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ARCHITECTURE OF THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K12

I. ACTION OF PHOSPHOLIPASES A_2 AND C ON WILD TYPE STRAINS AND OUTER MEMBRANE MUTANTS

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Summary

Phospholipids in whole cells of wild type *Escherichia coli* K12 are not degraded by exogenous phospholipases, whereas those of isolated outer membranes are completely degraded. It is concluded that the resistance of phospholipids in whole cells is due to shielding by one or more other outer membrane components. The nature of the shielding component(s) was investigated by testing the sensitivity of whole cells of a number of outer membrane mutants. Mutants lacking both major outer membrane proteins b and d or the heptose-bound glucose of their lipopolysaccharide, are sensitive to exogenous phospholipases. Moreover, cells of a mutant which lacks protein d can be sensitized by pretreatment of the cells with EDTA. From these results and from data on the chemical composition of the outer membranes, it is concluded that proteins b and d, the heptose-bound glucose of lipopolysacccharide and divalent cations are responsible for the inaccessibility of phospholipids to exogenous phospholipases.

Introduction

The cell envelope of gramnegative bacteria, which contains all the phospholipids of the cell [1], consists of three layers: the cytoplasmic membrane, a peptidoglycan layer and the outer membrane [2]. The latter membrane contains protein, phospholipid and lipopolysaccharide [3,4].

The structural organization of the phospholipids in the outer membrane is unknown. X-ray diffraction studies suggest that part of the phospholipids is organized as a mono- or bilayer [5]. Freeze fracture studies strongly suggest that part of the phospholipids is organized as a bilayer [6,7].

Zwaal et al. [8] have approached the lipid organization in erythrocyte membranes by using phospholipases. From these studies the distribution of phospholipids over both membrane halves could be deduced. A similar approach was used by Duckworth et al. [9]. They found that the phospholipids in whole cells of *Escherichia coli* B are neither degraded by phospholipase A_2 (porcine pancreas) nor by phospholipase C (*Bacillus cereus*).

In this paper we describe the action of exogenous phospholipases on cells of both wild type strains and of a wide variety of outer membrane mutants. The results will be discussed in relation to the chemical composition of the outer membrane.

Materials and Methods

Chemicals and radiochemicals

Chemically pure phospholipase A_2 from porcine pancreas (EC 3.1.1.4) [10] was a generous gift of Dr. G.H. de Haas. Purified phospholipase C (EC 3.1.4.3) from *B. cereus* [11] and phospholipase A_2 from bee venom (*Apis Mellifica*), mellitin-free [12], were kindly provided by Dr. R.F.A. Zwaal.

Sodium [2-^{14}C]acetate, specific activity 58 Ci/mol, was obtained from the Radiochemical Centre, Amersham, U.K.

Bacterial strains and growth conditions

Various parent strains derived from *E. coli* K12 as well as mutants with defects in lipopolysaccharide and/or major outer membrane proteins were used. The strains and their relevant characteristics are listed in Table I. As the possibility existed that degradation of the phospholipids resulted in lysis of the cells, *lacI* derivatives were used in order to allow us to measure leakage of the cytoplasmic enzyme β-galactosidase. The *lacI* marker was introduced by crossing with strain AM 1001 (Hfr Cavalli, *lacI*), which was a generous gift of Dr. H.E.N. Bergmans [19]. Recombinants were selected for the *leu* marker.

The composition of yeast broth and brain heart has been described previously [15]. If indicated these media were supplemented with 0.3 M NaCl (high salt) in order to prevent the synthesis of protein b. The nomenclature of proteins is the one described by Lugtenberg et al. [22].

In order to label the lipids the growth medium was supplemented with 1 mM [2-^{14}C]-acetate, specific activity 0.5 Ci/mol. Cells were grown at 37°C and harvested at the end of the logarithmic growth phase, washed with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.5 mM $MgCl_2$ and resuspended in 1/20 volume of the same buffer without $MgCl_2$. For incubation with phospholipases the resulting suspension was used either directly or after preincubation with EDTA. Preincubation was carried out as described by Duckworth et al. [9], except that Tris buffer was replaced by 0.1 M phosphate, pH 7.8.

