Envelope alteration of *Escherichia coli* HB101 carrying pEAP31 caused by Kil peptide and its involvement in the extracellular release of periplasmic penicillinase from an alkaliphilic *Bacillus*

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The plasmid pEAP31 contains the colicin E1 *kil* gene. Peptidoglycan and outer-membrane components (lipopolysaccharide, proteins and phosphatidylethanolamine) decreased concurrently in the envelope fraction from *Escherichia coli* HB101 carrying pEAP31 during the stationary phase of growth. At almost the same time, D-alanine residues in peptidoglycan decreased. The Kil peptide is suggested to affect, directly or indirectly, the turnover of peptidoglycan in stationary phase and, as a result, to cause partial exfoliation of the outer membrane. Periplasmic proteins are liberated from *E. coli* HB101 (pEAP31) probably because of the exfoliation of outer membrane.

INTRODUCTION

The plasmid pEAP31 contains the penicillinase gene of alkaliphilic *Bacillus* and the originally dormant *kil* gene derived from a vector, pMB9 (Kato *et al.*, 1983). The penicillinase produced by *Escherichia coli* HB101 (pEAP31) firstly accumulates in the periplasm and then is released from the producer cells into the culture medium as a result of the action of mature Kil peptide (Aono, 1988b, 1989a). Kil peptide is one of the bacteriocin-releasing peptides and is known to be involved in release of the colicin E1 from producer cells (Suit *et al.*, 1983; Zhang *et al.*, 1985). Similar genes for colicins are known as gene *H* in *pCloDF13* (Hakkaart *et al.*, 1981), *celB* in *pColE2* (Pugsley & Schwartz, 1983), *hic* or *lysis* in *pColE3* (Jakes *et al.*, 1984; Watson *et al.*, 1984) and *cal* in *pColA* (Cavard *et al.*, 1985).

The function of the bacteriocin-releasing peptides has been genetically studied. Originally, genes for the peptides are under the control of a tandem SOS promoter-operator (which can be activated with SOS inducers such as mitomycin C). The genes have been cloned under the control of a *lac*, *lpp-lac* or *tac* promoter-operator system (Pugsley & Schwartz, 1983; Altieri *et al.*, 1986; Lührink *et al.*, 1987). The peptides induced with mitomycin C or isopropyl β -D-thiogalactopyranoside in *E. coli* cells in the exponential phase of growth cause activation of outer-membrane phospholipase A, release of colicins from producer cells, so-called 'pseudo-' or 'quasi-lysis' of the cells and death of the cells (Pugsley & Schwartz, 1984).

The *kil* gene on the plasmid pEAP31 is activated by an accidental insertion of a weak promoter region existing on a cloned alkaliphilic *Bacillus* sp. DNA fragment. *E. coli* HB101 (pEAP31) released the penicillinase without a requirement for administration of the promoter-activator, such as SOS inducer or β -galactoside. The penicillinase is slowly released from the organism in the stationary phase of growth, but not in the exponential phase of growth. The physiology of cells in the stationary phase must be different in many aspects from those in

the exponential phase of growth. Therefore the mode by which periplasmic proteins are released from HB101 (pEAP31) may be at least partially different from that found in young cells possessing the strong promoters inducible with the agents.

Previously I observed and documented materials released into the medium from *E. coli* HB101 carrying pEAP31 (Aono, 1989b). Outer-membrane components, i.e., lipopolysaccharide (LPS), phosphatidylethanolamine (PE) and some membrane proteins, are simultaneously released together with periplasmic proteins such as the penicillinase and alkaline phosphatase from HB101 (pEAP31). These results suggest that some alteration of the outer membrane is caused by Kil and is involved in the release of penicillinase. On the other hand, lysophosphatidylethanolamine (LPE) is not found in chloroform/methanol extracts of the organism immediately after harvest. It has been suggested that the phospholipase was not so strongly activated in HB101 (pEAP31) that its product accumulated in the outer membrane, probably because the promoter activity was weak. Moreover, 'quasi-lysis' and death of HB101 (pEAP31) are comparatively weak, probably because the 'quasi-lysis' is caused in the stationary phase of growth (Aono, 1989b).

Therefore the extracellular production of the penicillinase may be mainly due to a still-unclear alteration in the outer membrane of the penicillinase producer without accumulation of LPE. The presumed alteration may be closely related to the release of the outer-membrane components. However, the chemical nature of the presumed alteration has been unclear. Moreover, it is not clear whether the envelope of HB101 (pEAP31) is really altered or not when the envelope constituents and penicillinase are released.

This paper presents analyses of the envelope constituents of *E. coli* HB101 and shows that the outer membrane composition was truly altered by Kil. The content of peptidoglycan was lowered and the composition of peptidoglycan was altered in the stationary-phase cells of *E. coli* HB101 (pEAP31), which began to release the penicillinase.

Abbreviations used: LPS, lipopolysaccharide; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; KDO, '2-keto-3-deoxyoctanic acid' (3-deoxyoctulosonic acid); sarcosyl, sodium dodecyl sarcosinate; PG, phosphatidylglycerol; CL, cardiolipin.

