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Release of periplasmic proteins induced in *E. coli* by expression of an N-terminal proximal segment of the phage fd gene 3 protein

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We used the enzymes β-lactamase and alkaline phosphatase to quantitatively evaluate the release of periplasmic proteins from *E. coli* cells transformed by plasmids harboring gene 3 of phage fd. Different deletion mutants of gene 3 released varying fractions of the enzymes. From these results we conclude that essentially the amino-terminal proximal part, upstream of the first glycine-rich region but not this region itself, is responsible for the excretion of periplasmic proteins in *E. coli* cells expressing the gene 3 protein of phage fd.

Filamentous phage; Adsorption protein; Glycine-rich region

1. INTRODUCTION

The Ff phages (fd, f1, M13) are filamentous, single stranded DNA phages which infect *E. coli* cells carrying the F episome. The phage recognizes the host's F-pilus via its gene 3 product (g3p) which is located on one end of the phage coat [1,2]. G3p is synthesized as a 424 amino acid long preprotein with a signal sequence 18 amino acids in length [3]. Besides its host recognition function, the protein is probably also involved in the process of DNA transfer into the host [4]. Furthermore, the protein gives rise to a pleiotropic effect in the host which results in higher detergent sensitivity, resistance to superinfection with pilus-specific phage, tolerance to certain colicins, and increased release into the medium of periplasmic proteins [5–7]. This report deals with a quantitative evaluation of the latter effect for two marker-enzymes of the periplasm (β-lactamase and alkaline phosphatase), and aims at a precise assessment of the site in the primary structure of g3p responsible for the leaky phenotype. This phenomenon has been already observed by Boeke et al. [6], but no quantitative data were presented. In their report the authors concluded that the first 98 amino acids are responsible for the pleiotropic effect of g3p and emphasized the importance of a glycine-rich domain present in this region (an involvement of the second glycine-rich domain, located at the middle of the polypeptide chain, was excluded). The present report stresses on the primary importance of the N-terminal amino acids, upstream of the first glycine-rich region, for the leakage effect.

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2. MATERIALS AND METHODS

2.1 *Bacteria and plasmids*

The genotypes of the *E. coli* strains used are: BRZ329 (TL45, phoR::Tn10), TL45 (TS100, delta (glpT-glpA)593; gyrA; [8]) and KK2186 (delta lac-pro, thi, strA, supE, endA, sbcB15, hsdR4, [F', traD36, proAB, lacI^q, lacZ delta M15]); all were obtained from Dr W. Boos, Konstanz. Strain BRA100 (phoR::Tn10) expressing alkaline phosphatase constitutively was constructed by P1-transduction [9] into strain KK2186. Transductants (blue colonies) were selected on LB-agar [9] containing tetracycline and 5-bromo-chloroindolyl-phosphate.

Plasmid pUC4K was described by Vieira and Messing [10]. PUA76K is a derivative of plasmid pUC8.1 [11]. It contains the coding sequence for g3p of phage fd without the promoter-terminator-region upstream of gene 3, and the kanamycin cartridge of plasmid pUC4K. Expression of gene 3 is controlled by the *lac*-promoter. For overproduction of the cloned g3p, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added. This plasmid was constructed by X. Garcés (Diplomarbeit, Konstanz, 1986).

Plasmids pIX445K, pIX249K and pIX200K [12] are basically the same as pUA76K, but with various deletions at the N-terminus of the gene 3 protein (Fig. 1). Each new N-terminus was provided with a synthetic signal sequence, identical with the native signal sequence of g3p. Plasmid pUB85K carries the coding sequence for the first N-terminal 85 amino acids of g3p including the signal sequence (Fig. 1). This gene 3 mutant was constructed using the polymerase chain reaction PCR [13]. To this end two different oligonucleotides complementary to sequences on opposite strands of the template DNA (pUA76K) were used as primers: The M13 reverse primer and a mutagenic primer which introduces a *Sful*-site 3' at the codon for amino acid 85 of g3p. The two primers flank the gene 3 segment that is to be amplified. The PCR product was digested with *Eco*RI and *Sful*, so that the resulting DNA codes for the N-terminal amino acids upstream of the first glycine-rich region, and was cloned into pUC8.1 digested with *Eco*RI and *Acc*I. Subsequently the kanamycin cartridge of pUC4K was introduced as a *Pst*I fragment into the plasmid.