Incubation with phospholipases

Samples of 200 μl of concentrated cell suspension, sometimes pretreated with EDTA, were incubated in a waterbath at 37°C. After 5 min cofactor was added: 10 μl 0.1 M $CaCl_2$ in case of phospholipase A_2 of porcine pancreas, 5 μl 0.1 M $CaCl_2$ in case of phospholipase A_2 from bee venom and 10 μl 0.1 M

TABLE I

STRAINS AND RELEVANT PROPERTIES

The defects refer to cells grown in yeast broth unless otherwise indicated.

Strain designation	Parent strain, relevant characteristics	Defects in outer membrane		Source, reference(s)
		Lacking major outer membrane proteins (if not completely lacking % decrease is indicated)	Defects in lipopolysaccharide [b]	
PC 1349	also known as JC 7620	None	None	Phabagen collection and ref. 13
PC 2040	PC 1349	b	Heptoseless	Phabagen collection and ref. 13
CE 1052	*lacI* derivative of AB 1859	None	None	This paper and refs. 6 and 14
CE 1056	*lacI* derivative of CE 1036	c	None	This paper and refs. 6, 14 and 15
CE 1054	*lacI* derivative of CE 1034	d	None	This paper and refs. 6, 14 and 15
CE 1058	*lacI* derivative of CE 1038	c, d	None	This paper and refs 14
CE 1053	*lacI* derivative of CE 1032	b	Heptoseless	This paper and refs. 6 and 14
CE 1057	*lacI* derivative of CE 1037	b, c	Heptoseless	This paper and ref. 14
CE 1055	*lacI* derivative of CE 1035	b, d	Heptoseless	This paper and ref. 14
CE 1059	*lacI* derivative of CE 1041	b, c, d	Heptoseless	This paper and ref. 14
CE 1023	CE 1022	b	Heptoseless	Laboratory collection
CE 1021	*lacI* derivative of CE 1018	b (50%)	Glucoseless	This paper and ref. 6
CE 1002 [a]	*galE* derivative of CE 1001	None	Galactoseless	Laboratory collection
D21e7	*lpsA* derivative	b (75%)	Galactoseless and lacking heptose-bound phosphate	Boman and refs. 16 and 15
P 400 [a]		None	None	Reeves and refs. 17 and 15
P 460 [a]	P 400	d	None	Reeves and refs. 17 and 15
P 460 pr [a]	partial revertant of P 460 to d^+	d (50%)	None	This paper
CE 1071 [a]	Phage K3 sensitive derivative of P 692 2dI	b, c	None	This paper
P 692 2dI [a]	Phage II * (resistant derivative of P 692	b, c, d	None	Henning and ref. 18

[a] Growth in Brain heart.

[b] The tentative structure is given in Fig. 1.

$ZnCl_2$ in case of phospholipase C from *B. cereus*. The reaction was started by the addition of 4 I.U. of enzyme. Unless otherwise indicated incubations were carried out for 1 h. The reaction was stopped with 40 µl saturated EDTA solution. Control incubations were carried out without exogenous enzyme. At the end of the incubation cells were harvested and resuspended in 0.1 M phosphate buffer, pH 7.8. Lipids were extracted from the resuspended pellet and analyzed as described by Duckworth et al. [9]. As a reference the phospholipid composition of non-incubated cells was determined.

Determination of release of cellular components and of viable cell count

In a number of experiments it was determined whether leakage of β-galactosidase or release of outer membrane components occurred due to the action of phospholipases. In these experiments the reaction was terminated with equimolar amounts of EDTA.

After centrifugation the pellet was resuspended and the supernatant was filtered through a Millipore filter, pore size 0.45 µm. β-galactosidase was determined according to Slein and Logan [21]. Release of lipopolysaccharide and protein was tested by assaying both the filtrate and the cell envelopes isolated from the resuspended cells [22]. Lipopolysaccharide was determined by determination of 3-deoxy-D-manno-octulosonic acid as described previously [14]. The assay yields a value of 11 weight percent of 3-deoxy-D-manno-octulosonic acid in lipopolysaccharide of wild type *E. coli* K12, purified according to Galanos et al. [23]. Release of protein was determined according to Lowry et al. [24] and by polyacrylamide gel electrophoresis [22]. Release of lipids was examined by analyzing the filtrate as described for the resuspended cells, in the previous paragraph. Viable cell count was determined as colony forming units after appropriate dilution of the suspension and incubation on solid medium for 18 h at 37° C.

