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(54) **Title:** PROTEIN, THEIR FRAGMENT AND USE THEREOF

(57) **Abstract:** The isolated protein present in the extract of bacterial outer membrane proteins and its fragments suitable for using in medicine and pharmacy, especially for producing vaccines and diagnostic tests.

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Protein, their fragment and use thereof

The subject of this invention is method of isolation of diagnostic preparation, which is bacterial protein purified from bacterial mass of enterobacteria and also the method of its using in test for the determination of specific anti enterobacterial immunoglobulin deficiencies as an indicator of antibacterial immunity, which play a main role in pathogenesis of infectious diseases, by direct using of this protein preparation for the determination of specific anti-enterobacterial antibodies in human sera, also the method of using of this preparation in affinity chromatography for isolation of specific protective immunoglobulins as a therapeutic agent in immunodeficiencies, and also the method of using this preparation as a protein carrier in conjugate vaccines.

Outer membrane proteins (OMP) of Gram-negative bacterial cell wall participate in maintaining of bacterial cell integrity, its adaptation to the environment and in interactions with host cells [Koebnik R. et al. Mol. Microbiol., 2000, 37, 239-253]. These proteins named porins, exposed on the bacterial cell surface, are immunologically important molecules in many Gram-negative bacteria. These proteins may affect the physiological functions of tissues and participate in mechanisms of pathogenicity, progression of infections and in development of inflammatory response [Lin J. et al. Microbes and Infection, 2002, 4, 325-331]. In many inflammation processes caused by Gram-negative as well as Gram-positive bacteria, antibodies against bacterial proteins were found [Biswas T., FEMS Immunol. Med. Microbiol. 2000, 29, 129-136]. These specific features of bacterial proteins (OMP) and their accessibility to the host immune system make them attractive as components of vaccines, convenient carriers for carbohydrate antigens and

also as immunodiagnostic markers [Roy S. et al., Infect. Immun. 1994, 62, 4333-4338]. Shigellosis, salmonellosis and other diseases caused by representatives of *Enterobacteriaceae* family are still very important medical problem, especially in developing countries.

In our earlier studies on OMP from different serotypes of *S. flexneri* [Witkowska D. et al. FEMS Microbiology Letters 1982, 13, 109-111], *S. sonnei* [Adamus G. et al. Arch. Immunol. Ther. Exp. 1986, 34, 513-516] and *Hafnia alvei* [Witkowska D. et al. Arch. Immunol. Ther. Exp. 1988, 36, 711-715] we have shown almost identical electrophoretic profile of OMPs with two characteristic 33 K and 36 K major proteins [Witkowska D. et al. FEMS Microbiology Letters 1982, 13, 109-111]. In the outer membrane of *S. sonnei* a heat modifiable 33 K protein and two other, 35 K and 37 K associated with peptidoglycan proteins were present [Adamus G. et al. Arch. Immunol. Ther. Exp. 1986, 34, 513-516]. Moreover, we also observed the distinct immunogenic and protective properties of OMPs extracts from *Salmonella*, *Shigella* and *Hafnia* [Adamus G. et al. Infect. Immun. 1980, 30, 321-324; Mulczyk M. et al. Arch. Immunol. Ther. Exp. 1981, 29, 85-90; Mulczyk M. et al. Arch. Immunol. Ther. Exp. 1984, 32, 631-63512]. Immunization of animals with OMP from different serotypes of *Shigella* induced the cell and humoral mediated immunity, protected guinea pigs against keratoconjunctivitis shigellosa induced by homological and heterological strains of bacteria, protected mice for a long time against lethal dose of homological and heterological strains of bacteria [Adamus G. et al. Infect. Immun. 1980, 30, 321-324; Witkowska D. et al. Arch. Immunol. Ther. Exp. 1985, 33, 625-628; Witkowska D. et al. Arch. Immunol. Ther. Exp. 1986, 34, 499-504]. Complete protection against bacterial challenge was observed on fourth day after immunization, and the highest level of specific antibodies against OMP was observed on 10th-21th days after immunization. It was also shown that immunity in mice induced with OMP may be transferred passively to nonimmunized animals by the serum of immunized animals [Witkowska D. et al. Arch. Immunol. Ther. Exp. 1986, 34, 499-504]. It was also found that OMPs isolated from *Shigella flexneri* exhibit immunomodulatory properties, as small doses of OMPs stimulated delayed hypersensitivity to sheep red blood

