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Fusion of Genes Encoding *Escherichia coli* Heat-Stable Enterotoxin and Outer Membrane Protein OmpC

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The OmpC outer membrane protein of *Escherichia coli* was used as a carrier molecule for the nonimmunogenic heat-stable enterotoxin STa. Two fragments of different lengths of the gene encoding STa were fused in vitro to the 3′ terminus of the truncated *ompC* gene. The resulting OmpC-STa hybrid proteins could be detected by L-[^{35}S]cysteine labeling, and they were processed and thus exported. All synthesized hybrid protein remained cell bound and was found by fractionation mainly in the periplasm. Immunoblot analysis showed that the hybrid proteins reacted in vitro both with anti-OmpC and anti-STa antibodies, and immunization of rabbits evoked an antibody response to either of these proteins.

The heat-stable enterotoxins (ST) and heat-labile enterotoxins produced by enterotoxigenic *Escherichia coli* are among the most important factors responsible for diarrhea in humans and farm animals (11, 16). *E. coli* ST toxins can be classified into STa and STb because of their host specificities and protein structures (1, 7). STa enterotoxins which are associated with diarrheal disease in humans and animals are small extracellular polypeptides of 18 or 19 amino acids, including six cysteine residues; they cause fluid secretion by stimulating guanylate cyclase in intestinal cells following binding to the brush border membrane receptor (3).

Because of their small size, STa enterotoxins are nonimmunogenic by themselves but can behave as haptens. Hence, a promising approach for development of immunoprophylactic agents against diarrhea caused by STa is the coupling of this protein to larger carrier molecules by chemical (5, 23, 24) or genetic (4, 18–20) means. We have studied the possibility of using the *E. coli* OmpC outer membrane protein (12) as a carrier protein for STa. Genetic fusion of genes encoding STa and OmpC proteins should result in cell-bound hybrid protein molecules with a possibly immunogenic STa epitope, and accordingly, a recombinant *E. coli* expressing such a gene fusion could provide novel whole-cell vaccine candidates for protection against diarrhea caused by enterotoxigenic *E. coli*.

In this report, we describe the fusion of STa to the carboxy end of a truncated OmpC protein. Plasmids pJS006 and pJS007 (18) were used as sources of the STa gene for fusions to the *ompC* gene. The gene in pJS006 encodes STa with 5 additional amino acids at the amino end of the protein, and the gene in pJS007 encodes an STa with a 35-amino-acid extension at its amino end. Both of these amino acid extensions originate in the natural precursor peptide of STa (21). Elimination of the single-stranded DNA termini generated by *Ava*I digestion of pJS006 or pJS007 with Klenow enzyme and digestion with *Hin*dIII provided two STa-encoding gene fragments. These fragments were then ligated with *Eco*RV-*Hin*dIII-digested plasmid pHSK48 (Fig. 1). The resulting fused genes encode the mature OmpC protein up to amino acid 293, followed by either 6 or 37 amino acids (pSS486 and pSS487, respectively; Fig. 1) from the fusion joint and the ST precursor peptide, as well as the 19 amino acid residues of mature ST.

The initial analysis of the hybrid proteins encoded by plasmids pSS486 and pSS487 was made by using the maxicell technique (17), which allows almost exclusive radiolabeling of plasmid-encoded proteins. Protein labeling was done with L-[^{35}S]cysteine supplied in M9 (9) minimal medium. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (6) and autoradiography of whole-cell lysates of the labeled maxicells of *E. coli recA ompR* strain PK235 (8) carrying either plasmid pSS486 or pSS487 revealed protein bands with approximate M_rs of 34,000 and 38,000, respectively (Fig. 2). These values corresponded closely to the M_r values predicted for the two hybrid proteins on the basis of known DNA sequence. Since native OmpC contains no cysteines (10) while STa has six cysteines in its primary structure (19), these L-[^{35}S]cysteine-labeled bands were considered most likely to represent the desired OmpC-STa hybrid proteins.

The immunological properties of the hybrid proteins were subsequently characterized by immunoblotting. Cell pellets obtained from 37°C overnight cultures of strains PK235(pSS486) and PK235(pSS487) were washed and lysed in electrophoresis sample buffer by being boiled. Lysates were electrophoresed and electrotransferred (2) to nitrocellulose filters for reaction with polyclonal anti-OmpC serum or monoclonal anti-STa antibodies (24), followed by addition of alkaline phosphatase or peroxidase conjugate and the required substrate. Incubation with anti-STa (Fig. 3) or anti-OmpC (data not shown) developed protein bands with M_r values corresponding to the predicted sizes for the hybrids encoded by plasmids pSS486 and pSS487. The values obtained also agreed with the estimates based on the experiments with radiolabeled proteins, confirming that the 34,000- and 38,000-M_r bands actually were protein products of the constructed gene fusions.

