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Molecular cloning and expression in *E. coli* of a *Salmonella typhi* porin gene

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Immunoscreening of a *Salmonella typhi* cosmid library in *E. coli* allowed the detection of clones producing a 36 kDa porin from *S. typhi*. The gene is efficiently expressed in an *E. coli* porin-less mutant and the protein is exported to the outer membrane envelope. Two clones which markedly differ in their level of expression have been isolated.

Outer membrane gene; Cosmid library; Porin expression; Typhoid fever; (*Salmonella typhi*)

1. INTRODUCTION

Porins are bacterial outer membrane proteins capable of forming pores that are involved in the permeability of low molecular mass substances of hydrophilic nature [1,2]. Porins from *E. coli* and *Salmonella typhimurium* have been extensively studied [3,4]. These proteins have also been described in other microorganisms [5] and have recently been reported as constitutive elements of some eukaryotic outer membrane organella [6,7] and curiously, as a bacteriophage-encoded protein [8]. Three porin genes have been isolated and characterized in *E. coli*: *ompF, ompC* and *phoE* [9–11]. In *S. typhimurium*, four porins have been reported: OmpF, OmpC, OmpD [4] and recently, a PhoE-like protein [12]. For *S. typhi*, the causal agent of typhoid fever, two porins of 35 and 36 kDa have been isolated [13] but no information regarding their gene structure is available. Since it has been demonstrated that *S. typhi* porins induce high titer antibodies in typhoid patients [14] and that *S. typhimurium* porins have a protective effect in mice under challenge infection [15,16], it appears very attractive to explore their use as a diagnostic tool for typhoid fever and in the development of a vaccine for humans. Toward this end we have initiated studies on the structure of these proteins and their genes. We report here the isolation and preliminary characterization of an *S. typhi* porin gene encoding a 36 kDa protein, which is expressed in the outer membrane of *E. coli*.

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2. MATERIALS AND METHODS

2.1. *Construction of an* S. typhi *genomic library*

High molecular mass DNA from *S. typhi* Ty2 was prepared according to Marmur [17]. The DNA was partially digested with *Hin*dIII and fractionated on a sucrose gradient [18]. DNA fractions ranging between 20 and 40 kb were pooled (approx. 5 μg) and ligated to 5 μg pHC79 (Bethesda Research Laboratories) linearized with *Hin*dIII and treated with calf intestine alkaline phosphatase [18]. The ligation was carried out overnight at 14°C. 2 μg concatemerized DNA were packaged using the GigaPack Gold System from Stratagene, following the supplier's instructions. As a host strain we used *E. coli* VCS 257 provided by the Stratagene packaging kit. A collection of 4500 ampicillin-resistant colonies was obtained.

2.2. *Library screening by colony immunoblotting*

After amplification, the library was screened with rabbit antiporin serum. NZY agar plates containing 10 g NZ amin (ICN), 5 g yeast extract, 2 g $MgSO_4 \cdot xH_2O$ and 15 g agar per l with 50 μg/ml ampicillin were inoculated with about 250 colonies from the library and grown overnight at 37°C. The colonies were transferred to moist nitrocellulose filters (Schleicher & Schuell) and left on fresh plates for an additional 3 h. Filters

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were then washed with 1 × PBS (50 mM phosphate buffer, pH 7.5; 0.15 M NaCl) containing 0.1% Tween 20. Filter blockage was performed with 3% BSA in 1 × PBS for 30 min at room temperature with continuous shaking. A 1:1000 dilution (in 1 × PBS-1% BSA) of the first antibody (previously absorbed with a crude sonicate of strain VCS 257) was added to the filters, and incubated for 45 min at room temperature. Filters were washed with 1 × PBS-0.1% Tween 20 and incubated for 45 min with a 1:2500 dilution of protein A-peroxidase (Amersham). After washing as above, filters were developed with a Bio-Rad HRP color developer kit. Positive colonies from master plates were isolated, grown in NZY medium and stored frozen at −70°C.

2.3. *Outer membrane preparation*

Bacterial cells were grown in 100 ml Luria broth and the required antibiotic until late exponential growth phase. Cells were pelleted, resuspended in 3 ml of 10 mM Tris-HCl, pH 8.0, and sonicated on ice three times for 1 min. The cell lysate was separated from intact cells by centrifugation at 12000 × *g* for 20 min. The supernatant was then ultracentrifuged (Beckman, Ti65) at 40000 × *g* for 30 min. The pelleted membrane preparation was thoroughly resuspended in 10 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$ and 2% Triton X-100, and then incubated at 37°C for 45 min with continuous shaking. The suspension was centrifuged at 80000 × *g* for 90 min. The membrane pellet was finally suspended in 100 μl of 100 mM Tris-HCl, pH 8.0, with 1% SDS.