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MATERIALS AND METHODS

Bacterial strains, plasmids and cultivation conditions

E. coli HB101 [*hsdS20 (hsdR hsdM) recA13 proA2 leuB leuBI ara14 lacY1 galK2 xyl-5 mtl-1 supE44 endA rpsL20 (str^r) F⁻*] was used. Plasmids used were pEAP4 and pEAP31, which contain the penicillinase gene from the alkaliphilic bacterium *Bacillus cereus* no. 170. The *kil* gene is active in pEAP31, but inactive in pEAP4, owing to the lack of a promoter region (Kato *et al.*, 1986; Kobayashi *et al.*, 1986). The medium used was LG broth containing 10 g of Bacto tryptone, 5 g of Bacto yeast extract, 2 g of glycerol, 1 g of glucose and 10 g of NaCl per litre of deionized water. *E. coli* HB101 containing pEAP4 or pEAP31 was grown in LG broth containing ampicillin (50 µg/ml) at 30 °C overnight. Host cells were grown without ampicillin. The cultures were diluted 100-fold with fresh LG broth without ampicillin. The subcultures were incubated on a reciprocal shaker at 30 °C (Aono, 1989a).

Preparation of the envelope fraction

Cells were centrifuged (7000 *g*, 10 min, 4 °C) and kept at -20 °C. The envelope fraction was prepared from the frozen cells by sonication, as described previously (Aono, 1988b). The fraction was suspended in 10 mM-Hepes/NaOH buffer, pH 7.4, and kept at -20 °C.

Preparation of an SDS-insoluble wall fraction

The envelope fraction was incubated in the Hepes/NaOH buffer containing 0.5% sodium *N*-dodecanoyl sarcosinate ('sarcosyl') for 30 min at room temperature at a protein concentration of 3 mg/ml (Filip *et al.*, 1973). The suspension was centrifuged at 100000 *g* for 45 min at 15 °C. The sarcosyl-insoluble fraction was subsequently extracted with, sequentially, 0.5% SDS, boiling 1% (w/v) SDS and boiling 1% (w/v) SDS/0.5 M-NaCl. Insoluble matter was washed with 0.1% NaN₃ and water and kept at -20 °C.

Preparation of a peptidoglycan fraction

The wall fraction was treated with trypsin in 30 mM-Tris/HCl, pH 8.0, in the presence of a few drops of toluene at 37 °C overnight. The peptidoglycan fraction was recovered by the centrifugation as described above and washed sequentially with hot 1% (w/v) SDS, 0.1% NaN₃ and water.

Analyses

(1) Protein. Protein was determined by the method of Lowry *et al.* (1951), with BSA as standard. Protein in culture medium was determined by the method of Bradford (1976). SDS/PAGE was carried out in phosphate buffer as previously described (Aono, 1988b).

(2) Amino acids and amino sugars. Samples were hydrolysed in 4 M-HCl in sealed tubes at 105 °C for 15 h. HCl was removed *in vacuo* over NaOH at 42 °C. Hydrolysates were analysed with an automatic-amino-acid analyser. Amino sugar was determined by the Morgan-Elson reaction (Spiro, 1966). D-Alanine was determined with D-amino acid oxidase, L-lactate dehydrogenase and catalase (Grassl & Supp, 1985). N-Terminal amino acid residues were determined by dinitrophenylation, with alanine as the standard (Aono, 1987).

(3) Neutral sugar. Total neutral sugar was estimated by the phenol/H₂SO₄ method and expressed as glucose equivalents (Spiro, 1966). D-Glucose released by hydrolysis of samples with 1 M-HCl at 100 °C for 2 h was assayed with hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer *et al.*, 1974).

(4) Phosphorus. Samples were ashed in the presence of Mg(NO₃)₂ and hydrolysed in 0.5 M-HCl. Phosphorus was determined by the molybdate method (Ames, 1966).

(5) Reducing groups. Reducing groups were determined with ferricyanide using glucosamine as the standard (Park & Johnson, 1949).

(6) Phospholipid. Samples were extracted by the method of Bligh & Dyer (Rouser & Fleischer, 1967). The phospholipid content was estimated on the basis of phosphorus content, assuming that 1 µmol of phosphorus is equivalent to 0.7 mg of phospholipids. Lipids were developed on a silica-gel thin-layer plate in chloroform/methanol/acetic acid (65:25:8, by vol.) at room temperature. Compounds were located with iodine vapour. The iodine-positive area was scraped off. Phosphorus was determined as described above without extraction from the silica powder.

(7) LPS. 3-Deoxyoctulosonic acid [the so-called '2-keto-3-deoxyoctanoic acid (KDO)], which is a peculiar component of LPS, was determined with thiobarbituric acid reagent after weak hydrolysis and periodate oxidation (Weissbach & Hurwitz, 1959). LPS was estimated on the basis of the KDO content by using an LPS preparation from HB101 as a reference (Aono, 1989b). Liberated LPS in the culture broth was determined by a colorimetric *Limulus* test (Aono, 1989b). LPS was subjected to SDS/PAGE and stained as described previously (Aono, 1989b).

(8) Peptidoglycan. Diaminopimelic acid was determined on an amino acid analyser after hydrolysis as described above. Peptidoglycan was determined by assuming that the peptidoglycan of HB101 is composed of *N*-acetylglucosamine, *N*-acetylmuramic acid, alanine, isoglutamine and diaminopimelic acid (1:1:2:1:1 molar ratio) and that 1 µmol of diaminopimelic acid corresponds to 0.92 mg of peptidoglycan.