Inhibition of signal peptide cleavage by ethanol addition (15) before radiolabeling with L-[^{35}S]cysteine resulted in protein bands with slightly higher M_r values than those of the mature proteins (data not shown). This suggests that the signal peptides were cleaved at the OmpC-processing site, and thus the hybrid proteins were apparently exported to a

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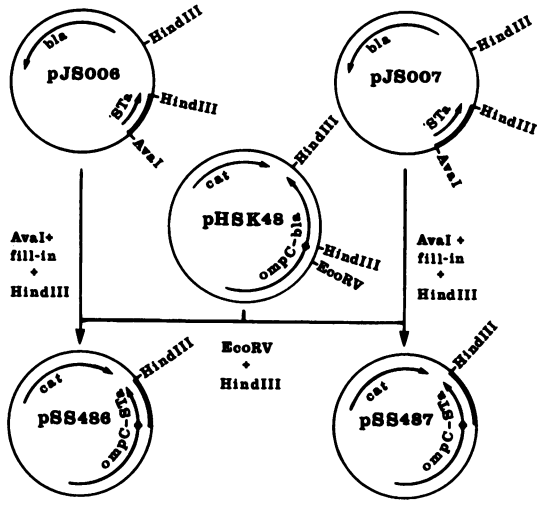

sequence of the fusion joints:

	ompC			STa		
	+292	+293	•	-5	-4	-3
pSS486	GAA	GAT	CCG	GGT	CCT	GAA
	+292	+293	•	•	-35	-34
pSS487	GAA	GAT	CCG	GGT	CCT	CAG

FIG. 1. Construction of plasmids pSS486 and pSS487. Plasmids pJS006 and pJS007 were digested with *Ava*I, the single-stranded termini were filled in with the Klenow fragment of DNA polymerase I, and the linearized plasmids were ligated with an *Eco*RV digest of pHSK48 at a high concentration of DNA. The ligated DNA was cut with *Hin*dIII, followed by a second ligation at a low DNA concentration to favor circularization of plasmid molecules. The nucleotide sequences at the gene fusion joints of the resulting plasmids, pSS486 and pSS487, were determined by the dideoxy-chain termination method in M13 (13).

noncytoplasmic location. The hybrid proteins appeared to be exclusively cell bound; no bands corresponding to the hybrid proteins or their degradation products could be detected in the immunoblots with anti-STa (Fig. 4A) or anti-OmpC (data not shown) from the culture supernatants. However, we also examined the supernatants and the cell-associated proteins for free (active) STa by using an enzyme-linked immunosorbent inhibition assay (24) which specifically detects free but not coupled or fused STa. The supernatants contained low

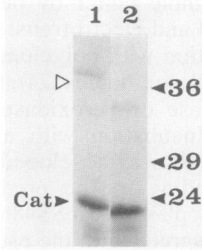

FIG. 2. Analysis of plasmid-encoded proteins. Maxicells were prepared from the fusion-carrying strains by the method of Sancar et al. (17). Samples labeled with 25 μCi of L-[^{35}S]cysteine were electrophoresed in an SDS–12% polyacrylamide gel. Protein bands were visualized by autoradiography of dried gels. Lanes: 1, PK235(pSS487); 2, PK235(pSS486). The position of the chloramphenicol acetyltransferase (Cat) encoded by the plasmids is indicated. The position corresponding to the M_r of the native OmpC protein is shown by an open arrowhead. The M_r markers indicated (Sigma MW-SDS-70L Kit) are expressed in thousands.

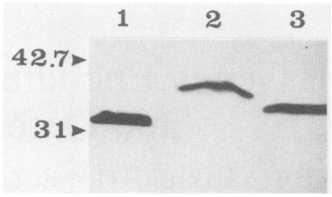

FIG. 3. Detection of OmpC-STa hybrid proteins with anti-STa antibodies. Total-cell sonic extracts were electrophoresed in an SDS–13.5% polyacrylamide gel, electrotransferred to nitrocellulose, and reacted with anti-STa monoclonal antibodies. Lanes: 1, LTA-STa hybrid protein (18) with an approximate M_r of 32,800; 2, PK235(pSS487); 3, PK235(pSS486). The arrowheads indicate the positions of the molecular weight markers carbonic anhydrase (M_r, 31,000) and ovalbumin (M_r, 42,700).

levels of STa activity, in contrast to the cell-associated fractions, which showed no activity at all. We interpret this STa activity to represent material released from the degraded OmpC-STa fusion protein.