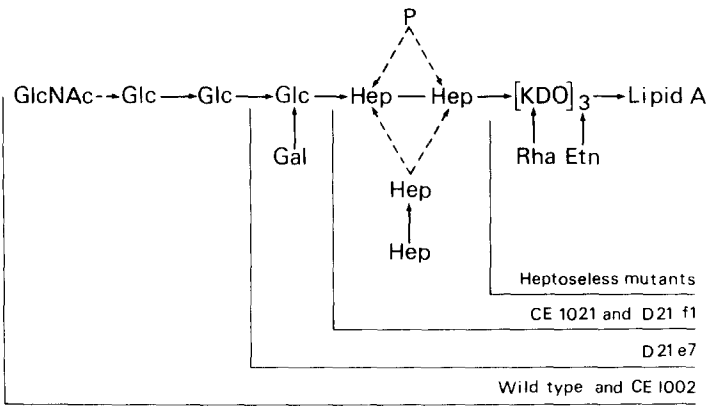

Fig. 1. Tentative structure of *E. coli* K12 lipopolysaccharide [20]. The length of the oligosaccharide chain of various mutants is indicated. Deficiencies in branches are not shown. Abbreviations: GlcNAc, *N*-acetylglucosamine; Glc, glucose; Gal, galactose; Hep, L-glycero-D-manno-heptose; Rha, rhamnose; KDO, 3-deoxy-D-manno-octulosonic acid; Etn, ethanolamine; P, phosphate.

Isolation and analysis of outer membranes

Outer membranes were isolated in order to determine the relative amounts of outer membrane components per unit cell surface. As no differences were observed in the shape of the cells of the studied strains by interference phase contrast microscopy, it was assumed that the cell surface area was proportional to total cell protein. The determination was carried out in two steps. (i) The amounts of total cell protein and lipopolysaccharide were determined quantitatively from desintegrated cells and cell envelopes respectively, resulting in the ratio of lipopolysaccharide over total cell protein. (ii) The amounts of phospholipid and protein relative to lipopolysaccharide were determined in isolated outer membranes. The experiments were carried out as follows. The cells were divided into two portions. The first portion was desintegrated by ultrasonic treatment. A sample of the desintegrated cells was boiled for 5 min in 1% sodium dodecyl sulphate and used for the determination of total cell protein [24]. Cell envelopes were isolated quantitatively [22] from the remainder of the desintegrated cells and used for the determination of 3-deoxy-D-manno-octulosonic acid. The second portion of cells was used for the separation of cytoplasmic and outer membrane by the procedure of Osborn et al. [4], modified as described earlier [25]. The quality of this separation was checked by determination of 3-deoxy-D-manno-octulosonic acid and NADH oxidase, which are specific for outer and cytoplasmic membrane respectively [4]. The amount of phospholipid was calculated after determination of lipid-phosphate [26], using an average molecular weight of 700. Thin-layer chromatography of the lipid fraction showed that phospholipid degradation due to the isolation procedure had not occurred. As the Lowry et al. method might not be reliable for membranes of mutants [14], both the Lowry et al. method as well as scanning of sodium dodecyl sulphate polyacrylamide gels [22], with bovine serum albumin as a standard, were used for protein determination. The amount of lipopolysaccharide was calculated using the mentioned value of 11 weight percent of 3-deoxy-D-manno-octulosonic acid in lipopolysaccharide. The values obtained for the amounts of protein, phospholipid and lipopolysaccharide were related to total cell protein in order to obtain data for the relative amounts of outer membrane components per unit cell surface.

Results

Chemical characterization of outer membranes

The chemical composition of the outer membranes of a number of lipopolysaccharide mutants of *Salmonella typhimurium* has recently been characterized. It was reported, that glucoseless and heptoseless mutants contain decreased amounts of protein and increased amounts of phospholipid per unit cell surface compared with a galactoseless strain, whereas the lipopolysaccharide content did not change [27]. As such data are not available for mutants which lack one or more major outer membrane proteins, the composition of the outer membranes of strain P400 and its derivatives was determined. Table II shows that the values for protein depend on the assay method. The lack of correlation between these two methods has been observed earlier [14]. From recent work of Schweizer et al. [28] it can be concluded that the values obtained with the

TABLE II

COMPOSITION OF OUTER MEMBRANES OF THE P400 SERIES

Cells were grown in Brain heart. Part of the cells was used for the determination of the ratio lipopolysaccharide over total cell protein. From another part outer membranes were isolated and the weight ratio's of lipopolysaccharide, protein and phospholipid were determined. Protein was determined by two methods. Finally the amounts of outer membrane constituents were expressed per mg of total cell protein. As described in Materials and Methods, these values can be considered to represent the amounts of outer membrane constituents per unit surface area.