(9) Glucan. The D-glucose content was enzymically determined as described above. The glucan content in samples was estimated by subtraction of D-glucose content found in LPS from that in the sample.

(10) Dry weight. The dry weight of samples was measured by heating at 45 °C *in vacuo* over NaOH until a constant weight was obtained.

(11) Penicillinase activity. Penicillinase was assayed by the colorimetric method with benzylpenicillin (Aono, 1988b).

Materials

Bacto tryptone and Bacto yeast extract were products of Difco Laboratories, Detroit, MI, U.S.A. Egg-white lysozyme (EC 3.2.1.17), D-amino acid oxidase (EC 1.4.3.2), L-lactate dehydrogenase (EC 1.1.1.27), catalase (EC 1.11.1.6), hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and proteinase K were purchased from Boehringer Mannheim G.m.b.H., Mannheim, Germany. KDO was purchased from Sigma.

RESULTS

Preparation of an envelope fraction from cells producing penicillinase either intracellularly or extracellularly

An envelope fraction was prepared from HB101 (pEAP31) cells at various stages. Table 1 summarizes the intracellular and extracellular penicillinase activities, the content of extracellularly

Table 1. Growth and production of penicillinase when cells were harvested to prepare an envelope fraction

E. coli HB101 carrying or not carrying the plasmid was aerobically grown in the LG broth at 30 °C overnight. HB101 carrying plasmid pEAP4 or pEAP31 was grown in the presence of ampicillin (50 µg/ml). The cultures were diluted 100-fold with fresh LG broth without ampicillin. Subcultures were incubated at 30 °C. An aliquot of the culture was withdrawn and divided into two. One part was sonicated at 30 W (20 kHz) for 1 min. The sonicated lysate was directly analysed for total penicillinase activity. The other portion of culture fluid was centrifuged at 7000 *g* for 10 min at 4 °C. The supernatant fluid was analysed for extracellular materials and activity of the enzyme. Abbreviation: NT, not tested.

Plasmid	Time (h)	<i>A</i> ₆₆₀	Penicillinase activity (units/ml)		Content of extracellular materials		
			Total	Extracellular	Protein (µg/ml)	LPS (µg/ml)	Phospholipid (nmol/ml)
None	4	1.2	NT	NT	3	NT	0.03
	13	4.5	NT	NT	8	0.4	0.36
	23	5.3	NT	NT	34	NT	0.39
	39	5.9	NT	NT	49	1.9	0.91
pEAP4	12	4.3	17	< 1 (3 %)	2	0.6	0.40
	36	5.9	74	5 (7 %)	42	2.1	0.81
pEAP31	5	1.3	3	< 1 (3 %)	11	0.8	0.07
	14	4.6	54	4 (7 %)	24	5.9	0.78
	23	5.2	73	5 (7 %)	34	3.7	0.94
	29	5.1	119	63 (53 %)	167	18.8	2.82
	39	4.0	124	117 (95 %)	293	21.6	5.17

Table 2. Chemical composition of the envelope fraction from *E. coli* HB101

Envelope fractions were prepared from the cells grown as shown in Table 1. Protein was determined by the method of Lowry *et al.* (1951) before (whole) and after (Sar-sol) the extraction with 0.5 % sarcosyl. An amount of LPS was calculated on the basis of KDO determination. Phospholipid was estimated on the basis of chloroform-soluble phosphorus. Peptidoglycan was estimated from diaminopimelic acid determination. Glucan was estimated by subtraction of the D-glucose content found in LPS from the total D-glucose content found in the envelope fraction. Values refer to the dry weight of the envelope fraction and are given in mg/mg of envelope.

Plasmid	Time (h)	Content (mg/mg of envelope)					
		Protein		LPS	Phospho-lipid	Peptido-glycan	Glucan
		Whole	Sar-sol				
None	4	0.53	0.23	0.22	0.12	0.055	0.007
	13	0.53	0.23	0.19	0.13	0.057	0.033
	23	0.53	0.24	0.19	0.14	0.056	0.015
	39	0.54	0.25	0.19	0.16	0.058	0.006
pEAP4	12	0.53	0.27	0.19	0.12	0.058	0.038
	36	0.56	0.27	0.20	0.14	0.054	0.007
pEAP31	5	0.56	0.25	0.22	0.12	0.053	0.008
	14	0.59	0.29	0.18	0.12	0.044	0.019
	23	0.62	0.33	0.19	0.12	0.039	0.010
	29	0.68	0.38	0.19	0.13	0.040	0.007
	39	0.69	0.43	0.16	0.07	0.032	0.008

released materials and the stage of growth when the cells were harvested. The absorbance of the culture increased and the penicillinase accumulated intracellularly until 23 h. Slight 'quasi-lysis' was observed as a decrease of turbidity at 29 h, and approx. 60 % of the penicillinase was released from HB101 (pEAP31) cells. Large amounts of proteins, phospholipids and LPS were released at the same time. Thereafter the 'quasi-lysis' and release of penicillinase continued. Envelope fractions were also prepared from HB101, either containing or not containing pEAP4, as references.