All plasmids contain an ampicillin-resistance gene and therefore express β-lactamase.

2.2. *Growth conditions*

Bacteria carrying the different plasmids were grown in LB-medium [9] supplemented with the appropriate antibiotics. The cells were

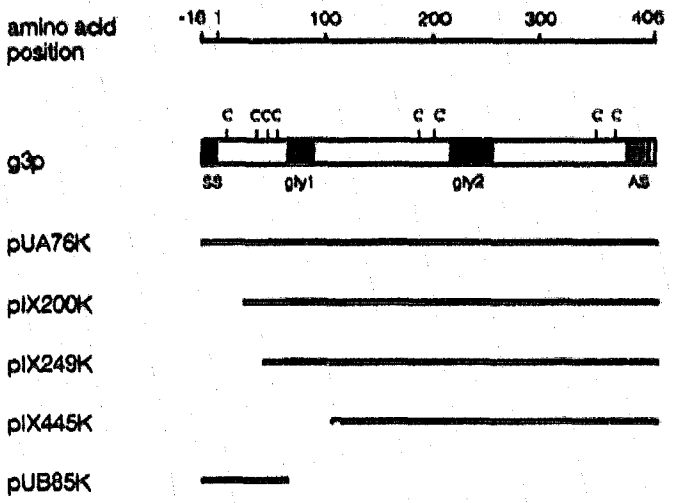

Fig. 1. Schematic representation of fd g3p as well as the deletion mutants constructed. Wild-type g3p is shown on the top. The scale refers to the amino acid positions. SS = signal sequence (18 amino acids); AS = membrane anchor sequence; gly = glycine-rich region; C = position of cysteine residues.

cultivated at 37°C with good aeration. Cultures of strains carrying a plasmid with gene 3 under the control of the *lac*-promoter were divided at an optical density (578 nm) of 0.2 and in one half gene 3 expression was induced with 1 mM IPTG.

Strain BRZ329 has no $lacI^q$-repressor gene and therefore the *lac*-operon (gene 3) is constitutively expressed.

2.3. *Enzyme assays*

β-Lactamase activity was determined according to Sawai [14] with benzylpenicillin as a substrate. At definite points during exponential and stationary growth, an aliquot of the cell suspension was spun down to remove whole cells and β-lactamase activity was determined in the supernatant. For quantitation of cell-bound β-lactamase, the pellet was resuspended in phosphate buffer and the cells were disrupted with toluene according to Miller [9].

The activity of alkaline phosphatase was determined colorimetrically at 405 nm using 4-nitrophenylphosphate as a substrate (1.5 M Tris buffer (pH 8.0) 14.5 mM nitrophenylphosphate, sample 1 ml each). Here, too, activities were measured both in the culture medium and in the cells (the pellet was resuspended in Tris buffer, pH 8.0, in this case).

2.4. *Detection of g3p by Western blotting*

To test if gene 3 is expressed after IPTG-induction of the *lac*-promoter, a Western blot was performed in each case. An aliquot of cell suspension was precipitated with trichloroacetic acid as described by Bross et al. [15], and electrophoresis of protein samples through SDS-polyacrylamide gels (12.5% w/v acrylamide) was done using the discontinuous buffer system of Laemmli [16]. The electrophoretic transfer to Immobilon membranes (Millipore) was performed with a semi-dry apparatus (Biometra) at recommended by the supplier. Bound anti-gene-3-antibodies (4) were stained as described by Blake et al. [17].

3. RESULTS

3.1. *Leakage induced by wild-type g3p*

To quantitatively investigate the leakage effect of wild-type g3p, *E. coli* strains BRZ329 and BRA100 were transformed with plasmid pUA76K and grown in L.B-kanamycin-medium. Bacteria transformed with pUC4K were used to quantitate the background release. PUC4K is identical to pUA76K except that it lacks gene 3, and therefore could be used as a negative control. As strain BRZ329 has no $lacI^q$-repressor gene, the *lac*-controlled gene 3 is constitutively expressed. Cultures of strain BRA100 transformed with plasmid pUA76K were divided at an optical density of 0.2 measured at wavelength 578 nm ($OD_{578} = 0.2$), and in one-half gene 3 expression was induced with 1 mM IPTG. Both cultures were grown further and samples were taken to evaluate β-lactamase and alkaline phosphatase activities as described in section 2. The results are summarized in Tables I and II for the exponential ($OD_{578} = 2.0$) and the stationary (after 24 h) growth phases.

