroform/methanol/acetic acid (65:25:8, by vol.) at room temperature. Compounds were located with I_2 vapour, a ninhydrin spray and the Dittmer-Lester reagent for phosphorus compounds (Rouser & Fleischer, 1967).

Antiserum against extracellular proteins from *E. coli* HB101 (pEAP31)

Extracellular proteins were recovered from spent medium of *E. coli* HB101 (pEAP31) grown at 30°C for 38 h as cold-5%-(w/v)-trichloroacetic acid-insoluble materials. The materials were washed with cold acetone and then used as an antigen without further purification.

Subcellular fractionation of bacterial proteins

E. coli HB101 was cultured at 30°C for 14 h. The culture was centrifuged at 7000 *g* for 10 min at 4°C . The supernatant was retained as the extracellular fraction. The pellet of cells was divided into two parts. One was treated with lysozyme in 25% (w/v) sucrose/1 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.5. The resulting spheroplast suspension was centrifuged as described above. The supernatant was retained as the periplasmic fraction. The pellet was washed with the same buffer and sonicated briefly in 10 mM-Tris/HCl buffer, pH 7.5. The sonicated lysate was centrifuged at 100000 *g* for 45 min at 4°C . The supernatant was retained as the cytoplasmic fraction and the pellet was taken as the envelope fraction. Inner-membrane proteins were solubilized from this envelope fraction by treatment with 0.5% sodium *N*-lauroylsarcosinate at room temperature for 30 min (Filip *et al.*, 1973). The outer-membrane fraction was washed with the *N*-lauroylsarcosinate solution by centrifugation.

Table 1. Distribution of enzymes in *E. coli* HB101 carrying plasmids

E. coli HB101 carrying pEAP4 or pEAP31 was grown aerobically at the temperature indicated in the Table for 48 h. The culture (1 ml) was diluted 3-fold with distilled water and sonicated at 25 W (20 kHz) for 1 min in an ice/water bath. The sonicated lysate was analysed as a total fraction. The culture (5 ml) was centrifuged at 7000 *g* for 10 min at 4°C . The supernatant was analysed as an extracellular fraction. Total activity of the penicillinase is expressed as units/ml of the culture. Extracellular activities of penicillinase, alkaline phosphatase and β -galactosidase are expressed as percentages of respective total activities. Concentrations of proteins and LPS are expressed as $\mu\text{g/ml}$ of the culture supernatant. Phospholipid concentration is expressed as nmol of P/ml. Abbreviations: N.D., not detected; –, not tested.

Culture temperature (°C)	<i>A</i> ₆₆₀	Total penicillinase (units/ml)	Released from cells					Phospho-lipid (nmol/ml)
			Penicillinase (%)	Alkaline phosphatase (%)	β-Galact- osidase (%)	Protein (μg/ml)	LPS (μg/ml)	
HB101 (pEAP4)								
20	5.00	116	3	2	N.D.	42	2.0	—
22	4.80	121	12	8	N.D.	—	—	—
26	4.89	129	14	9	2	—	—	—
28	4.47	150	17	8	1	69	2.5	—
30	4.39	103	21	12	2	81	4.0	0.80
32	4.27	104	33	15	4	65	6.3	—
36	3.31	50	17	15	5	67	8.0	—
38	2.96	< 1	—	19	1	76	3.6	—
HB101 (pEAP31)								
20	5.74	174	1	2	N.D.	36	0.6	—
22	5.54	181	2	2	N.D.	—	—	0.78
24	5.71	206	5	4	2	51	1.6	—
26	5.60	206	17	11	N.D.	—	—	—
28	4.40	183	66	42	1	186	15.8	—
30	4.36	155	81	55	14	339	20.6	6.4
32	2.27	148	95	85	26	468	27.1	—
36	2.26	102	94	73	37	470	20.3	—
38	3.01	88	75	66	11	250	11.8	—
40	3.22	55	85	67	7	273	18.6	—
43	2.62	< 1	—	43	N.D.	44	14.3	—

The other *E. coli* HB101 cells from the culture were broken by sonication (Aono, 1988b). Soluble and envelope fractions were prepared by centrifugation. Inner-membrane and outer-membrane fractions were prepared with the use of *N*-lauroylsarcosinate as described above.