Strain	Lacking outer membrane protein(s)	Outer membrane component over total cell protein (μg/mg) [a]			
		Protein		Phospholipid	Lipopoly-saccharide
		Lowry et al. method	Gel scanning		
P400	None	62 ± 4	32 ± 3	17 ± 2.0	38 ± 4
P460	d	64 ± 5	26 ± 3	30 ± 3.6	45 ± 4
CE 1071	b, c	62 ± 4	29 ± 3	27 ± 3.2	39 ± 4
P692 2dI	b, c, d	43 ± 3	14 ± 2	36 ± 4.3	53 ± 5

[a] For all strains the membrane separation was good in that the amount of middle band was small and the outer membrane always contained less than 2% of the NADH-oxidase activity of the total membrane fraction.

Lowry method are too high. Table II shows that the mutants P460 and CE1071 have only a slight reduction in protein content as the lack of a certain protein in a mutant is compensated for by increased amounts of other major outer membrane proteins [14,15]. A strong reduction in outer membrane protein was observed in strain P692 2dl, which lacks all three proteins. A significant increase of the amount of phospholipid was found for all three mutants. The two phospholipase sensitive strains P460 and P692 2dl have the largest amount of phospholipid. For all strains outer membranes were enriched in phosphatidylethanolamine whereas cytoplasmic membranes were enriched in phosphatidylglycerol. These observations confirm results recently reported for other *E. coli* strains [25]. In addition to an increased amount of phospholipid strain P692 2dl also contains a significantly increased amount of lipopolysaccharide. The buoyant density of the outer membrane of P692 2dl was only slightly lower than that of strain P400 (1.217 vs 1.225 g/cm^3).

Phospholipase action on strains with wild type outer membrane (Table III)

Strains which are wild type with respect to their outer membrane contained 71—73% phosphatidylethanolamine, 20—22% phosphatidylglycerol and small amounts of diphosphatidylglycerol, free fatty acids and lysophospholipids.

When strains with wild type outer membrane, e.g. PC1349 and CE1052 (grown in yeast broth) as well as P400 (grown in brain heart) were incubated with phospholipase A_2 (porcine pancreas), extensive degradation of phospholipids did not occur (Table III). The slight degradation observed in strain P400 is probably significant. Similar results were obtained with phospholipase C from *B. cereus* and phospholipase A_2 from bee venom, which are active up to a much higher surface pressure [29]. When cell envelopes of CE1052, isolated after mild desintegration of the cells with the French press, were used as a substrate for these enzymes, 95% of the radioactivity of the lipids was found in the

TABLE III

EFFECT OF PHOSPHOLIPASE A_2 (PORCINE PANCREAS)

Cells were grown in yeast broth unless otherwise indicated, washed and resuspended. After prewarming incubation with enzyme in the presence of 5.0 mM $CaCl_2$ was carried out for 1 h at 37°C. As a control on the effect of endogenous phospholipase part of the suspension was incubated without enzyme. Part of the cells was neither pretreated nor incubated and served as a reference. Recovery of lipid was always more than 90%. n.d., not determined.

Strain	Defects in outer membrane		Radioactivity in lysophospholipids plus free fatty acids (% of total lipids)		
	Lacking major outer membrane proteins (if not completely lacking % decrease is indicated)	Defects in lipopolysaccharide	Incubation		
			None	Without enzyme	Complete
PC 1349	None	None	4	3	3
PC 2040	b	Heptoseless	11	21	88
CE 1052	None	None	3	6	4
CE 1052 [a]	b	None	3	3	13
CE 1056	c	None	4	2	4
CE 1054	d	None	4	13	17
CE 1054 [a,b]	b,d	None	2	25	64
CE 1058	c,d	None	4	13	17
CE 1058 [a,b]	b,c,d	None	3	21	47
CE 1053	b	Heptoseless	35	34	80
CE 1021	b (50%)	Glucoseless	3	20	84
D21e7	b (75%)	Galactoseless and lacking heptose-bound phosphate	2	n.d.	4
CE 1002 [b]	None	Galactoseless	5	4	5
P 400 [b]	None	None	3	3	10
P 460 [b]	d	None	5	17	68
P 460 pr [b]	d (50%)	None	n.d.	17	15
CE 1071 [b]	b,c	None	2	5	10
P 692 2dI [b]	b,c,d	None	5	22	84

[a] Growth medium supplemented with 0.3 M NaCl.