Composition of the envelope fraction

Table 2 shows alterations in amount of various constituents found in the envelope fractions of the organisms according to

cultivation time. Protein accounted for 53–56 % of the dry weight of the envelope preparations from HB101 containing, or not containing, pEAP4. The protein content was almost constant in the fractions from both organisms. Protein content was high in HB101 (pEAP31) and gradually increased during cultivation. It accounted for about 70 % of the dry weight at 29 or 39 h, but 56 % at 5 h. The increase in the protein content in the envelope preparation from HB101 (pEAP31) was essentially caused by an increase in sarcosyl-soluble proteins. Sarcosyl-insoluble proteins were almost constant. It is suggested that a proportion of the sarcosyl-soluble proteins in the envelope proteins of HB101 (pEAP31) continued to increase gradually.

The content of peptidoglycan was low in the fraction prepared from HB101 (pEAP31). The peptidoglycan content decreased

Fig. 1. SDS/PAGE of envelope proteins from *E. coli* HB101

E. coli HB101 carrying or not carrying the plasmid was grown aerobically in the LQ broth at 30 °C. The cells were harvested as shown in Table 1. The envelope fraction was extracted with 0.5% sodium *N*-dodecanoyl sarcosine at room temperature for 30 min (3 mg/ml). The sarcosyl-soluble or -insoluble proteins (2 mg/ml) were solubilized in 1% (w/v) SDS/1% (v/v) 2-mercaptoethanol/15% (w/v) sucrose/10 mM-sodium phosphate buffer (pH 7.2) at 100 °C for 5 min. (a) The sarcosyl-soluble fraction from the envelope containing 50 µg of protein was electrophoresed on a polyacrylamide gel composed of 15% (w/v) acrylamide, 0.32% *N,N*'-methylenebisacrylamide, 0.5% SDS, 8 M-urea and 0.1 M-sodium phosphate buffer, pH 7.2. (b) The sarcosyl-insoluble fraction from the envelope containing 50 µg of protein was electrophoresed on a gel composed of 10% (w/v) acrylamide, 0.5% *N,N*'-methylenebisacrylamide, 0.5% SDS, 8 M-urea and 0.1 M-sodium phosphate buffer, pH 7.2. The gels were stained with Coomassie Brilliant Blue R-250. M_r markers used were CNBr fragments of myoglobin and oligomers of cytochrome *c*. Arrows indicate the proteins discussed in the text.

slightly in the envelope of HB101 (pEAP31) with growth in the stationary phase (the content decreased to only 3% at 39 h). On the other hand, the peptidoglycan content was 6% in the envelope fraction from HB101, even at 39 h.

The content of phospholipids in the fraction from HB101 (pEAP31) was not so very different from that of the other samples. However, it decreased significantly from 29 to 39 h. The LPS content expressed on a dry-weight basis was almost constant among the samples.

A small amount of unidentified glucan was found as an additional component in the fraction. The glucan content was high at 12–14 h in all the organisms and thereafter decreased. The decrease was rapid in HB101 (pEAP31).

To summarize the results obtained for the constituents, the envelope of HB101 (pEAP31) was relatively rich in sarcosyl-soluble proteins and depleted in other components, the quan-

titative alteration becoming significant during the stationary phase after 14 h.

Composition of proteins

There was no significant qualitative difference among the sarcosyl-soluble proteins of any of the envelope preparations irrespective of cultivation time or the presence of Kil (Fig. 1a). Stationary-phase cells of HB101 (pEAP31) were non-specifically rich in sarcosyl-soluble proteins of over 20 kDa, attributable to large amounts of sarcosyl-soluble proteins in the envelope fractions. A protein of apparently 29 kDa was found in HB101 (pEAP4) as a major band and in HB101 (pEAP31) as a minor band. The mature penicillinase corresponds to the 29 kDa band.

Sarcosyl-insoluble proteins were different in composition among the samples (Fig. 1b). A protein of 19 kDa appeared in HB101 during the stationary phase. This protein increased earlier

Table 3. Composition of phospholipids in the envelope fraction from *E. coli* HB101

Phospholipids extracted from the envelope preparation shown in Table 2 were developed on a silica-gel thin-layer plate. Phosphorus in the iodine-positive area was determined. The value of PE is shown as the sum of determinations of PE and LPE. The LPE content found in the extract is shown in parentheses. Each value represents the molar percentage (mol %).

Plasmid	Time (h)	Content (mol %)			
		PE	(LPE)	PG	CL
None	4	89	(11)	11	< 1
	13	86	(6)	14	< 1
	23	88	(5)	12	< 1
	39	85	(5)	12	3
pEAP4	12	87	(9)	13	< 1
	36	85	(8)	13	2
pEAP31	5	85	(13)	15	< 1
	14	82	(8)	17	2
	23	79	(6)	19	2
	29	77	(6)	22	1
	39	64	(3)	32	4

Table 4. Composition of cellular phospholipids from *E. coli* HB101

Phospholipids were extracted from cells immediately after harvesting. The procedures that followed were the same as those in Table 3. In this case LPE was not detected.