2.4. *Porin purification*

Porin purification from *S. typhi* Ty2 was carried out as described by Calderón et al. [13]. This procedure results in the copurification of both porins. For isolation of single porins, purification was started from outer membrane preparation as described above, followed by a preparative 12% SDS-PAGE. The bands were cut and electroeluted at 75 mA for 6 h in a dialysis bag immersed in 200 ml of 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.3. The 36 kDa *S. typhi* porin obtained was free of contaminants and suitable for determination of amino acid composition and sequencing analysis.

2.5. *Porin antibodies*

Antisera against *S. typhi* porins were raised in rabbits according to standard procedures using the mixture of both *S. typhi* porins as antigens. Prior to immunization the copurified porins were denatured in 1% SDS.

2.6. *Gel electrophoresis and Western blotting*

Protein SDS-PAGE was carried out according to Laemmli [19] in a mini vertical gel apparatus. Usually, 12% polyacrylamide gels were run at 250 V for 30 min. High range prestained protein molecular mass standards (BRL) were used. Western transfers were performed according to Towbin et al. [20] using a miniblotting system, at 200 mA for 30 min. Nitrocellulose filters were blocked with 1 × PBS-1% BSA for 30 min at room temperature. Rabbit antiporin serum diluted 1:1000 (in 1 × PBS-1% BSA) was used as first antibody. Then, 1-min washes in 1 × PBS-0.1% Tween 20 were carried out. The second antibody added was either goat anti-rabbit IgG-peroxidase or protein A-peroxidase (Amersham) diluted 1:1000 and 1:2500, respectively, and incubated for 30 min at room temperature with continuous shaking. Other details were as described for the library screening. For DNA gel electrophoresis, 0.8 and 1.3% agarose gels were run in TAE buffer [18].

3. RESULTS

3.1. *Isolation of a 36 kDa* S. typhi *porin gene*

Colony immunoscreening of the library allowed us to isolate seven positive clones. No lysis of the colonies was necessary for antibody binding. Samples were prepared from total cellular extracts and from outer membrane preparations. These clones were analyzed by protein gel electrophoresis and Western blotting. Four clones, pSTP1-3, pSTP1-2, pSTP1-5 and pSTP2-1 are shown in fig.1. The blot indicated that OmpF and OmpC porins from *E. coli* VCS 257, as well as OmpA protein (lane 1) were recognized by the rabbit antiporin serum. Clones pSTP1-3, pSTP1-2, pSTP1-5 and pSTP2-1 (lanes 2–5) presented an extra immunoreactive band in addition to those from the host. Porins can be easily detected in both cell lysates and outer membrane preparations. Based also on an electrophoretic mobility similar to that of the *S. typhi* porins (see fig.4) we conclude that these clones encode a 36 kDa porin. One of these clones, pSTP2-1, presented a markedly lower level of expression. Altered electrophoretic mobilities when samples were not heated before loading the gel were also indicative of the porin nature of these proteins (not shown). We selected pSTP1-3 and pSTP2-1 for further studies because of their different level of expression.

3.2. *Characterization of the DNA from clones pSTP1-3 and pSTP2-1 and subcloning in pUC19*

DNA from both clones was extracted by the alkaline lysis method [18], digested with *Hin*dIII and analyzed in a 0.8% agarose gel (fig.2A). A four-*Hin*dIII-band pattern for pSTP1-3 (21, 12, 10, 6.4 kb) was obtained. A slightly different four-band pattern for pSTP2-1 (20, 11.6, 10.5, 6.4 kb) was observed. The 11.6 and 10.5 kb fragments appear as a doublet in the gel (fig.2A). Both clones included the 6.4 kb pHC79 vector fragment. In both clones the 36 kDa porin gene is contained in the larger *Hin*dIII fragment, since they release the 800 bp fragment after *Kpn*I digestion, as mention-

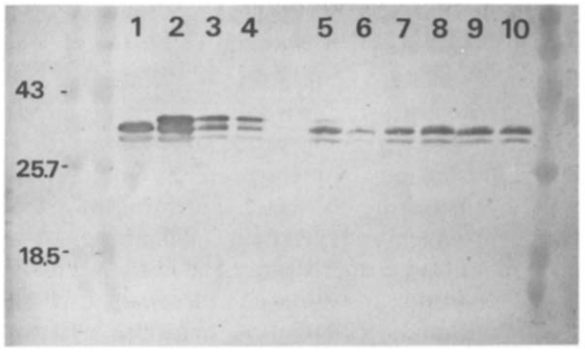

Fig.1. Detection of positive clones by Western blotting. Outer membrane preparations were separated in a 12% gel and screened with rabbit antiporin serum. Lanes: 1, *E. coli* VCS 257; 2, pSTP1-3; 3, pSTP1-2; 4, pSTP1-5; 5, pSTP2-1; 6–10, negative clones in VCS 257. Numbers indicate prestained molecular mass markers in kDa.