cells, whereas higher doses suppressed this type of immune response. Immunochemical analysis of OMPs from *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* performed by Roy et all. showed similar electrophoretical profiles in all *Shigella* species with the major protein bands around 34-38 kDa, indicating them to be porins and confirmed our earlier results [Roy S et al. Microbios, 1994, 79, 318, 55-64]. Analysis of antigenic properties of OMPs from four *Shigella* species showed cross-reactivity of their major protein with molecular mass 38 kDa. In sera of mice immunized with formalinized bacteria of four *Shigella* species, specific antibodies of IgM and IgG class were shown, but in IgG class dominating was IgG1 subclass [Roy S et al. Microbios, 1994, 79, 318, 55-64].

Immunoglobulins are important effectors of specific humoral immunity. Different classes and subclasses of immunoglobulins exhibit distinct functions. The predominant subclasses of immunoglobulins G (IgG) elicited during natural infection could influence the capacity of the humoral response to provide an adequate defense of the host. Bacterial proteins are the antigens preferentially inducing IgG1 response, with minor contribution of IgG3 and IgG4 [Islam D. et al. Infect. Immun. 1995, 63, 2054-2061]. It was reported that acquired immunity to *Shigella* bacilli is mainly connected with the production of local secretory immunoglobulin A (IgA) and serum IgG, which are specific to some bacterial virulence proteins, which are the major bacterial cell surface components [Sansonetti P. and Phalipon A., Res. Immunol. 1996, 147, 595-602]. In our earlier studies we observed immunogenic and protective properties of OMP from *Shigella, Salmonella* and *Hafnia* [Mulczyk M. et al. Arch. Immunol. Ther. Exp. 1981, 29, 85-90]. In an animal model of shigellosis it was shown that the 38 kDa protein and three other outer membrane proteins were the major antigens in the induction of protective immune response [Witkowska D. et al. Arch. Immunol. Ther. Exp. 1992, 40, 119-124] and thus these proteins may be the excellent candidates as antigens or carriers in conjugate vaccines and may serve as immunodiagnostic markers. In order to investigate the reactivity of OMP with human sera from healthy donors and of immunodeficiency patients, we isolated these OMP from cell wall of *Shigella flexneri* bacilli and few other Gram-negative bacterial strains. Interestingly, we observed in immunoblotting the reactivity of OMPs with umbilical cord

plasma. This important observation of such beneficial reactivity indicates that cord plasma antibodies may be protective, because antibacterial protective antibodies are transferred from the mother to fetus. That reactivity was present in all examined children, adolescents and adults sera. Specific antibodies of IgA and IgG classes interacted primarily with 38 kDa protein, in similar way for several studied enterobacterial strains, but different for *Pseudomonas aeruginosa* from *Pseudomonadaceae* family. This indicates for the specificity towards *Enterobacteriaceae* strains, the major components of intestinal microbial flora, which translocate to the circulation and are responsible for the development of antibacterial immunity. Reactivity was then determined of sera of several groups of children with 38 kDa protein from *Sh. flexneri* purified by preparative electrophoresis. The reactivity of this protein with sera in ELISA was age dependent, decreased in infants below 4 months of life, increased from 12 months of life to 5 years and next in teenagers the ratio of reactivity became like in adults. These beneficial results show that 38 kDa protein is a major enterobacterial antigen recognized by human immune system and plays a protective role against enterobacterial infection. This allows to expect that this protein is non-toxic, non-pathogenic and may be useful as an antigen carrier in construction of conjugate vaccines. The specific reactivity of IgG and IgA estimated as normal, physiological is transferred from the mother to blood stream of fetus and thus protects newborn to infection during the first period of his life.