[b] Brain heart medium instead of yeast broth.

degradation products. Incubation of these cell envelopes without enzyme but with cofactor, resulted in 39% degradation.

The phospholipids of *E. coli* B become susceptible to degradation by phospholipases after pretreatment of the cells with EDTA [9], a result which was confirmed by our experiments (not shown). However, this pretreatment has hardly any influence on the sensitivity of *E. coli* K12 strains.

Phospholipase action on cells of LPS mutants (Table III)

The phospholipid composition in heptoseless strains derived from AB1859 is remarkable in that 25-34% of the phospholipids are lysophospholipids. Slightly increased amounts of degradation products were also found in the two other heptoseless strains PC2040 and CE1023. The outer membrane associated phospholipase A_1 [30] is apparently activated in heptoseless derivatives.

In contrast to the situation in the wild type strains, cells of heptoseless mutants are sensitive to exogenous phospholipase A_2 (porcine pancreas), resulting in degradation of phospholipids to a large extent (Table III). Comparison

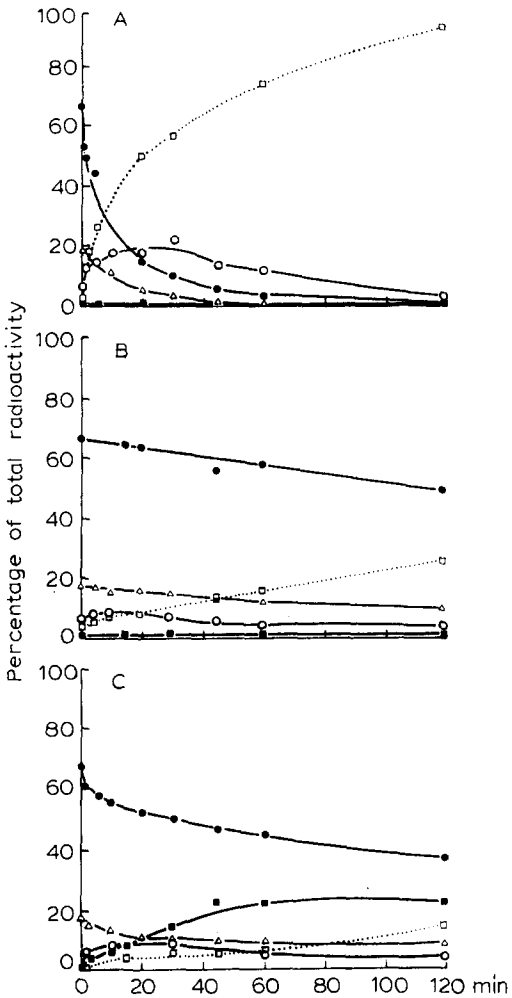

Fig. 2. Effects of phospholipases A_2 (porcine pancreas) and C (*B. cereus*) on the phospholipids of the heptoseless strain PC2040 *lacI*. Cells were grown in the presence of [2-^{14}C]acetate, harvested, washed and resuspended as described. $CaCl_2$ (A and B) or $ZnCl_2$ (C) was added as a cofactor. After prewarming the cells at 37°C phospholipase A_2 (20 I.U./ml) (A), no enzyme (B) and phospholipase C (20 I.U./ml) (C) was added. Samples were taken at various times and lipids were isolated and analyzed. The amount of phosphatidylethanolamine, (●———●); phosphatidylglycerol, (△———△); diphosphatidylglycerol, free fatty acids, (□———□); lysophospholipids, (○———○); and diglycerides, (■———■) are expressed as percentages of total recovered radioactivity. The percentage of diphosphatidylglycerol is not shown. It was always less than 5%.

with a suspension incubated without enzyme shows that, although an endogenous phospholipase is very active in some heptoseless strains, most of the degradation is caused by the exogenous enzyme. By testing the susceptibility of other LPS mutants it was shown that *E. coli* K12 is sensitive when the LPS is glucose deficient (e.g. strain CE1021), whereas strains lacking galactose (strain CE1002) or galactose plus heptose-bound phosphate (strain D21e7) are resistant (Table III). Pretreatment with EDTA hardly changed the results. Incubation with phospholipase C or the bee venom enzyme gave the same results as

described for phospholipase A_2 (porcine pancreas) (not shown).