Plasmid	Time (h)	Phospholipid (mol %)		
		PE	PG	CL
None	4	86	14	< 1
	13	89	11	< 1
	24	81	16	2
	36	81	14	4
	48	82	14	4
pEAP31	4	84	16	< 1
	13	83	16	1
	24	75	21	4
	36	73	22	5
	48	67	28	5

in HB101 (pEAP31) than in HB101 and had already appeared by 14 h. OmpA and OmpC proteins were present in slightly lesser amounts in the stationary-phase cells of HB101 (pEAP31) than in those of HB101 containing or not containing pEAP4.

SDS/PAGE of proteins indicated that the increase in sarcosyl-soluble proteins found in HB101 (pEAP31) (Table 2) is not due to cross-contamination between the sarcosyl-soluble and -insoluble proteins, but due to the fact that inner-membrane proteins increase, or outer membrane proteins decrease, in the envelope fraction from HB101 (pEAP31) in the stationary phase.

Composition of phospholipids

Phosphatidylglycerol (PG), cardiolipin (CL), PE and LPE were found in each envelope fraction (Table 3). LPE had probably been produced from PE by phospholipase A artificially activated through dissociation of envelope during freeze-thaw of cells and preparation of the envelope fractions by sonication, because LPE was not found in intact cells immediately after harvest from cultures, as shown in Table 4 (Aono, 1989b). Therefore contents of LPE plus PE are considered as a true content of PE (Table 3).

Table 5. Chemical composition of the LPS fraction from *E. coli* HB101

The envelope fraction shown in Table 2 was extracted with warm phenol and water, after removal of lipid with chloroform/methanol (Rouser & Fleischer, 1967). The water layer was washed with diethyl ether three times before the determinations were made. Neutral sugar was determined by the phenol/H₂SO₄ reaction as glucose. Amino sugar was determined by the Morgan-Elson reaction as glucosamine. Recovery of KDO is a percentage of that found in the original envelope fraction. Contents of phosphorus, neutral sugar and amino sugar are represented as molar ratios to that of KDO.

Plasmid	Time (h)	Recovery of KDO (%)	Molar ratio of compound in phenol extract		
			Phosphorus	Neutral sugar	Amino sugar
None	4	96	4.7	3.0	1.2
	13	88	4.9	5.6	1.2
	23	88	5.1	4.6	1.3
	39	93	4.8	2.9	1.1
pEAP4	12	100	4.4	5.5	1.2
	36	86	4.8	3.1	1.1
pEAP31	5	88	5.1	3.2	1.1
	14	78	5.0	4.6	1.1
	23	69	5.1	3.9	1.1
	29	70	5.2	3.3	1.1
	39	79	4.7	2.9	1.2

In fact, the sum is similar to the content of PE found in phospholipids immediately after extraction from cells.

PE and PG were the major phospholipids in *E. coli*, and respectively accounted for 85–89% and 11–14% of phospholipids in the host cells. Similar contents were found in HB101 (pEAP4). In HB101 (pEAP31) the relative PG content gradually increased in the stationary phase. Conversely, the PE content decreased. The contents of PE and PG were respectively 64 and 32% at 39 h. The same alterations in composition were found in phospholipids extracted immediately from intact cells (Table 4). CL was a minor phospholipid and increased in all the organisms at late stationary phase. The increase of CL seemed to be earlier in HB101 (pEAP31) than in HB101 containing or not containing pEAP4.

Chemical composition of LPS

The envelope fractions were washed with chloroform/methanol and then extracted with warm phenol (Westphal & Jann, 1965). LPS washed with diethyl ether three times was analysed without further purification.

The amount of LPS extracted from the envelope of HB101 (pEAP31) after 14 h seemed to be somewhat low on the basis of KDO recovery (Table 5). The molar proportions of KDO, phosphorus, neutral sugar and amino sugar were approx. 1:5.3:6:1 in all the extracted samples. The ratio found in an LPS reference from the host cells was 1.0:3.4:2.5:1.3 (Aono, 1989b). The neutral-sugar content varied among the samples, because the LPS fractions contained the unidentified glucan solubilized with warm phenol (Table 2). The presence of excess phosphorus might suggest a slight contamination with RNA in the envelope preparations. It is suggested that the LPS preparations were not altered in chemical composition except for glucose content, although unextractable LPS still remained in HB101 (pEAP31).

LPS molecules present in the envelope preparations were analysed on SDS/polyacrylamide gels without phenol extraction (Fig. 2). No detectable change was found in the electrophoretic

Fig. 2. SDS/PAGE of envelope LPS from *E. coli* HB101

The envelope fraction was prepared from *E. coli* HB101 carrying or not carrying the plasmid grown at 30 °C, as shown in Table 1. Envelope fractions containing 30 pmol of KDO were solubilized in 1% (w/v) SDS/15% (w/v) sucrose/5 mM-Tris/HCl, pH 8.0, at 100 °C for 5 min. Proteins were completely digested with proteinase K. Samples were electrophoresed on a polyacrylamide gel composed of 12% (w/v) acrylamide, 0.25% *N,N'*-methylenebisacrylamide, 0.5% SDS, 6 M-urea and 0.1 M-sodium phosphate buffer, pH 7.2. LPS was oxidized with periodic acid and revealed by silver staining.

mobility of LPS bands among the envelope preparations. Also there was no difference between the mobilities of LPS extracted with warm phenol and that of LPS present in the envelope fraction (results not shown). The electrophoretic analyses suggest that the LPS molecules were not altered in size, although small changes in LPS could not be ruled out.