Further localization of the OmpC-STa hybrid proteins was made by fractionation of the gene fusion-carrying cells by isopycnic sucrose density gradient centrifugation (14). The

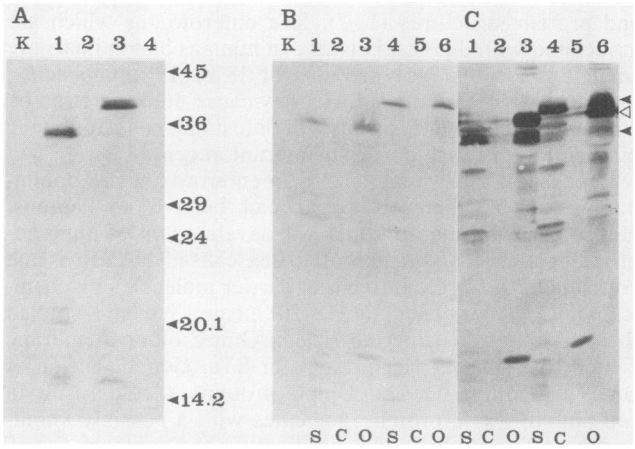

FIG. 4. Subcellular localization of OmpC-STa hybrid proteins. (A) Cells were grown to the early stationary phase in liquid L medium and collected by centrifugation, and equivalent amounts of cells and culture supernatant were subjected to electrophoresis in a SDS–12% polyacrylamide gel before electrotransfer to a nitrocellulose filter and reaction with anti-STa monoclonal antibodies. Lanes: 1, PK235(pSS486) cells; 2, PK235(pSS486) supernatant; 3, PK235(pSS487) cells; 4; PK235(pSS487) supernatant; K, control PK235 cells and supernatant. The M_r values of the standard proteins used (Sigma MW-SDS-70L Kit) are expressed in thousands. (B) Cells grown to the early stationary phase in liquid L medium were harvested, washed, and disrupted with a French pressure cell. The soluble fraction was collected by pelleting the cytoplasmic and outer membranes by ultracentrifugation, followed by separation of the two membranes in an isopycnic sucrose density gradient. The different subcellular fractions were subjected to immunoblot analysis with anti-STa antibodies as for panel A. Lanes: 1 to 3, PK235(pSS486); 4 to 6, PK235(pSS487); K, control PK235 cells. S, C, and O at the bottom correspond to the cytoplasmic-periplasmic, cytoplasmic membrane, and outer membrane fractions, respectively. (C) Samples in lanes 1 to 6 were prepared as for panel B, except that the protein bands were reacted with anti-OmpC serum. The positions of the hybrid proteins are indicated by filled arrowheads, and that of the native OmpC protein is indicated by an open arrowhead.

membrane and periplasmic-cytoplasmic fractions obtained by this procedure were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot reactions. Use of either anti-STa antibodies (Fig. 4B) or anti-OmpC serum (Fig. 4C) led to the identification of bands of the expected M_rs for the corresponding OmpC-STa hybrids in the soluble (periplasmic-cytoplasmic) and outer membrane fractions but gave no reaction in the cytoplasmic membrane fraction. It is worth noting that the level of expression of the gene fusions in the OmpR$^-$ background used (PK235) appeared to be comparable to that of the single chromosomal *ompC* gene (Fig. 4C), thus yielding roughly 10^4 hybrid protein molecules per cell.

The immunogenicity of the OmpC-STa hybrid proteins described has been tested only after immunization of rabbits with whole cells expressing the described gene fusions. Three subcutaneous immunizations with 1×10^9 to 5×10^9 *E. coli* cells per dose harboring either pSS486 or pSS487 evoked an antibody response to STa, and the anti-STa titer in a GM1 enzyme-linked immunosorbent assay (22, 24) was slightly higher for the shorter of these OmpC-STa hybrid proteins (a titer of 290 compared with 190 for the larger hybrid). The immune sera obtained also contained antibodies reacting with OmpC in immunoblot analyses (data not shown). However, the anti-STa antibody levels attained by these immunizations were quite low (i.e., insufficient to neutralize STa activity) in comparison with those of our highest-titered anti-ST immune sera (anti-STa titer, 10,000), which were obtained after repeated immunizations with STa chemically coupled to cholera subunit B (23, 24). This is not surprising in view of the much lower content of specific antigen in the whole-bacterial-cell immunizations (in terms of STa hapten, calculated as less than 0.04 to 0.2 μg per dose compared with ca. 10 μg per dose for STa coupled to cholera subunit B). It is quite possible, therefore, that immunizations with the purified proteins can give rise to both anti-OmpC and anti-STa antibodies at high titers which are neutralizing. We are also developing new hybrids containing all of OmpC so as not to miss protein sequences crucial for outer membrane localization. Some of the hybrids will carry short STa-related antigenic peptides lacking toxicity (23). Immunization with the latter proteins or with cells bearing them holds greater promise in terms of vaccine safety than the use of hybrids carrying a native STa.

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