Assay of enzymes

Penicillinase was assayed by the iodometric method in the presence of 0.3 mM-ZnSO₄, with benzylpenicillin as a substrate. One unit of the enzyme hydrolyses 1 μ mol of benzylpenicillin/min at 30 °C (Aono, 1988b). Alkaline phosphatase was assayed with 4-nitrophenyl phosphate at room temperature (Kreuzer *et al.*, 1975). β -Galactosidase was assayed with 2-nitrophenyl β -D-galactopyranoside at room temperature (Miller, 1972). Activities of these two enzymes were measured from changes in absorbance at 420 nm.

Miscellaneous determinations

The concentration of protein released from the cells into the culture broth was determined by the method of Bradford (1976), with bovine serum albumin as reference standard. Protein in the other samples was determined by the method of Lowry *et al.* (1951), also with bovine serum albumin as reference standard. Phosphorus content was determined by the molybdate method after samples were washed with Mg(NO₃)₂ (Ames, 1966).

Materials

Proteinase K was a product of E. Merck. Low-*M_r* markers were CNBr-cleavage fragments of myoglobin obtained from BDH Chemicals. High-*M_r* markers were obtained from Boehringer Mannheim. A silver staining kit was purchased from Daiichi Pure Chemicals, Tokyo, Japan.

RESULTS

Release of LPS from cells

The content of LPS released from *E. coli* HB101 carrying pEAP4 or pEAP31 into the spent medium was determined (Table 1). *E. coli* HB101 (pEAP31) released a considerable amount of LPS into the culture medium when the organism was grown at over 28 °C, but the organism grown at low temperature released a smaller amount of LPS. On the other hand, *E. coli* HB101 (pEAP4), in which the *kil* gene product is not expressed, released only a small amount of LPS, irrespective of the culture temperature. These results indicated that the *kil* gene was involved in the release not only of the penicillinase but also of LPS from the cells.

The amount of extracellular LPS was periodically measured. The released LPS increased suddenly and rapidly after 24 h when *E. coli* HB101 (pEAP31) was grown at 30 °C (Fig. 1). The extracellular LPS did not increase rapidly after 24 h when grown at 22 °C, although it increased slightly after 38 h. Also, such a rapid release of LPS was not found in cultures of *E. coli* HB101 or *E. coli* HB101 (pEAP4) grown at 30 °C, as suggested in Table 1 (results not shown).

The extracellular LPS released from *E. coli* HB101 (pEAP31) was periodically analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2). Three bands of LPS were detected with silver staining in all the samples. Migration rates of the respective bands were identical in all the samples. No detectable change in electrophoretic

Fig 1. Release of LPS and phospholipids during culture of *E. coli* HB101 (pEAP31)

E. coli HB101 (pEAP31) was grown in 50 ml of LG broth containing 2 mg of ampicillin at 22 °C for 24 h. The culture was diluted 100-fold with fresh LG broth. The subculture was grown aerobically at 22 °C (○ and △) or at 30 °C (● and ▲). Samples (7 ml) of the culture broth were periodically withdrawn from the culture. The culture broth was centrifuged at 7000 *g* for 10 min at 4 °C. The concentration of LPS in the culture supernatant was assayed by the colorimetric Limulus test (○ and ●). An LPS reference was prepared from the host cells. The culture supernatant (1.6 ml) was extracted by the method of Bligh & Dyer. The extract was washed with distilled water and dried with a stream of N₂. Phosphorus content was determined by the molybdate method (△ and ▲).

mobility of LPS derived from *E. coli* HB101 (pEA231) (both cell-associated and extracellular material) and from *E. coli* HB101 (pEAP4) was found, although small changes in LPS cannot be ruled out. These electrophoretic analyses suggested that the LPS molecules were released from the cells without alteration in their sizes.