Table 7. Chemical composition of the SDS-insoluble wall fraction from *E. coli* HB101

Aspartic acid, diaminopimelic acid and glucosamine were determined with the amino acid analyser. Lipoprotein was calculated on the assumption that 1 mol of the protein should release 14 mol of aspartic acid on hydrolysis. Glucose was determined by the phenol/H₂SO₄ reaction. Values are given molar ratios, taking diaminopimelic acid (DAP) as 1.0.

Plasmid	Time (h)	Content (mol/mol of DAP)	
		Lipoprotein	Glucose
None	4	0.052	0.67
	13	0.056	3.5
	23	0.052	1.9
	39	0.059	0.13
pEAP4	12	0.056	3.7
	36	0.061	0.22
pEAP31	5	0.065	0.69
	14	0.075	2.0
	23	0.072	0.49
	29	0.073	0.14
	39	0.081	0.14

Composition of the SDS-insoluble wall fraction

The wall fraction contained peptidoglycan, protein and glucan. Several amino acids arose from both the peptidoglycan and protein. Thus it was hard to determine accurately serine, glutamic acid and alanine derived from the protein in the wall fraction, because the latter two amino acids were also constituents of peptidoglycan and because serine was co-eluted with muramic acid derived from peptidoglycan under the conditions used for the amino acid analysis. Table 6 shows the molar proportions of the obviously 'proteinaceous' amino acids. The ratios were similar to the theoretical ones expected for the lipoprotein binding to peptidoglycan in all of the samples, except for methionine. The wall fraction was electrophoresed on an SDS/

Table 6. Composition of 'proteinaceous' amino acids in wall fraction from *E. coli* HB101

Wall fractions was prepared from the envelope fraction by washing with detergents and hydrolysed with HCl. Amino acids were determined with an amino acid analyser. The molar ratios of amino acids obviously derived from protein are shown, taking the aspartic acid value as 14. Alanine, glutamic acid and serine were also found in the hydrolysates. The theoretical values for amino acids found in lipoprotein and Kil peptide are also shown.

Plasmid	Time (h)	Molar ratio of amino acid								
		Asp	Thr	Val	Met	Ile	Leu	Tyr	Lys	Arg
None	4	14	2.1	4.0	0.8	1.3	4.4	1.0	4.3	3.6
	13	14	3.3	3.5	0.5	1.2	4.1	0.9	4.0	3.3
	23	14	2.0	3.6	0.7	1.1	4.1	0.9	4.3	3.6
	39	14	2.1	3.7	1.1	1.0	3.9	1.0	4.1	3.6
pEAP4	12	14	2.1	3.6	0.8	0.9	4.0	0.9	4.1	3.4
	36	14	2.0	3.9	1.6	1.1	4.2	1.2	4.2	3.7
pEAP31	5	14	2.2	4.1	0.9	1.3	4.2	0.9	4.2	3.7
	14	14	2.0	3.8	0.7	1.1	4.2	1.0	4.4	3.8
	23	14	2.0	3.9	0.8	0.8	4.0	1.0	4.2	3.6
	29	14	2.3	4.3	2.1	1.2	4.7	1.1	5.2	4.4
	39	14	2.2	3.8	0.8	0.8	4.0	1.0	4.2	3.8
Theoretical values:										
Lipoprotein		14	2	4	2	1	4	1	5	4
Kil peptide		14	14	14	0	21	7	7	7	7

Table 8. Chemical composition of the peptidoglycan fraction from *E. coli* HB101

The wall fraction shown in Table 6 was digested with trypsin and washed with hot SDS. Amino acids, amino sugars and glucose were determined as shown in Table 6. *N*-Terminal amino acids (*N*-) were determined by the dinitrophenylation method as dinitrophenylalanine. Reducing terminal residues (Red-) were determined with ferricyanide as glucose. Values are molar ratios with respect to diaminopimelic acid (DAP). Mur, muramic acid.

Plasmid	Time (h)	Content (mol/mol of DAP)							Terminal	
		Mur	GlcN	Glu	Ala	D-Ala	Lys	Glc	<i>N</i> -	Red-
None	4	0.96	0.93	1.00	1.71	0.71	0.06	0.05	0.67	0.011
	13	0.96	0.95	1.04	1.75	0.72	0.08	0.03	0.70	0.011
	23	0.96	0.96	1.03	1.69	0.68	0.08	0.03	0.65	0.010
	39	0.98	0.97	1.06	1.64	0.69	0.08	0.04	0.70	0.012
pEAP4	12	0.98	0.94	1.00	1.65	0.70	0.08	0.03	0.66	0.011
	36	0.92	0.93	0.97	1.62	0.63	0.09	0.05	0.62	0.012
pEAP31	5	0.93	0.93	0.96	1.61	0.68	0.08	0.04	0.72	0.014
	14	0.89	0.92	0.97	1.52	0.51	0.08	0.02	0.62	0.011
	23	0.90	0.93	0.92	1.44	0.45	0.08	0.02	0.72	0.011
	29	0.95	0.97	0.94	1.42	0.43	0.08	0.02	0.67	0.010
	39	0.91	0.92	0.97	1.41	0.42	0.09	0.02	0.63	0.010

polyacrylamide gel before and after digestion with lysozyme. Protein bands were essentially the lipoprotein in all of the samples (results not shown). These results suggest that only lipoprotein exists in each of the wall fractions. Therefore, the amount of lipoprotein bound to peptidoglycan could be calculated on the basis of the content of aspartic acid, which was a major amino acid in the protein (Table 7). The amount of lipoprotein was higher in the wall fraction from HB101 (pEAP31) than in that of other samples.