The subject of this invention is bacterial surface protein beneficially recognized by antibodies of healthy human without active infection, what indicates that correct level of protective antibodies is very important. The OMP preparations were obtained from *Shigella flexneri* strain and other Gram-negative strains, and reactivity of human sera was determined with 38 kDa protein isolated from *Shigella flexneri* strain 3a. Oczyszczone korzystnie z preparatu białkowego otrzymanego z masy bakteryjnej białko, będące przedmiotem wynalazku, było obecne w 27 szczepach należących do 9-ciu gatunków bakterii reprezentujących rodzinę *Enterobacteriaceae*. Wydajność preparacji mogą charakteryzować przykładowo wyniki, gdzie z 90 g masy bakteryjnej *Shigella flexneri* 2a uzyskano 535 mg preparatu białek błonowych (0.60%), z 85 g *Shigella flexneri* 3a

otrzymano 465 mg białek (0.55%) a ze 120 g masy bakteryjnej *Hafnia alvei* uzyskano 900 mg białek (0.75%). Wydajność preparacji (OMP) z masy bakteryjnej 9 serotypów *Sh. flexneri* wahała się w granicach od 0.3 % do 0.8%. Otrzymane preparaty białek charakteryzowano w elektroforezie na żelu poliakrylamidowym w obecności SDS i stwierdzono, że w przypadku *Shigella flexneri* są to mieszaniny białek, zróżnicowane w zależności od serotypu. Jednakże, spośród dwóch głównych białek o ciężarze cząsteczkowym 35 kDa i 38 kDa, charakterystycznych dla wszystkich badanych serotypów różnych gatunków z rodziny *Enterobacteriaceae* takich jak *Hafnia alvei* i *Salmonella*, lecz nie u *Pseudomonas aeruginosa*, stwierdzono wyraźną immunoreaktywność przeciwciał typu IgG i IgA w surowicach ludzi zdrowych tylko z jednym z głównych białek (OMP), o ciężarze cząsteczkowym 38 kDa. Immunoreaktywność taka występowała w przypadku białek ze szczepów: *Sh. flexneri, Klebsiella pneumoniae, Escherichia coli, Citrobacter, Proteus* i *Hafnia*, z mniejszą intensywnością w przypadku białek *Sh. sonnei*. Enterobakteryjne białko 38 kDa było rozpoznawane przez przeciwciała klasy IgG, IgM i IgA obecne w surowicach ludzkich i co jest bardzo ważną nowością, stwierdzano wyraźną reaktywność przeciwciał klasy IgG osocza krwi pępowinowej z tym głównym białkiem. Dotychczas badacze analizowali białka OMP w aspekcie właściwości ochronnych na modelu zwierzęcym w zakażeniach i rzadziej na surowicy ludzkiej w fazie chorobowej i w czasie rekonwalescencji, natomiast nasze podejście polegało na analizie krwi pępowinowej, surowicy dzieci i ludzi zdrowych. Ta ostatnia reaktywność w krwi pępowinowej wskazuje na ochronną rolę swoistych przeciwciał skierowanych na immunogenny epitop bakteryjny, który nie powinien być szkodliwy dla człowieka. Otrzymane rezultaty skłoniły nas do oczyszczenia tego białka i przeprowadzenia jakościowych badań jego immunoreaktywności z innymi surowicami ludzi dorosłych i dzieci w różnym wieku, także pacjentów z niedoborami immunologicznymi. Badania strukturalne tego białka metodą spektrometrii masowej fragmentów trypsynowych i ich identyfikacją z odpowiednimi sekwencji w bazach danych wykazały jego tożsamość z białkiem OmpF *E. coli*.