A time course of the phospholipid degradation in the heptoseless mutant PC2040 *lacI* by phospholipases A_2 (porcine pancreas) and C is shown in Fig. 2. Incubation of these cells with phospholipase A_2 initially resulted in the fast appearance of lysophospholipids due to degradation of all phospholipid species. After 2 h almost all radioactivity was recovered in free fatty acids (Fig. 2A), indicating that lysophospholipids are further degraded, probably by the outer membrane-bound phospholipase A_1 [30]. It can be concluded that the degradative action of phospholipase A_2 is not restricted to the outer membrane or its outer leaflet as already was observed by Duckworth et al. [9] for *E. coli* B. In a control experiment without exogenous enzyme some phospholipid degradation occurred, although at a much slower rate than in the presence of exogenous enzyme (Fig. 2B). After 2 h 44% of the phosphatidylglycerol and 26% of the phosphatidylethanolamine was degraded.

Degradation of phospholipids by phospholipase C takes place at a slower rate (Fig. 2C). During the incubation the radioactivity in diphosphatidylglycerol increased, which is probably due to conversion of phosphatidylglycerol to diphosphatidylglycerol. In a control experiment without exogenous enzyme no degradation occurred. Again only an increase in the amount of diphosphatidylglycerol and a slight decrease in phosphatidylglycerol were found.

During incubation with phospholipase (porcine pancreas) a strong decrease in the number of viable cells was observed, coinciding with the degradation pattern. Degradation of the phospholipids did not result in lysis of the cells as never more than 0.7% of the cellular β-galactosidase, a cytoplasmic enzyme, could be detected in the sypernatant after harvesting the cells. Moreover, after degradation of the phospholipids in strain PC2040 *lacI* with phospholipase A_2 (porcine pancreas) no release of phospholipid degradation products, LPS or protein could be measured. Washing of phospholipase treated cells with phosphate buffer containing fat free bovine serum albumin (10 mg/ml) resulted only in the release of free fatty acids from the cells.

Phospholipase action on strains lacking outer membrane proteins (Table III)

The lipid composition of major membrane protein mutants did not differ from that of wild type strains.

Strain CE1052, grown in yeast broth high salt and therefore phenotypically lacking protein b, is resistant or only slightly sensitive towards phospholipase A_2. Strain CE1056, lacking protein c, is resistant towards the enzyme. In strains lacking protein d, e.g. CE1054 and P460, the endogenous enzyme is active during incubation with cofactor. Strain CE1054 is resistant towards exogenous phospholipase whereas P460 is sensitive. This effect is not due to the difference in growth medium as P460 is also sensitive when grown in yeast broth (not shown). As the partial revertant P460 pr is resistant, the sensitivity of strain P460 is caused by the lack of protein d. The difference in sensitivity between strains CE1054 and P460 must therefore be caused by strain differences. In order to obtain a better insight in the role of protein d in protecting the cell against exogenous phospholipase, four d-less strains of various parents were tested. The degradation of phospholipids due to exogenous enzyme varied from 1—9%. It thus can be concluded that strain P460 is an exception.

The additional lack of protein c in strains lacking either b or d does not increase the sensitivity towards exogenous enzyme (compare the pair CE1052 grown in yeast broth high salt and CE1071 and the pair CE1054 and CE1058, both grown in yeast broth). Comparison of a strain lacking both b and d, e.g. CE1054 grown in brain heart high salt, with isogenic strains lacking only protein b or protein d, e.g. CE1052 (grown in yeast broth high salt) or CE1054 (grown in yeast broth), shows that the lack of both b and d has a dramatic effect on the susceptibility towards phospholipase A_2. Of course strains lacking all three proteins are also sensitive, e.g. CE1058 (grown in brain heart high salt) and strain P692 2dl. The additional lack of various proteins in mutants with heptoseless lipopolysaccharide (CE1057, CE1055 and CE1059) is overshadowed by the effect of the heptose-deficiency (not shown).