Release of PE

Phospholipids in the spent medium of *E. coli* HB101 carrying pEAP4 or pEAP31 were extracted with chloroform (Table 1). The content of phospholipids in the spent medium was found to be significant for *E. coli* HB101 (pEAP31) grown at 30 °C, but was low in the other samples (Table 1). The release of the phospholipids from *E. coli* HB101 (pEAP31) grown at 30 °C occurred after 24 h, concurrently with the release of LPS (Fig. 1). These results suggested that the mature Kil peptide was also involved in the release of phospholipids.

The extracellular phospholipids were analysed by silica-gel t.l.c. (Fig. 3). A major phosphorus-positive spot, whose *R_F* value was 0.41, was commonly found in each extract. This spot was also I₂-positive and ninhydrin-positive. The *R_F* value of the spot corresponded to that of authentic PE prepared from the host cells but not to that of LPE. Therefore the major phospholipid was identified as PE. A large spot near the solvent front

Fig. 2. SDS/polyacrylamide-gel electrophoresis of LPS released during culture of *E. coli* HB101 (pEAP31)

E. coli HB101 (pEAP31) was grown at 30 °C as described in the legend to Fig. 1. LPS in the supernatant was sedimented in cold 80% (v/v) ethanol. Proteins in the precipitate were denatured by heating in 1% (w/v) SDS/1% (v/v) 2-mercaptoethanol and digested with proteinase K. Samples from 10 μ l of the supernatant were electrophoresed on a 0.5% SDS/6 M-urea/12% (w/v) polyacrylamide gel. LPS was oxidized with periodic acid and detected with silver staining. The numbers at the top of the gel represent the times at which the supernatant was obtained. The number with a prime represents the sample from the culture at 22 °C. 'S' represents LPS (90 ng) from the host cells.

contained no phosphorus and was probably free fatty acids charred with H_2SO_4 .

The phospholipids released from *E. coli* HB101 (pEAP31) grown at 30 °C were separated by silica-gel t.l.c. Phosphorus in the I_2 -positive spots was determined. The contents of PE and phosphatidylglycerol were respectively 97% and 3% of the phospholipids in the spent medium. Only trace amounts of cardiolipin or LPE were detected in the spent medium.

Phospholipids in intact cells

Lipids in the cells of *E. coli* HB101 either carrying or not carrying pEAP31 were periodically analysed by silica-gel t.l.c. (Fig. 4). If the activation of the phospholipase A_1 - A_2 had been essential for release of the penicillinase, more amounts of LPE and fatty acids would have accumulated in *E. coli* HB101 (pEAP31) cells grown at 30 °C than in *E. coli* HB101 (pEAP31) grown at 22 °C or in the host cells grown at 30 °C. The amount of LPE was slight in all of the samples. The Kil peptide did not cause LPE to accumulate in the membrane of *E. coli* HB101 (pEAP31). LPE was not released from the organism, as described above. The ratio of free fatty acid content to phospholipid content increased at the late stationary phase in each culture. The ratio increased earlier at 30 °C than at 22 °C, i.e. fatty acids accumulated after 13 h when grown at 30 °C but not even after 24 h at 22 °C. However, the earlier accumulation of fatty acids at 30 °C was not specific for *E. coli* HB101 (pEAP31), and was

Fig. 3. T.l.c. of phospholipids released from *E. coli* HB101 containing or not containing plasmids