Istotą wynalazku jest izolacja immunoreaktywnego białka 38 kDa na przykład z *Sh. flexneri*, korzystnie stosując elektroforezę preparatywną na żelu poliakrylamidowym w obecności SDS i elucję ciągłą, oraz opracowanie korzystnie testu immunoenzymatycznego. Do testu używa się wyizolowanego białka korzystnie opłaszczonego na płytkach. Test okazał się przydatny w celu potwierdzenia roli białka OMP-38 we wrodzonej i nabytej odporności, gdyż poziom reaktywności przeciwciał klasy IgG i IgA jest znacząco niższy u dzieci niż u dorosłych, zgodnie z klasycznym obrazem rozwoju odporności. W przypadku surowic pochodzących od pacjentów z niedoborami IgA i IgG, w obydwu grupach poziom swoistych przeciwciał był znacząco niższy.

It should be emphasized that crude preparations of outer membrane proteins isolated from cells of *Sh. flexneri*, *K. pneumoniae*, *Hafnia* and *Citrobacter* comprise a multi-component mixture of proteins. The efficient method for purification of 38 kDa protein from *Sh. flexneri* appeared the preparative electrophoresis, beneficially one step process. Similarity between bacterial antigens and host antigens, called molecular mimicry, is considered as one of pathogenic factors in many autoimmunological diseases. Knowledge on the occurrence of such similarity between bacterial antigens and their host antigens, allows to exclude some bacterial proteins as potential components of safe and effective antibacterial vaccines. Identification of anti 38 kDa protein antibodies in cord blood plasma indicates that the mimicry phenomenon does not occur here and the protein may be used as an early marker of innate immunity, which is especially important for protection against intestinal bacilli. Moreover, bacterial protein 38 kDa may be considered as a potential marker for detection of immune deficiencies, and also as indicator used for monitoring of development of antibacterial immunity, also during therapy. Our observations indicate for the protective role of antibodies anti 38 kDa OMP. Such antibacterial protection is transferred from mother to fetus. The level alters of protective antibodies anti 38 kDa OMP and depends on age, what corresponds to classic pattern of development of immune response, resembling the development of antibacterial immunity typical to humoral response.

The presented invention permits beneficially to prepare the affinity column with chemically linked OMP 38 kDa protein and using this column enables beneficially purifying the specific antibodies from animal and human origin of anti enterobacterial specificity. Such antibodies have diagnostic and therapeutic value. Preparations of human immunoglobulins may be applied to the affinity column, thus producing beneficially the fraction of specific anti enterobacterial antibodies which bind to the affinity gel and also beneficially the fraction of non bound immunoglobulins with total value activity, for example antiviral, for further therapeutic applications. The column is used beneficially for several times. The use of substitutions with high doses of immunoglobulins may be harmful [Bernatowska E., Post. Hig. Med.Dośw., 2002, 56, Supl., 23-31], and the present invention permits beneficially for significant lowering of effective doses of immunoglobulin substitutions. There is urgent need for administering the preparations of specific antibacterial immunoglobulins to newborns [Gajewska E. et al., Post. Hig. Med. Dośw. 2002, 56, Supl., 103-126], children and adults in several cases [Wróbel G. et al., Post. Hig. Med. Dośw. 2002, 56, Supl. 59-68 and other articles in his volume], therefore the prezent invention will have broad application in therapy.

The present invention, thanks to obtained results of studies, enables beneficially also to apply the 38 kDa OMP protein as carrier for conjugate vaccines, useful and safe for its use as active for inducing the formation of protective antibodies.

Examples

1. Preparation of extract of bacterial outer membrane proteins (OMP).

Wet bacterial mass from 7 hours culture in liquid BHI medium at 37°C was obtained by centrifugation and washing with 10mM Tris-HCl buffer pH 7.6, containing 10 mM $MgSO_4$. Bacteria were suspended in the same buffer, containing 20 μg of RNase and 20 μg of DNase per ml, and then disrupted in an ultrasonic disintegrator for 10 min. The disrupted cell suspension was centrifuged at 7000 x g to remove undisrupted cells, and then the resulting supernatant was centrifuged at 150,000 x g for 1 hour to separate the envelope fraction. The cell envelope sediment was extracted twice with 10 mM Tris-HCl, pH 7.6, containing 10 mM MgSO4 and 2% Triton X-100 at room temperature to dissolve

the cytoplasmic membrane. After centrifugation at 150,000 x g, the sediment was extracted twice with the same buffer containing 2% Triton X-100 and 5 mM EDTA. The supernatant obtained after centrifugation at 160,000 x g contained OMPs, which were precipitated with 2 volumes of 95% ethanol. The OMP fraction was analysed by SDS-PAGE which showed to contain about 20 proteins. Fractions OMP contained less than 5% lipopolysaccharide (LPS), as calculated from 3-deoxyoctulosonic acid measurement.

