HYDROLYSIS OF PHOSPHOLIPIDS BY PHOSPHOLIPASE C IN INTACT CELLS OF WILD-TYPE AND ENVELOPE MUTANTS OF *ESCHERICHIA COLI K12*

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1. Introduction

The penetration of lipophilic molecules across the envelope of gram-negative bacteria is controlled by the outer membrane. The selective permeability of the outer membrane is attributed to the presence of lipopolysaccharides (LPS) and of membrane proteins integrated in the phospholipid layer [1]. Hydrophilic pores formed by porin proteins allow the nonspecific penetration of small molecules (M_r 600–800) across the outer membrane [2]. Several larger molecules (e.g. siderochromes, maltodextrins, vitamin B_{12}) are transported by specific mechanisms but many other molecules, either hydrophilic or lipophilic, are excluded [2]. Thus, gram-negative bacteria are generally resistant to lipophilic antibiotics such as actinomycin D, rifampicin, novobiocin or clindamycin.

However, several categories of mutants of *Salmonella typhimurium* and *Escherichia coli* hypersensitive to lipophilic drugs have been selected [2–4]. They are either 'deep rough', i.e. their LPS core is glucose-less or heptose-less, or some of their outer-membrane proteins are affected (some *omp* mutants). Moreover, some *pls* mutants with their phospholipid synthesis perturbed are also hypersensitive [5], as well as certain envelope mutants without any apparent modification of outer-membrane proteins of LPS [6–9].

It has been suggested that lipophilic molecules enter cells of deep rough and *omp* mutants by dissolving in phospholipids exposed on the outer surface of the outer membrane [10]. Indeed, phospholipids of whole cells of deep rough *S. typhimurium* [11] and of *E. coli* [12] are accessible to phospholipase C, while wild-type cells are resistant to that treatment.

The aim of the present work was to test the presence of the hydrophobic pathway of penetration in *env* (envelope) mutants of *E. coli* hypersensitive to lipophilic antibiotics.

2. Materials and methods

2.1. *Bacterial strains and growth media*

The characteristics and growth conditions of *E. coli* K12 strains used have been described previously [8]. Strains D31-m4 and C35 are heptose-less mutants kindly provided by Drs Boman [13] and Beacham [14], respectively.

2.2. *Sensitivity to antibiotics*

Sensitivity to antibiotics, detergents, and dyes was determined by the size of the inhibition zone around a disk, using agar plates as described by Coleman and Leive [9].

2.3. *Sensitivity to phospholipase C*

Sensitivity to phospholipase C was followed essentially as described by Kamio and Nikaido [11]. ^{32}P-Labelled cells (0.2 mg dry weight ml^{-1}) grown in 10 ml of nutrient broth containing 1 μCi of $^{32}PO_4^{3-}$ at 37°C were washed twice and resuspended in 10 mM Hepes buffer, pH 7.4, at 0°C. The assay was supplemented with 20 μg of phospholipase C ml^{-1} (from *Bacillus cereus;* Boehringer) and incubated at 28°C. Duplicate 0.2-ml samples were added at the times indicated to 0.2 ml of cold 10% trichloroacetic acid in Eppendorf tubes and centrifuged. Sedimented cells were extracted twice with 1.5 ml of ethanol–ether (3:1). After evaporation, the radioactivity in the extract was determined with an Intertechnique scintillation spectrometer, by using PPO-POPOP-toluene fluid (4 g:0.5 g:1000 ml).

2.4. *EDTA pretreatments*

Where indicated, ^{32}P-labelled cells were first incubated in ice-cold 30 mM Tris–HCl buffer, pH 7.3, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), for 10 min, washed in 10 mM Hepes buffer, pH 7.4, and treated with phospholipase as above.

3. Results and discussion

It has been shown previously [6,7] that some envelope mutants of *E. coli* K12 affected in the division process grow in chains (D22 *envA;* PM61 *envC*) or display irregular morphology (PM31). They are all more sensitive to various antibiotics and other antibacterial agents than their respective parents (D21; P678) [8,15]. It should be noted that the LPS of these strains appears not to be modified [16,17].

Thus, the first series of experiments was designed to see whether hypersensitive envelope mutants have phospholipid head groups exposed on the surface of the outer membrane. Furthermore, two heptose-less mutants of *E. coli* K12, i.e. strains D31 [13], and C35 [14] were added to the set of strains tested. Table 1 shows that only PM61, PM31 and C35 phospholipids were digested to about 30–40%. Other strains were insensitive to phospholipase C treatment. Interestingly enough, parent strains P678 and D21, as well as *env* mutants PM61 and D22, pretreated with EDTA showed a partial digestion of phospholipids (~20–30%). EDTA-pretreated PM61 lost less phospholipids than PM61 without EDTA, perhaps because of traces of EDTA adsorbed to the outer membrane and not removed by washing.