ed below (not shown). In addition, pSTP1-3 and pSTP2-1 were digested with *Kpn*I and *Bam*HI for subcloning in pUC19. *Kpn*I digestion products analyzed in a 1.3% agarose gel revealed six common fragments (20, 16, 2.1, 1.7, 0.9, 0.8 kb). Digestion of pSTP1-3 revealed the presence of a seventh band of 7.8 kb while digestion on pSTP2-1 resulted in two extra fragments being obtained (8.8, 1.6 kb) in addition to the six common fragments (fig.2B). For subcloning experiments, DNA from pSTP2-1 was separately digested with *Kpn*I and *Bam*HI, ligated to pUC19 linearized with the respective enzyme and used to transform *E. coli* DH5α. All positive clones resulted from *Kpn*I end ligations which were initially detected by colony immunoscreening. All synthesized a truncated protein of about 30 kDa when they were analyzed by Western blotting. Two of them, pSTP2K2 and pSTP2K3 are shown in fig.3 (lanes 2,3). The *Bam*HI constructions gave negative results (not shown). DNA from one of the *Kpn*I subclones, pSTP2K2, was isolated and when digested with *Kpn*I released an 800 bp insert (fig.2B, lane 4). These results, besides their different level of expression, suggest that the clones pSTP1-3 and pSTP2-1 are different in some way. However, both contain the same 800 bp *Kpn*I fragment present in pSTP2K2.

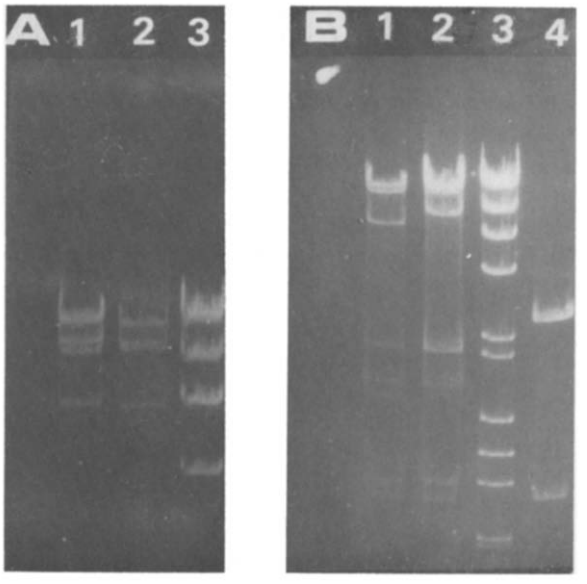

Fig.2. Restriction analysis of two clones containing an *S. typhi* porin gene. (A) DNAs from clones pSTP1-3 (lane 1) and pSTP2-1 (lane 2) were digested with *Hin*dIII and separated in a 0.8% agarose gel. (B) *Kpn*I digestions of pSTP1-3 (lane 1); pSTP2-1 (lane 2) and subclone pSTP2K2 (lane 4) analyzed in a 1.3% agarose gel. λ-*Hin*dIII and ϕX174-*Hae*III digested DNAs were included as standards (lane 3).

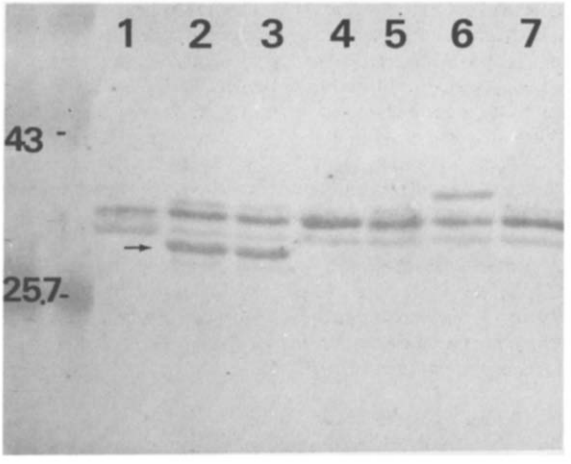

Fig.3. Expression of the clones encoding the 36 kDa porin and the truncated protein. Outer membrane protein preparations were analyzed in a 12% polyacrylamide gel and the bands transferred by Western blotting. The porins were detected using rabbit antiserum according to section 2. Lane 1 contained porins from *E. coli* VCS 257; lane 2, from subclone pSTP2K2; lane 3, subclone pSTP2K3; lane 4, *E. coli* DH5α; lane 5, a negative VCS 257 clone; lane 6, pSTP1-3; lane 7, pSTP2-1. The arrow indicates the 30 kDa truncated product. Molecular mass markers as in fig.1.