2. Preparation of 38 kDa protein immunoreactive with human serum.

The preparative electrophoresis of the outer membrane proteins was performed beneficially using Prep Cell 491 apparatus (BioRad) with a 37-mm ID tube and 80 ml of 10% or 12.5% resolving gel and 20 ml of 5% stacking gel and buffer containing 25 mM Tris, 0.192 M glycine, and 1% SDS, pH 8.3, for both electrophoresis and elution. After application of the 30- to 40-mg sample of OMPs extract to the top of the stacking gel, electrophoresis was run at the maximum setting of 260 V and 109 mA. Elution was started when the bromophenol blue indicator band reached the base of the separating gel. Fractions of 1.4 ml were collected according to the protein, continuously monitored with an UV detector set at 280 nm, and checked with SDS-PAGE and immunoblotting. Fractions beneficially with the appropriate proteins were dialyzed against water, pooled, and concentrated using vacuum centrifugation. The OMP fractions were characterized in polyacrylamide gel at reducing conditions using 10% or 12,5% gels with standard methods.

3. Determination of the level of specific anti enterobacterial immunoglobulins in human serum.

The level of protective antibodies against enterobacteria was determined in human serum with ELISA test on plates coated with protein antigen. The polystyrene 96-wells plates were coated with a solution of the 38 kDa OMP from *Shigella flexneri* 3a cell wall (1 µg/100 µl antigen) in carbonate buffer, pH 9.6, at 37°C for 3 h and then at 4°C overnight. The plates were then blocked with 1% BSA in water. After blocking, wells were washed three times using 250 µl TBS-T per well (TBS-T is a solution of 20 mM Tris-HCl, 50 mM NaCl buffer, pH 7, containing 0.05% Tween 20). The wells were then filled with

serial dilutions of human sera and the plates were left at room temperature for 2.5 h. After washing three times with TBS-T, 100 µl of an alkaline phosphatase-labeled goat anti-human IgG or IgA diluted 1:10,000 in PBS was added to each well. Following incubation at room temperature for 1h, the plates were washed three times with TBS-T (250 µl/well) and then 200 µl/well of pNPP substrate (Sigma) was added. After 30 min the reaction was stopped by 50 µl of 3M NaOH and the optical density was read at 405 nm using a Dynatech MR 5000 Microplate Reader.

4. Fractionation of antibodies with the method of affinity chromatography.

The affinity chromatography column was prepared, namely with bound 38 kDa protein. Agarose gel, for example Sepharose 4B (Pharmacia) (10-20 ml) was activated with CNBr (Fluka) according to the standard procedure, preferentially for about 30 min at room temperature at a constant pH of 11. The excess of cyanogen bromide was washed with water and 0.1 M $NaHCO_3$ of pH 8.2. To the activated gel the protein 38 kDa OMP was added (30 mg) in 5 ml 0.1 M $NaHCO_3$ of pH 8.2 and gel was incubated for 2 hours with gentle rotation. Then the mixture was left at room temperature for 2 hours with equal volume of 1 M ethanolamine (POCh), and then at 4 °C overnight with gentle rotation. The gel was washed with water and left for 30 min with 2 M K_2HPO_4, then with water again. Finally, glass column (1 x 10 cm) was filled with gel suspended in the PBS without Mg^{2+}, Ca^{2+} as eluent.