The organisms were grown aerobically for 38 h. The chloroform extract of the spent medium was filtered through Whatman 1PS filter paper and concentrated in a rotary evaporator at 35 °C. The sample from 12 ml of the spent medium was analysed by silica-gel t.l.c. in chloroform/methanol/acetic acid (65:25:8, by vol.). Compounds were located with Dittmer-Lester reagent. Standards used were prepared from an envelope fraction of *E. coli* HB101, in which phospholipase A_1 - A_2 was activated by sonication. LPE was purified by silica-gel t.l.c. and identified by determination of phosphorus, ethanolamine and acyl group. The following observations are used: F, fresh medium; -, spent medium of the host cells; 4, spent medium of *E. coli* HB101 (pEAP4) grown at 30 °C; 31', spent medium of *E. coli* HB101 (pEAP31) grown at 22 °C; 31, spent medium of *E. coli* HB101 (PEAP31) grown at 30 °C.

found also in the host cells. The ratio of fatty acids was slightly higher in *E. coli* HB101 (pEAP31) than in the host cells after 24 h, probably because phospholipid content was decreased after 24 h as a result of the release of PE from *E. coli* HB101 (pEAP31) grown at 30 °C. The accumulation of fatty acids was not directly affected by either the precursor or mature Kil peptide. These results suggested that the phospholipase A_1 - A_2 was not activated in the outer membrane of *E. coli* HB101 (pEAP31).

Extracellular release of proteins

Total and extracellular activities of the penicillinase produced by *E. coli* HB101 carrying either of the two plasmids and grown at various temperatures are listed in Table 1. The penicillinase and several other proteins were efficiently released from *E. coli* HB101 (pEAP31) grown at the higher temperature. *E. coli* HB101 (pEAP4) always released small amounts of proteins, including the penicillinase.

The penicillinase produced by *E. coli* HB101 (pEAP31) grown at 30 °C initially accumulated in the periplasm (Aono, 1989). The intracellular penicillinase was about

Fig. 4. T.l.c. of phospholipids in intact *E. coli* HB101 cells containing or not containing pEAP31

E. coli HB101 or *E. coli* HB101 (pEAP31) cells were grown aerobically at 22 °C or 30 °C. Samples of the culture were periodically withdrawn and centrifuged (7000 *g* for 10 min at 4 °C). The cells were immediately extracted with methanol/chloroform. The chloroform extract was concentrated with a stream of N₂. Samples containing 0.15 µmol of phosphorus were analysed by t.l.c. as described in the legend to Fig. 3. Lipids were located with I₂ vapour.

Fig. 5. Release of proteins, penicillinase, alkaline phosphatase and β -galactosidase during culture of *E. coli* HB101 (pEAP31)

E. coli HB101 (pEAP31) cells were grown at 30 °C as described in the legend to Fig. 1. The culture (1 ml) was diluted 3-fold with distilled water and then sonicated at 25 W (20 kHz) for 1 min in an ice/water bath. The sonicated lysate was analysed for total activity of each

enzyme. The culture (5 ml) was centrifuged at 7000 *g* for 10 min at 4 °C. The supernatant was analysed for protein concentration (○) and extracellular activity of each enzyme. Extracellular alkaline phosphatase (▲) and β -galactosidase (■) are each expressed as percentages of the respective total activities. Total (●) and extracellular (△) penicillinase activities are represented as units/ml.

93 % of total activity at 20 h (Fig. 5). The organism began to release the penicillinase into the culture medium at 24 h. At the same time the amount of extracellular proteins began to increase. About 90 % of the penicillinase was recovered in the culture medium at 44 h. Alkaline phosphatase is one of the periplasmic enzymes in *E. coli*. At 24 h a considerable amount of alkaline phosphatase was released from *E. coli* HB101 (pEAP31) grown at 30 °C (Fig. 5). Release of the enzyme continued during the rapid release of other proteins. At 44 h 63 % of the alkaline phosphatase was recovered in the culture medium. β -Galactosidase, a cytoplasmic enzyme, was released at 32 h. The release of β -galactosidase was less significant at 30 °C. The release of both enzymes was also dependent on the culture temperature (Table 1). The organism grown at 36 °C released 37 % of β -galactosidase at 48 h (Table 1), suggesting that some of the cells were probably lysed at much higher temperatures.