Pretreatment with EDTA hardly influences the sensitivity of strains lacking proteins b or c. This pretreatment causes a slight activation of the endogenous phospholipase as well as a slight increase in sensitivity to exogenous enzymes. Pretreatment with EDTA has a striking effect on strain CE1054 (grown in yeast broth). After incubation of such cells without or with exogenous enzyme 30 and 58%, respectively of the radioactivity was found in the degradation products. This effect is due to the lack of protein d, as CE1052 is not influenced by EDTA. The effect of EDTA pretreatment on CE1054 cannot be attributed to release of lipopolysaccharide [31], as only 3% of the total 3-deoxy-D-manno-octulosonic acid was released.

Discussion

All tested *E. coli* K12 strains which are wild type with respect to the composition of their outer membrane are resistant to the action of exogenous phospholipases. Several explanations can be given for the resistance of *E. coli* phospholipids to the degradative action of phospholipase A_2. (i) Phospholipids are absent from the outer leaflet of the outer membrane of *E. coli* K12, as is assumed by Smit et al. [27] for *Salmonella typhimurium*. However, the presence of smooth areas in the outer fracture face of the outer membrane of *E. coli* K12 observed with the freeze fracture technique [6,7] suggests that at least a small amount of phospholipid is present in the outer leaflet. (ii) A too high surface pressure of the lipids in the outer leaflet prevents the degradation by exogenous phospholipases. This possibility is unlikely as none of the tested phospholipases, which vary considerably in the maximal surface pressure that limits their action, degrades the phospholipids. (iii) The phospholipids in the outer leaflet are bound to proteins or lipopolysaccharide, such that they cannot be degraded by phospholipases. This explanation is unlikely as almost all phospholipids can be degraded in isolated membranes. (iv) Phospholipids are present in the outer leaflet but shielding by lipopolysaccharide and/or protein prevents their degradation in intact cells. This explanation is supported by our observation that some mutants with deficiencies in lipopolysaccharide or outer membrane proteins are sensitive to exogenous enzymes. However, a straightforward conclusion with respect to the nature of the shielding component(s) is not possible as the chemical composition of the outer membranes of wild type and mutant cells can differ dramatically (Table II).

Phospholipase sensitive mutants have in common that they lack major outer membrane protein which is compensated for by an increase in phospholipid content, indicating that the lacking protein(s) occupy some space in the hydrophobic area in the wild type outer membrane. Smit et al. [27] calculated that the amount of phospholipid in the outer membrane of galactoseless mutants is barely enough to cover one side of the membrane. The increase in phospholipid content in the outer membranes of the sensitive mutants therefore indicacates that at least some phospholipid is present in the outer leaflet of the outer membrane of the sensitive strains. However, an increase in the phospholipid content of the outer membrane as observed in sensitive mutants, is not sufficient to explain the sensitivity to phospholipases as strain CE1071, which contains strongly increased amounts of phospholipids (Table II), is resistant (Table III). Therefore resistance of wild type cells must be caused by shielding by lipopolysaccharide and/or protein.

The heptose-bound glucose of lipopolysaccharide is crucial for the resistance of *E. coli* K12 cells against exogenous phopholipases (Table III). Similar results have recently been reported for *S. typhimurium* [32]. The observation that strain P692 2dl, which lacks b, c and d but contains increased amounts of wild type lipopolysaccharide (Table II), is sensitive, shows that lipopolysaccharide alone is not able to protect the cell. The results with mutants deficient in outer membrane proteins (Table III) show that the lack of both proteins b and d makes phospholipids accessible to phospholipases. A prerequisite for a role of proteins b and d in shielding is that at least some amino acids of these proteins are located at the outside of the hydrophobic area. Evidence for such a localization has been presented. Protein b probably interacts with the inner core region of lipopolysaccharide [15]. Protein d probably is the receptor for the phages Tu II* [18] and K3 [17,13] as well as for the F-pilus mediated conjugation [17,13]. Strain CE1054, which lacks protein d, can be sensitized to phospholipase by pretreatment with EDTA, which suggests that divalent cations play a role in protecting the phospholipids, at least in strains lacking d. The results obtained with the outer membrane mutants show that the heptose-bound glucose of lipopolysaccharide, the outer membrane proteins b and d and divalent cations play a role in the protection of wild type cells against exogenous phospholipases.

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