Serum (20 ml) diluted two fold with PBS was precipitated with small portions of ammonium sulfate (9.6 g) and incubated at 4°C for 2 hours. Then the pellet was centrifuged at 3000 r/min for 30 min at 4°C. The pellet was solubilized in 10 ml PBS and dialyzed to PBS at 4°C with several changes of buffer. The salted-out antibodies were concentrated by ultrafiltration to a volume of 3 ml

Then the salted-out antibodies were fractionated of the affinity column with immobilized protein. Antibodies were applied onto the gel and column was washed with PBS without Mg^{2+}, Ca^{2+}. Fractions 2 ml were collected and checked for the protein content by measuring the absorbance at 280 nm. After washing out the proteins not bound with gel, the antibodies of low affinity to the antigen were eluted with 1 M NaCl in PBS without

Mg^{2+}, Ca^{2+}. The fraction of antibodies of high affinity to immobilized antigen to the gel was obtained by elution with using 3M KCNS (Reachim) in PBS without Mg^{2+}, Ca^{2+}. Finally, column was thoroughly washed with PBS without Mg^{2+}, Ca^{2+}. Material bound to the gel and then eluted was dialyzed to several changes of PBS and concentrated. Specific antibodies were concentrated to a volume of about 1 ml and stored at -20°C in 50% glycerol.

5. Preparation of conjugate

Antigen or hapten, for example carbohydrate (2-4 mg) and bacterial protein OMP 38 kDa as protein carrier (1-2 mg) were mixed together in water (about 0.2ml), lyophilized, and then heated at a temperature up to 130°C for several tens minutes, beneficially for 15-60 minutes. After the reaction the product was solubilized and dialyzed to PBS, and stored at -20°C.

Patent claims

1. An isolated protein occurring in extract of bacterial outer membrane proteins possessing molecular mass about 38 kDa, as well comprising the amino acid sequence which contains not less than 80% of identity to any sequence shown on fig. 1.
2. The protein of claim 1 wherein it is isolated from bacteria belonging to the family of *Enterobacteriaceae.*
3. The protein of claim 1 wherein it was isolated from bacteria belonging to the species *Shigella flexneri.*
4. A fragment of protein of claims 1-3 wherein it possesses an amino acid sequence selected among sequences underlined on fig. 1 but longer than 4 amino acids.
5. The application of protein of any claims 1 to 3 or its fragment of claim 4 for the producing of immunochemical tests.
6. The application of claim 5 wherein the produced tests serve for the analysis of specific antibodies in immunological deficiencies.
7. The application of protein of any claim of 1 to 3 or its fragment of claim 4 for producing of vaccines.
8. The application of claim 7 wherein the protein or its fragment are utilized for the producing of conjugate vaccines, beneficially as carriers.
9. The application of protein of any claim from 1 to 3 or its fragment of claim 4 for producing of carriers for affinity chromatography.
10. A method for producing of enterobacterial outer membrane proteins with molecular mass abort 38 kDa wherein from the extract of outer membrane proteins obtained from bacterial mass of *Enterobacteriaceae*, beneficially *Shigella flexnerii*, is obtained a single protein by preparative electrophoresis in denaturing conditions.

11. Affinity columns wherein they have the protein of any claim from 1 to 3 or its fragment of claim 4 covalently bound to chromatographic gel.
12. The metod of producing of animal antibodies protective against enterobacteria wherein the animals are immunized with protein of any claim from 1 to 3 or its fragment of claim 4 and from the obtained serum an immunoglobulin fraction is salted out, beneficially with ammonium sulfate.
13. The metod of producing therapeutic specific antibodies protective against enterobacteria wherein from human serum or from preparation of human immunoglobulins or from fraction of animal antibodies obtained with the metod of claim 12 are isolated on the affinity column of claim 11 a specific antibodies.
14. The metod of producing diagnostic conjugates or conjugate vaccines wherein protein of any claim from 1 to 3 or its fragment of claim 4 is covalently linked as carrier protein with antygen or hapten.