Immunological classification of extracellular proteins released from *E. coli* HB101 (pEAP31)

The proteins of the host cells were subjected to subcellular fractionation, electrophoresed on an SDS/polyacrylamide gel and immunologically detected with antiserum against the extracellular proteins released from *E. coli* HB101 (pEAP31) grown at 30 °C (Fig. 6). The extracellular bacterial antigenic proteins released from

enzyme. The culture (5 ml) was centrifuged at 7000 *g* for 10 min at 4 °C. The supernatant was analysed for protein concentration (○) and extracellular activity of each enzyme. Extracellular alkaline phosphatase (▲) and β -galactosidase (■) are each expressed as percentages of the respective total activities. Total (●) and extracellular (△) penicillinase activities are represented as units/ml.

Fig. 6. Immunological classification of extracellular proteins released from *E. coli* HB101 (pEAP31)

E. coli HB101 cells were cultured at 30 °C for 14 h. The bacterial proteins were fractionated with lysozyme treatment into extracellular (E), periplasmic (P), cytoplasmic (C) and envelope (M) fractions. Inner-membrane (I) and outer-membrane (O) fractions were prepared from the envelope fraction with sodium *N*-lauroylsarcosinate. The bacterial proteins were also fractionated by sonication into soluble (S) and envelope (M') fractions. The envelope fraction was further fractionated into the inner-membrane (I') and outer-membrane (O') fractions with the detergent, as described in the Materials and methods section. (a) Samples containing 40 µg of protein (E, P, C, M, S and M') or samples obtained from the envelope fractions (40 µg of protein; I, O, I' and O') were electrophoresed on a 0.5% SDS/6 M-urea/12% (w/v) polyacrylamide gel. Proteins in the gel were stained with Coomassie Brilliant Blue R250. (b) Samples (20 µg of protein) were electrophoresed on the gel. The gel was washed with 50% (v/v) methanol for 30 min after the electrophoresis. Proteins were blotted on a nitrocellulose filter and were treated with antiserum against the extracellular proteins released from *E. coli* HB101 (pEAP31) grown at 30 °C for 38 h. The proteins that cross-reacted with the extracellular proteins were detected with horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies obtained from a commercial source.

Fig. 7. Number of viable cells and turbidity during culture of *E. coli* HB101 (pEAP31)

E. coli HB101 (pEAP31) cells were cultured as described in the legend to Fig. 1. Cell growth was measured by the absorbance at 660 nm (Δ and ▲) and by a number of the viable cells (○ and ●). The culture was diluted with

E. coli HB101 (pEAP31) could be localized at original locations in the host cells before their release. It was concluded that proteins of apparent M_r 82000, 74000, 65000, 62000, 38000 and 32000 were derived from the periplasm of the host cells, that proteins of apparent M_r 78000 and 21000 were derived from the cytoplasm, that proteins of apparent M_r 3900 and 3400 were derived from the inner membrane, and that proteins of apparent M_r 39000, 19000, 16000, 7200, 6500 and 5500 were derived from the outer membrane. Among these proteins, OmpA protein (M_r 39000) and the major outer-membrane lipoprotein (M_r 5500) could be identified. LPS bands were detected at M_r 2000. These results indicated that *E. coli* HB101 (pEAP31) preferentially released proteins from the outer membrane and the periplasm.

Decline of viable cells and turbidity

The organism could grow well in the LG broth at 22 °C and 30 °C irrespective of the presence of the plasmid (Fig. 7). Turbidity measured at 660 nm and the number of viable cells reached their maxima at 28 h when the organism was grown at 22 °C. The values were almost constant up to 44 h (A_{660} 6.0; 5×10^9 viable cells/ml at 44 h). On the other hand, the values reached their maxima at 16 h when the organism was grown at 30 °C. The number of viable cells rapidly decreased after

sterilized 1% NaCl and plated on L broth agar. Δ and ○ represent values at 22 °C, and ▲ and ● those at 30 °C. As a reference, *E. coli* HB101 (pEAP4) was grown at 30 °C and the absorbance at 660 nm (□) and number of viable cells (■) were monitored.