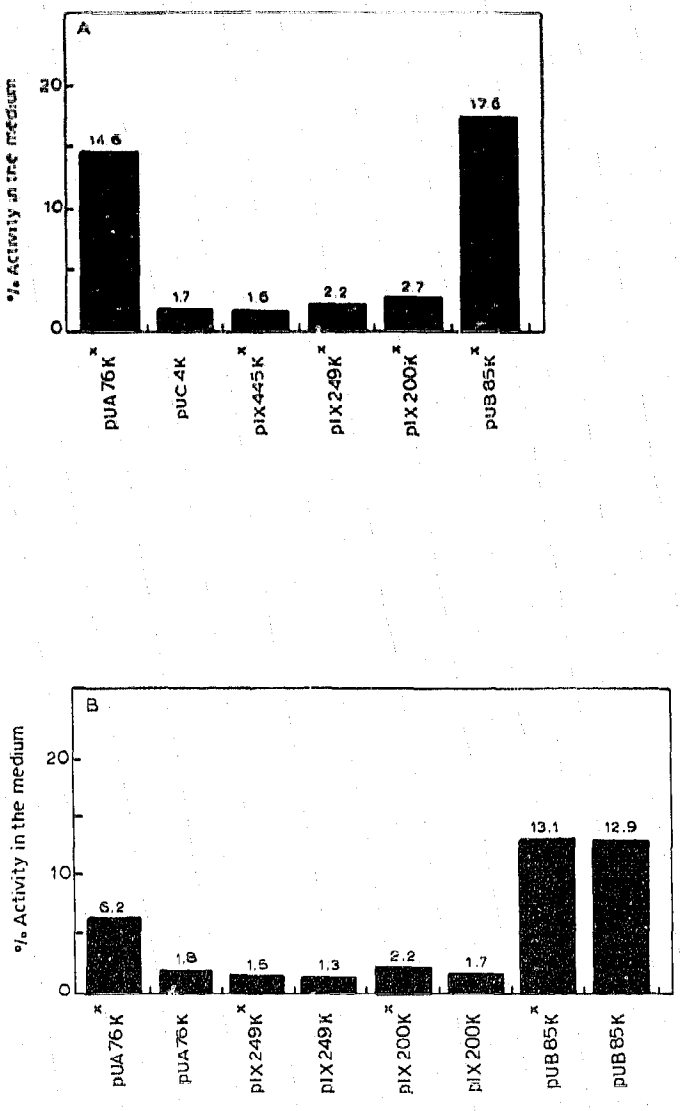

Fig. 2. Excretion of alkaline phosphatase from *E. coli* strains BRZ329 (A) and BRA100 (B) at the exponential growth phase ($OD_{578} = 2$). * g3p expression induced with 1 mM IPTG

During exponential growth, BRA100/pUA76K cells release about twice as much β-lactamase (a plasmid encoded protein) and 3 times more alkaline phosphatase (a chromosomally encoded protein) into the medium when g3p synthesis is induced. In strain BRZ329/pUA76K the secretion effect is even more pronounced: approximately 8 times more alkaline phosphatase and 4–5 times more β-lactamase are detectable in the medium when compared to BRZ329 transformed with pUC4K (no g3p).

In the stationary growth phase it is difficult to assess the leakage effect because cells begin to lyse.

3.2. *Leakage induced by g3p deletion mutants*

Using the experimental approach described above, the amount of the marker enzymes released into the medium in the presence of plasmids pIX445K, pIX249K and pIX200K (Fig. 1) was quantitatively determined.

BRZ329 cells carrying plasmid pIX445K, which codes for a gene 3 protein lacking 106 N-terminal amino acids (of the mature protein) including the first glycine-rich region (Fig. 1), shows essentially no increased release of both marker enzymes: the observed enzyme activities (Figs. 2A and 3A) in the culture medium correspond to the background leakage of these enzymes.