Table 1
Hydrolysis of phospholipids in whole cells of *E. coli* by phospholipase C

Strain	PLase	EDTA–PLase	Without enzyme
P678	95	72	95
PM61	68	74	94
PM31	56	n.d.	92
D21	96	76	100
D22	95	79	96
D31-m4	96	n.d.	100
C35	69	n.d.	100

^{32}P-Labelled cells (0.2 mg dry weight ml^{-1}) were incubated at 28°C in 10 mM HEPES buffer pH 7.4, containing 20 μg of phospholipase C ml^{-1}. The values represent per cent [^{32}P]-phospholipids remaining in the cells after 20 min of incubation. EDTA-pretreatment is described in section 2. n.d. = not determined

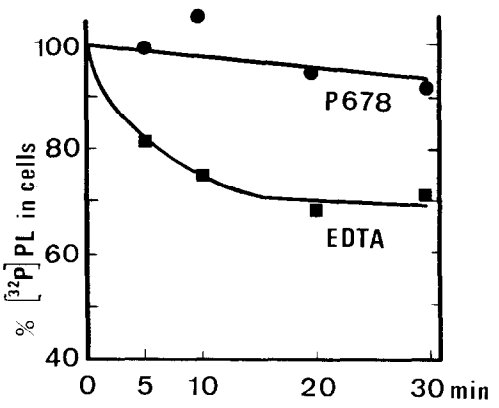

Fig.1. Effect of pretreatment with EDTA on the digestibility of phospholipids in wild-type *E. coli* P678. • = intact cells incubated with phospholipase C; ▪ = cells treated for 10 min in 0.1 mM EDTA–10 mM HEPES buffer pH 7.4 at 0°C, washed and incubated with phospholipase C.

Fig.1 shows the effect of EDTA-pretreatment on accessibility of phospholipids to phospholipase C in the parent P678 cells. It appears that a plateau is reached by 20 min. Similar kinetics were observed with EDTA-treated PM61 cells (results not shown). On the other hand, phospholipids in untreated PM61 were more rapidly digested, as shown in fig.2.

Of particular note is the phospholipase C sensitivity of PM61 and insensitivity of D22. Both strains have

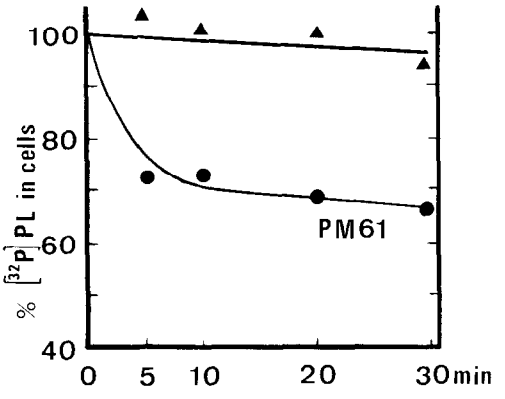

Fig.2. Hydrolysis of phospholipids by phospholipase C in *E. coli* PM61 (*envC*). At times indicated, ^{32}P-labeled cells incubated in the presence of phospholipase C were tested for undigested phospholipids as described in section 2. • = treated cells; ▲ = cells without enzyme.

Table 2
Antibiotics, detergents, and dye sensitivity of *E. coli* strains

Strain	CV	Acr	SDS	DOC	Act	Rif	Nov	Amp	Neo	Tet
P678	8	0	0	0	0	13	9	26	17	31
PM61	23	9	30	42	13	17	0	38	21	37
PM31	15	0	15	21	0	12	20	43	24	45
D21	0	0	0	0	0	12	11	14	17	30
D22	12	0	7	14	16	24	20	17	20	30
D31-m4	16	0	15	18	13	19	22	13	19	27
C35	16	0	20	23	16	30	25	29	20	37

Inhibition zone diameters (in mm) are average values of two tests. CV = crystal violet; Acr = acridine orange; SDS = sodium dodecyl sulfate; DOC = sodium deoxycholate; Act = actinomycin D; Rif = rifampicin; Nov = novobiocin; Amp = ampicillin; Neo = neomycin; Tet = tetracycline

complete LPS and are hypersensitive to many antibiotics. Also interesting is the observation that D31-m4, is phospholipase C-insensitive although another heptose-less deep rough C35 is sensitive. At this moment no explanation can be given for this difference. However, it cannot be ruled out that the modification of LPS core in deep-rough mutants is not always followed by the accessibility of phospholipids on the cell surface. On the other hand, phospholipase C-sensitive PM61 is not a deep-rough mutant and its outer membrane contains all major proteins (D. Karibian, unpublished results). The first question which thus remains is how lipophilic molecules penetrate through the outer membrane of phospholipase C-insensitive bacteria. The second question concerns the specificity or non-specificity of the lipophilic pathway.

Therefore, all strains used were tested for increased sensitivity to lipophilic and hydrophilic antibiotics, detergents and dyes. Table 2 shows that *env* mutants are hypersensitive to crystal violet and to detergents but their hypersensitivity to lipophilic antibiotics is not uniform. PM61 (*envC*) is hypersensitive to actinomycin D and rifampicin, PM31 only to novobiocin, and D22 (*envA*) to all three lipophilic antibiotics. The inhibition by hydrophilic antibiotics is stronger with all mutants tested but D31-m4.

Overall, the data indicate an increased sensitivity to lipophilic and hydrophilic drugs in all mutants tested, with the exception of heptose-less D31-m4, were hypersensitive to lipophilic agents only. However, a straightforward relation between phospholipase C sensitivity, LPS modification and antibiotic hypersensitivity does not seem to be a general rule. On the basis of the results presented in this paper it appears likely that the penetration of lipophilic molecules is not always related to the presence of heptose-less LPS in the outer membrane. One can speculate that the accessibility of phospholipids exposed on the cell surface might depend in some cases on the molecular weight of the interacting agent (phospholipase C = 20 000–25 000; actinomycin D = 1255). Moreover, the findings of Coleman and Leive [9], together with our results, lend support to a concept that the penetration of lipophilic molecules is more specific than supposed by Nikaido [18], and that it is controlled by several genes. The inverse effect of rifampicin and novobiocin on PM61 and PM31 is in agreement with this interpretation.

We are currently studying the phenotypic properties of other hypersensitive mutants. These studies, coupled with LPS and outer-membrane protein analysis, should produce further insight into the control of the lipophilic pathway.

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