The glucose content varied in the samples as well as in the envelope fractions described above (Table 2). The glucan accumulated transiently in the stationary phase of the growth. The disappearance of the glucan occurred earlier in HB101 (pEAP31) cells.

Chemical composition of peptidoglycan

Tryptic digestion removed lipoprotein and glucan from the wall fraction, although recovery of peptidoglycan was low, especially from the envelope fraction from HB101 (pEAP31) after 23 h. The composition of the peptidoglycan preparations was glucosamine, muramic acid, L-alanine, glutamic acid, diaminopimelic acid and D-alanine in the molar proportions of approx. 1:1:1:1:1:0.7–0.4 (Table 8). The results for contents of glucosamine, muramic acid and reducing terminal suggested that the glycan moiety of peptidoglycan did not differ among the samples.

Moreover, the content of *N*-terminal amino acids was almost constant, although its determination might be interfered with by remaining lysine. Peptidoglycans were dinitrophenylated and completely hydrolysed with 3 M-H₂SO₄ at 100 °C for 15 h. The yellow-coloured dinitrophenyl-amino acid derivative was acid-soluble and diethyl ether-insoluble, even under the strong acidic conditions used (results not shown). These facts indicated that the dinitrophenyl amino acid derivative was essentially a mono-dinitrophenyl substituent of diaminopimelic acid, but not a bis substituent of diaminopimelic acid or a dinitrophenyl derivative of another monovalent amino acid, because a bisdinitrophenyl substituent of a bivalent amino acid and a dinitrophenyl derivative of a univalent amino acid were diethyl ether-soluble under the conditions used. The mono substituent derivative was derived from diaminopimelic acid that had not been involved in cross-

linkage between the peptide moieties. Therefore the frequency of the cross-linkage of the peptide moieties should be constant. On the other hand, the content of D-alanine decreased in the peptidoglycan fraction prepared from HB101 (pEAP31) after 14 h. These results suggested that C-terminal D-alanine that was not involved in a cross-linkage might be removed in HB101 (pEAP31) after 14 h.

DISCUSSION

Extracellular production of the penicillinase by HB101 (pEAP31) is a unique system from four viewpoints: (1) release of periplasmic proteins is highly effective and significantly non-specific; (2) agents for activation of the *kil* promoter are not required; (3) the release occurs in the stationary phase of growth after prolonged incubation; (4) the release is extremely dependent on cultivation conditions, such as culture temperature, pH of medium, and the concentrations of salts and carbohydrates (Aono, 1988a, 1989a).

These features differ from the release of colicins. Components of the outer membrane are simultaneously released from HB101 (pEAP31) with the periplasmic penicillinase and alkaline phosphatase (Aono, 1989b). These facts suggest that sequential complex reactions must be mediated by Kil, directly or indirectly, and one of the reactions should be essential for release of periplasmic proteins from the stationary-phase cells. In order to understand the mechanism releasing proteins, it is important to distinguish between (i) a primary reaction caused by Kil in this system and (ii) a secondary alteration involved in the extracellular release of the penicillinase.

It has been proposed that Kil activates outer-membrane phospholipase A, and LPE accumulated via the activation makes the membrane permeable for proteins. The phospholipase A seems to be essential to 'quasi-lysis' by CelB peptide (Pugsley & Schwartz, 1984). However, moderate 'quasi-lysis' is caused by Cal peptide in the phospholipase-defective mutant (Cavard *et al.*, 1989). This fact might suggest that activation of the lipase should not be the primary site affected by the Cal peptide. In the exponential-phase cells, Kil might activate outer-membrane phospholipase A. However, LPE had not accumulated in the envelope of HB101 (pEAP31) releasing the penicillinase. The

Table 9. Alteration of envelope components of *E. coli* HB101

Weight ratios of the envelope components found in Table 2 were calculated taking sarcosyl-soluble proteins as 1.0.

Plasmid	Time (h)	Sar-insoluble protein	LPS	Components (weight ratio)				
				Phospholipid			Peptidoglycan	Glucan
				Total	PE	PG		
None	4	1.3	0.95	0.54	0.48	0.060	0.24	0.03
	13	1.3	0.83	0.57	0.49	0.081	0.25	0.14
	23	1.3	0.82	0.60	0.53	0.075	0.24	0.06
	39	1.1	0.75	0.63	0.54	0.075	0.23	0.02
pEAP4	12	1.0	0.71	0.45	0.39	0.059	0.22	0.14
	36	1.1	0.74	0.54	0.46	0.070	0.20	0.02
pEAP31	5	1.2	0.89	0.46	0.39	0.067	0.21	0.03
	14	1.0	0.62	0.42	0.34	0.071	0.15	0.07
	23	0.90	0.58	0.38	0.30	0.072	0.12	0.03
	29	0.78	0.51	0.35	0.27	0.076	0.10	0.02
	39	0.61	0.38	0.15	0.10	0.049	0.08	0.02

lipase can be artificially activated irrespective of the presence or absence of Kil during the disruption of young cells by sonication (Table 3). The activation by sonication was attenuated even in the cells of HB101 (pEAP31) in the stationary phase. The activation did not seem to be caused during growth in the stationary phase. The primary reaction might be different from that found in the exponential-phase cells treated with SOS activator, because the composition of outer-membrane proteins of the stationary-phase cells was different from that of exponential-growth-phase cells (Fig. 1b).