Only the marker enzyme alkaline phosphatase was measured in strains BRZ329 and BRA100 harboring plasmid pIX249K. In this plasmid 40 N-terminal amino acids of gene 3 were deleted, which means that the first glycine-rich region is now present. Here, too, no increased leakage could be observed (Fig. 2A,B).

When both strains were transformed with plasmid pIX200K, which lacks the 24 N-terminal amino acids of mature g3p the leakage of the marker enzymes is slightly higher than the background, but definitely much less than in the case of wild-type gene 3 (Figs. 2A,B and 3A,B).

In order to exclude that the glycine-rich sequence, together with the aminoterminal stretch of g3p is essential for the leakage phenomenon, we constructed the plasmid pUB85K, which codes for the first 67 amino acids of the mature g3p upstream of the first glycine-cluster. Cells transformed with this plasmid do leak the β-lactamase to about 59% in the exponential growth phase and the alkaline phosphatase to 13–18% (Figs. 2A,B and 3A,B). BRA100/pUB85K cells not induced with IPTG release periplasmic proteins into the medium to the same extent as induced cells. It seems that even the background expression of this latter mutant is sufficient to cause significant leakage (the *lac*-promoter is not completely repressed even without IPTG induction).

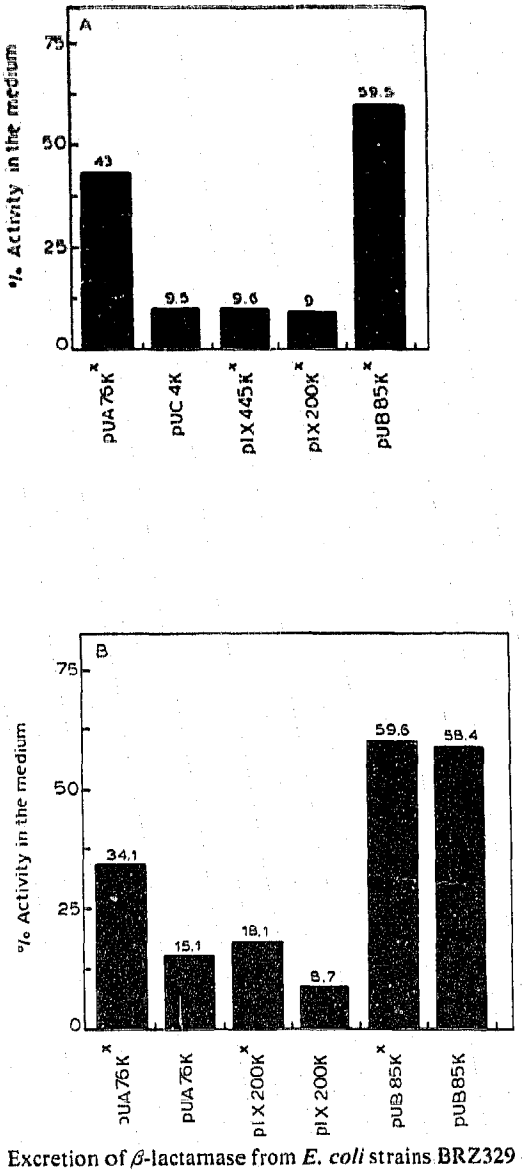

Fig. 3. Excretion of β-lactamase from *E. coli* strains BRZ329 (A) and BRA100 (B) at the exponential growth phase ($OD_{578} = 2$).
* g3p expression induced with 1 mM IPTG

4. DISCUSSION

To investigate the leakage induced by wild-type g3p or g3p fragments, the excretion of two periplasmic enzymes was quantitatively analysed: The plasmid encoded β-lactamase has a rather low molecular mass (29 000 Da), the second enzyme is the chromosomally encoded alkaline phosphatase (47 000 Da). As the *E. coli* strains used are phoR mutants, the latter enzyme is constitutively expressed. β-Lactamase could be measured only in those strains carrying a plasmid harboring the bla gene.

IPTG-induction of gene 3 in strains BRA100 and BRZ329 transformed with plasmid pUA76K (harboring wild-type gene 3) leads to an increased level of both enzymes in the culture medium (Tables I and II).