20 to 28 h. The numbers of viable cells were 2.0×10^9 /ml at 20 h, 5.5×10^8 /ml at 28 h and 2.5×10^8 /ml at 44 h. The turbidity was constant until 24 h and then gradually decreased (A_{660} 5.4 at 24 h and 3.3 at 44 h). The period in which the rapid decrease was found in the number of viable cells corresponded to that during which the LPS was rapidly released from the cells.

DISCUSSION

The outer membrane of *E. coli* is composed of proteins, phospholipids and LPS, and is normally a permeability barrier against various periplasmic proteins and large molecules in the outside of the cells. *E. coli* HB101 (pEAP31) grown at temperatures above 26 °C released several proteins, including the penicillinase (Table 1). Also, LPS and phospholipids were released at higher temperature but not at lower temperatures (Table 1 and Fig. 3). The maturation process of the Kil peptide is dependent on the culture temperature (Aono, 1988b). Moreover, *E. coli* HB101 (pEAP31) grown at 30 °C released the cell components described above concurrently after 24 h (Figs. 1 and 5). The results shown in Figs. 1, 2, 5 and 7 were obtained from the same culture. The time corresponded to that at which the mature Kil peptide appeared in the envelope (Aono, 1988b). These facts suggested that the mature Kil peptide was involved in the release of all the components.

The mature Kil peptide caused the following alterations of *E. coli* HB101 (pEAP31) cells under the optimum conditions for extracellular production of penicillinase. LPS was released from *E. coli* HB101 (pEAP31) without detectable alteration (Fig. 2). The amount of extracellular LPS was about 20% of the LPS bound to *E. coli* HB101 (pEAP31) cells grown at 22 °C on the basis of 2-keto-3-deoxyoctulosonic acid content in its envelope fraction (results not shown). It is not known whether the phospholipids released from the organism were derived from the outer membrane or the inner membrane of the organism. However, the composition of phospholipids found in the spent medium of *E. coli* HB101 (pEAP31) cells grown at 30 °C was similar to that of the outer membrane rather than that of the cytoplasmic membrane (Fig. 3; Mizushima & Yamada, 1975). Immunological classification of the extracellular proteins produced by *E. coli* HB101 (pEAP31) showed that the extracellular proteins included outer-membrane proteins of the host cells (Fig. 6), suggesting that the organism partially released the outer-membrane proteins.

These results indicated that all kinds of components of the outer membrane were released from *E. coli* HB101 (pEAP31), although the release was partial. Also, free fatty acids increased in amount relative to phospholipids (Fig. 4). Two possibilities were considered for the extracellular production of penicillinase. One is that the outer membrane might consequently become more permeable to proteins without accumulation of LPE. Periplasmic proteins could permeate through an outer membrane that was altered in its composition. The other is that the results concerning the release of the components might suggest that the altered membrane was unstable and that a local disruption of the outer membrane had occurred as a result of this instability. Periplasmic proteins could

pass through a hypothetical micro-porous outer membrane caused by such a disruption. The decrease in the number of viable cells might suggest instability of the outer membrane, although it was not clear whether the decrease was caused during culture or the dilution process (Fig. 7).

It is still unclear how the Kil peptide causes the release of components of the outer membrane of *E. coli* HB101 (pEAP31). However, it is established that the dormant phospholipase A_1 - A_2 in the outer membrane was not activated. Structural analysis of the membrane of the organism that was releasing these components might explain a mechanism through which the Kil peptide had its effect. However, the results presented in this paper clearly allow one to conclude that the release of outer-membrane components is responsible for the production of extracellular penicillinase.

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