Therefore it is essential to compare the compositions of (i) the envelope from HB101 (pEAP31), which is accumulating the penicillinase in its periplasm, (ii) that from the organism which is releasing the periplasmic penicillinase and (iii) that from the organism which has completed releasing the penicillinase (Table 1).

All the components altered quantitatively in the envelope possessing Kil (Tables 2–4 and 7). It cannot be easily concluded which is the primary or secondary effect of Kil. It may be absolutely necessary to consider the proportions of envelope components relative to a certain component which is not affected by Kil. Kil enhances the extracellular production of the penicillinase and does not aid excretion of the penicillinase through the inner membrane into the periplasm (Aono, 1989a,b). Presumably the inner membrane would not be affected by the Kil peptide. In Table 9 the data shown in Table 2 have been arranged and normalized with respect to sarcosyl-soluble proteins, which should give close approximate values of inner-membrane proteins.

The quantitative alterations in the envelope fraction from HB101 (pEAP31) are the concurrent decreases in peptidoglycan, sarcosyl-insoluble proteins, LPS and PE after 14 h. These results suggest that the concurrent decreases were probably caused by partial exfoliation or peeling of the outer membrane. Release of the envelope components was found at two stages. The release of these components during cultivation is accompanied by the extracellular production of the penicillinase. The release of the penicillinase and envelope components are suppressed by the addition of high concentration of NaCl or cultivation at low temperature, probably because the outer-membrane structure is stabilized (Aono, 1988a, 1989b). The release was also found during preparation of envelope fractions from the harvested cells. A considerable amount of LPS was released from the envelope of HB101 (pEAP31) grown for 14 or 23 h during

disruption of the cells by sonication and recovered in the cytoplasmic fraction (results not shown). This result suggested that Kil had already made the outer membrane fragile by 14 h. Therefore it seems very likely that exfoliation during cultivation causes the extracellular production of the penicillinase, as suggested previously (Aono, 1989b). However, the exfoliation of outer membrane during cultivation is considered to be a secondary reaction and not a primary reaction of Kil. Several components decreased markedly at 39 h, suggesting that 'true lysis' takes place after very long incubation periods. This lysis causes release of cytoplasmic proteins, including β -galactosidase (Aono, 1989b).

A qualitative alteration was found in the peptidoglycan of HB101 (pEAP31). The molar ratio of D-alanine to diaminopimelic acid decreased in HB101 (pEAP31) after 23 h (Table 8). The content of peptidoglycan decreased in HB101 (pEAP31) after 14 h (Table 9). Kil might affect the turnover of peptidoglycan in the stationary-phase cells of HB101 (pEAP31) (Goodell, 1985; Blasco *et al.*, 1988). Thereby the peptidoglycan was partially broken down, and interaction of the peptidoglycan and the outer membrane should be weakened, causing the outer membrane to be partially disassembled. 'Quasi-lysis' caused by Cal in autolysis-deficient mutants which show 60–65% of the wild-type level of peptidoglycan endopeptidase or *N*-acetylmuramidase has been examined (Howard *et al.*, 1987). 'Quasi-lysis' and colicin A release occur in the *N*-acetylmuramidase-deficient mutants as well as in the wild-type strain. On the other hand, 'quasi-lysis' is attenuated and colicin A release is decreased in the peptidoglycan endopeptidase-deficient mutant, as far as can be judged from the results presented by Howard *et al.* (1987), although they did not notice the attenuation and decrease. These facts might correspond to an effect of Kil on the peptide, but not glycan, moiety of the peptidoglycan of HB101 (pEAP31).

It is noteworthy that the sarcosyl-insoluble 19 kDa protein appeared earlier and was much enhanced in HB101 (pEAP31) compared with HB101 containing or not containing pEAP4 (Fig. 1). The protein should be encoded on the chromosomal DNA of the host cell, but not on the plasmid, because it appeared also in HB101 cells in the stationary phase. It has not been reported that bacteriocin-releasing peptides cause the accumulation of a peculiar protein encoded on host chromosomal DNA in the envelope. The function of this 19 kDa protein is not known. I suppose that the protein might be involved in the turnover of peptidoglycan or in the interaction between peptidoglycan layer

and the outer membrane. It is also noteworthy that lipoprotein binding to peptidoglycan increased in HB101 (pEAP31). The lipoprotein is known to be involved in the association of the outer membrane and the peptidoglycan layer. It is suggested that the Kil indirectly degrades peptidoglycan with the preferential removal of a region possessing no lipoprotein.

In conclusion, Kil seems to alter the envelope composition of *E. coli* HB101, as suggested by observation that outer-membrane components were simultaneously released into medium with penicillinase. Its mechanism is still unclear. However, lowering of D-alanine in peptidoglycan may be an important step in the partial exfoliation of the outer membrane from HB101.

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