			AA	MW
OMPC-ECOL6	Q8CVW1	MKVKVLSLLV*PALLVAGAAN*AAEVYNKDGN*KLDLYGKVDG*LHYFSDDKSV*	50	41225
OMPC SALTI	P0A264	MKVKVLSLLV*PALLVAGAAN*AAEIYNKDGN*KLDLFGKVDG*LHYFSDDKGS*		41239
OMPC KLEPN	Q48473	MKVKVLSLLV*PALLVAGAAN*AAEIYNKDGN*KLDLYGKIDG*LHYFSDDKDV*		39663
OMPF-ECOLI	P2931	MMKRNILAVI*VPALLVAGTA*NAAEIYNKDG*NKVDLYGKAV*GLHYFSKGNG*		39333
OMPF-SALTI	Q56113	MMKRKILAAV*IPALLAAATA*NAAEIYNKDG*NKLDLYGKAV*GRHVWTTTGD*		40106
Q83RY4-SHIFL	Q83RY4	MMKRNILAVI*VPALLVAGTA*NAAEIYNKDG*NKVDLYGKTV*GLHYFSKGNG*		41184
OMPC ECOL6	Q8CVW1	DGDQTYMRLG*FKGETQVTDQ*LTGYGQWEYQ*IQGNAPESEN*NSWTRVAFAG	100	
OMPC SALTI	P0A264	DGDQTYMRIG*FKGETQVNDQ*LTGYGQWEYQ*IQGNQTEGSN*DSWTRVAFAG*		
OMPC KLEPN	Q48473	DGDQTYMRLG*VKGETQINDQ*LTGYGQWEYN*VQANNTESSS*DQAWTRLAFA*		
OMPF-ECOLI	P2931	ENSYGGNGDM*TYARLGFKGE*TQINSDLTGY*GQWEYNFQGN*NSEGADAQTG*		
OMPF-SALTI	Q56113	SKNADQTYAQ*IGFKGETQIN*TDLTGFGQWE*YRTKADRAEG*EQQNSNLVRL*		
Q83RY4-SHIFL	Q83RY4	ENSYGGNGDM*TYARLGFKGE*TQINSDLTGY*GQWEYNFQGN*NSEGADAQTG*		
OMPC-ECOL6	Q8CVW1	LKFQDIGSFD*YGRNYGVVYD*VTSWTDVLPE*FGGDTYGSDN*FMQQRGNGFA	150	
OMPC SALTI	P0A264	LKFADAGSFD*YGRNYGVTYD*VTSWTDVLPE*FGGDTYGADN*FMQQRGNGYA*		
OMPC KLEPN	Q48473	GLKFGDAGSF*DYGRNYGVVY*DVTSWTDVLP*EFGGDTYGSD*NFLQSRANGV*		
OMPF-ECOLI	P2931	NKTRLAFAGL*KYADVGSFDY*GRNYGVVYDA*LGYTDMLPEF*GGDTAYSDDF*		
OMPF-SALTI	Q56113	AFAGLKYAEV*GSIDYGRNYG*IVYDVESYTD*MAPYFSGETW*GGAYTDNYMT*		
Q83RY4-SHIFL	Q83RY4	NKTRLAFAGL*KYADVGGSFDY*GRNYGVVYDA*LGYTDMLPEF*GGDTAYSDDF *		
OMPC-ECOL6	Q8CVW1	TYRNTDFFGL*VDGLNFAVQY*QGQNGSVSGE*NDPDFTGHGI*TNNGRKALRQ	200	
OMPC SALTI	P0A264	TYRNTDFFGL*VDGLDFALQY*QGKNGSVSGE*NTNGRSLLNQ*NGDGYGGSLT*		
OMPC KLEPN	Q48473	ATYRNSDFFG*LVDGLNFALQ*YQGKNGSVSG*EGATNNGRGA*LKQNGDGFGT*		
OMPF-ECOLI	P2931	FVGRVGGVAT*YRNSNFFGLV*DGLNFAVQYL*GKNERDTARR*SNGDGVGGSI*		
OMPF-SALTI	Q56113	SRAGGLLTYR*NSDFFGLVDG*LSFGIQYQGK*NQDNHSINSQ*NGDGVGYTMA*		