The efficiency of protein excretion seems to depend on the genotype of the strain used. Different *E. coli* strains release the same protein to different extents into

Table I

Leakage of alkaline phosphatase and β-lactamase activities in *E. coli* strain BRA100 transformed with plasmid pUA76K

g3p +/−	Growth phase[a]	Alkaline phosphatase activity			β-Lactamase activity		
		Total[b] (U/ml)	Cell-bound (%)	Medium (%)	Total[b] (U/ml)	Cell-bound (%)	Medium (%)
−	exp.	8.8×10^{-2}	98.2	1.8	4.4	84.9	15.1
−	stat.	7.3×10^{-2}	64.5	35.5	6.8	4.4	95.6
+	exp.	7.0×10^{-2}	93.8	6.2	5.0	65.9	34.1
+	stat.	6.6×10^{-2}	49.3	50.7	6.5	3.4	96.6

− = not induced and + = induced with 1 mM IPTG
[a]exp. = late exponential growth phase at OD (578 nm) = 2
[b]stat. = stationary growth phase after 24 h total enzyme activity (cell-bound + medium)
Values are in units (U = μmol/min) per 1 ml of culture

Table II

Leakage of alkaline phosphatase and β-lactamase activities in *E. coli* strain BRZ329 transformed with plasmid pUA76K and pUC4K respectively

g3p +/−	Growth phase[a]	Alkaline phosphatase activity			β-Lactamase activity		
		Total[b] (U/ml)	Cell-bound (%)	Medium (%)	Total[b] (U/ml)	Cell-bound (%)	Medium (%)
−	exp.	3.2×10^{-2}	98.3	1.7	28.1	90.5	9.5
−	stat.	3.0×10^{-2}	90.7	9.3	24.1	38.4	61.6
+	exp.	2.5×10^{-2}	85.4	14.6	2.6	57.0	43.0
+	stat.	2.6×10^{-2}	60.8	39.2	5.3	17.5	82.5

− = plasmid lacks gene 3 (pUC4K)
+ = plasmid harbors gene 3 (pUA76K)
[a,b]see legend to Table I

the medium when they concomitantly synthesize g3p (data not shown).

The higher background of β-lactamase (9–18% activity) found in the medium in the absence of g3p is probably a specific property of this enzyme, since alkaline phosphatase is invariably secreted only in minute amounts (less than 2%) by these cells.

In order to more precisely define the sequence elements of g3p involved in the leakage effect, strains BRA100 and BRZ329 were transformed with plasmids coding truncated g3p's:the pIX-plasmids 445, 249 and 200 code for g3p's with various deletions at the N-terminal part of the protein (Fig. 1). Since these g3-fragments were cloned behind a synthetic signal sequence, the products were translocated across the inner membrane [12]. Our results show that these gene 3 deletion mutants do not induce increased release of periplasmic enzymes. The enzyme activities detected in the medium correspond to the background values that were found in the absence of mutated protein.

Boeke et al. [6] stated that at least the 98 aminoterminal amino acids including the glycine-rich domain of gene 3 are necessary to induce the pleiotropic effect in *E. coli*. However, as far as the leakage phenomenon is concerned, our results clearly show that the deletion of as few as 24 N-terminal amino acids is sufficient to abolish leakage. In all of the 'non-leaky' mutants, the glycine-rich region is present, showing that this sequence motif is not solely, if at all, responsible for the leakage. In order to definitely assess the relevance of the glycine-rich domain for the leakage effect, a mutated gene 3 lacking this sequence was also constructed (plasmid pUB85K). This g3p mutant induces even more release than the wild-type g3p, thus demonstrating that only the N-terminal amino acids upstream of the first glycine-cluster are essential for the release of periplasmic proteins. We therefore suggest that these few amino-terminal amino acids induce membrane perturbation. (Cells expressing this g3p mutant are also sensitive to the detergent deoxycholate, whereas cells expressing the N-terminal deletion mutants of g3p are resistant; data not shown.) Perhaps the cystein residues included within the first N-terminal amino acids form a disulfid bridge(s) and consequently stabilize a specific structure arrangement (loop) which destabilizes the membrane.

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