3.3. *Expression of* S. typhi *porin gene in an* E. coli *mutant*

E. coli strain UH 302, a mutant which lacks both OmpF and OmpC porins [21], represented a suitable host in which to explore the expression of foreign porins and it was used to confirm that clones pSTP1-3 and pSTP2-1 indeed carry the *S. typhi* 36 kDa porin. Strain UH 302 was transformed with DNA from clones pSTP1-3 and pSTP2-1 and the recombinants were selected by rapid colony immunoscreening. Expression of the 36 kDa porin was confirmed by Western blot analysis of total cell extracts. The results were further checked by SDS-PAGE protein analysis of outer membrane preparations of the recombinants and Western blotting analysis (fig.4). It is interesting to note that pSTP1-3 DNA also expressed the 36 kDa porin at a much higher level in this host as compared to the expression level shown by pSTP2-1.

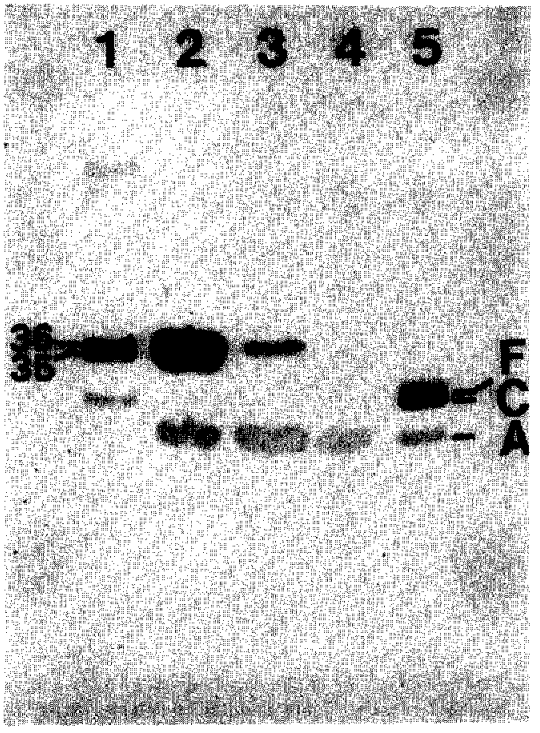

Fig.4. *S. typhi* porin expression of clones pSTP1-3 and pSTP2-1 in the porin-less mutant UH 302. Immunoblot of membrane preparations analyzed as in fig.2. Lanes: 1, *S. typhi* Ty2; 2, pSTP1-3; 3, pSTP2-1; 4, UH 302; 5, *E. coli* VCS 257. Bands marked F, C, and A correspond to OmpF, OmpC and OmpA, respectively. Numbers indicate positions of the 36 and 35 kDa porins.

4. DISCUSSION

We report here the isolation of two clones containing a gene expressing a 36 kDa porin gene from *S. typhi*. Our screening procedure was based on immunodetection of intact bacterial colonies. No lysis was necessary to detect expression of the *S. typhi* outer membrane protein, indicating that the protein is being properly processed and exported to the outer membrane in the *E. coli* strains used for cloning. Immunoscreening of intact bacterial colonies could therefore represent a simple and rapid general procedure to detect expression of outer membrane protein genes. Additional criteria to identify the expressed protein as a porin include its electrophoretic mobility, similar to that of *S. typhi* porins; its altered electrophoretic mobility if the protein is not heated prior to electrophoresis and the appearance of susceptibility to phage PH 221 [22] which uses *S. typhimurium* OmpC as a receptor. The phage also infects *S. typhi* Ty21A (Mora, G., personal communication), indicating that at least one of the *S. typhi* porins is also a receptor for phage PH 221. The fact that only clone pSTP1-3 was lysed by the phage PH 221 could be explained by the low level expression shown by clone pSTP2-1 or be due to the fact that the expressed protein is actually different. Although proteins from both clones show a similar electrophoretic mobility they may correspond to different outer membrane proteins. Electrophoretic mobility could be changed by an altered post-translation processing or changes in the interaction with LPS when these proteins are expressed in *E. coli*. Obtaining of the sequence of these clones, which is now under way, will help us to clarify this point and identify these genes.

The cloning and expression of these genes in *E. coli* will facilitate further studies to explore the use of porins in the diagnosis and as a potential antigen for a vaccine against typhoid fever.

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