Q83RY4-SHIFL	Q83RY4	FVGRVGGVAT*YRNSNFFGLV*DGLNFAVQYL*GKNERDTARR*SNGDGVGGSI*		
OMPC-ECOL6	Q8CVW1	NGDGVGGSIT*YDYEGFGVGA*AVSSSKRTWD*QNNTGLIGTG*DRAETYTGGL*	250	
OMPC SALTI	P0A264	YAIGEGFSVG*GAITTSKRTA*DQNNTANARL*YGNGDRATVY*TGGLKYDANN*		
OMPC KLEPN	Q48473	SVTYDIFDGI*SAGFAYANSK*RTDDQNQLLL*GEGDHAETYT*GGLKYDANNI*		
OMPF-ECOLI	P2931	SYEYEGFGIV*GAYGAADRTN*LQEAQPLGNG*KKAEQWATGL*KYDANNIYLA*		
OMPF-SALTI	Q56113	YEFDGFGVTA*AYSNSKRTND*QQDRDGNGDR*AESWAVGAKY*DANNVYLAAV*		
Q83RY4-SHIFL	Q83RY4	SYEYEGFGIV*GAYGAADRTN*LQEAQPLGNG*KKAEQWATGL*KYDANNIYLA*		
OMPC-ECOL6	Q8CVW1	KYDANNIYLA*AQYTQTYNAT*RVGSLGWANK*AQNFEAVAQY*QFDFGLRPSV*	300	
OMPC SALTI	P0A264	IYLAAQYSQT*YNATRFGTSN*GSNPSTSYGF*ANKAQNFEVV*AQYQFDFGLR*		
OMPC KLEPN	Q48473	YLATQYTQTY*NATRAGSLGF*ANKAQNFEVA*AQYQFDFGLR*PSVAYLQSKG*		
OMPF-ECOLI	P2931	ANYGETRNAT*PITNKFTNTS*GFANKTQDVL*LVAQYQFDFG*LRPSIAYTKS*		
OMPF-SALTI	Q56113	YAETRNMSIV*ENTVTDTVEM*ANKTQNLEVV*AQYQFDFGLR*PAISYVQSKG*		
Q83RY4-SHIFL	Q83RY4	ANYGETRNAT*PITNKFTNTS*GFANKTQDVL*LVAQYQFDFG*LRPSIAYTKS*		
OMPC-ECOL6	Q8CVW1	AYLQSKGKNL*GVVAGRNYDD*EDILKYVDVG*ATYYFNKNMS*PYVDYKINLL	350	
OMPC SALTI	P0A264	PSVAYLQSKG*KDISNGYGAS*YGDQDIVKYV*DVGATYYFNK*NMSTYVDYKI*		
OMPC KLEPN	Q48473	KDLNGYGDQD*ILKYVDVGAT*YYFNKNMSTY*VDYKINLLDD*NSFTRSAGIS*		
OMPF-ECOLI	P2931	KAKDVEGIGD*VDLVNYFEVG*ATYYFNKNMS*TYVDYIINQI*DSDNKLGVGS*		
OMPF-SALTI	Q56113	KQLNGADGSA*DLAKYIQAGA*TYYFNKNMNV*WVDYRFNLLD*ENDYSSSYVG*		
Q83RY4-SHIFL	Q83RY4	KAKDVEGIGD*VDLVNYFEVG*ATYYFNKNMS*TYVDYIINQI*DSDNKLGVGS*		
OMPC-ECOL6	Q8CVW1	DDNQFTRAAG*INTDDIVALG*LVYQF		207/375
OMPC SALTI	P0A264	NLLDKNDFTR*DAGINTDDIV*ALGLVYQF		189/378
OMPC KLEPN	Q48473	TDDVVALGLV*YQF		173/363
OMPF-ECOLI	P2931	DDTVAVGIVY*QF		162/362
OMPF-SALTI	Q56113	TDDQAAVGIT*YQF		133/363
Q83RY4-SHIFL	Q83RY4	DDTVAVGIVY*QF		164/362

Fig.1