Immunoactive chimeric ST-LT enterotoxins of *Escherichia coli* generated by in vitro gene fusion

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Two different lengths of the gene encoding *Escherichia coli* heat-stable toxin (STa) were fused to the carboxy end of the gene coding for the *E. coli* heat-labile toxin A-subunit (LT_A). The hybrid genes directed expression of chimeric LT_A-STa proteins. Association of these chimeras with native heat-labile toxin B-subunit (LT_B) resulted in protein complexes that bound to GM_1 ganglioside and thereby could be assayed in a GM_1 ELISA. The complexes reacted with monoclonal antibodies against either LT_A, LT_B or STa indicating that the STa and LT epitopes remained immunologically intact after fusion. Genetically constructed chimeric proteins exhibiting LT and STa antigens on the same molecule may represent a promising approach to development of broadly protective immunoprophylactic agents and/or useful immunodiagnostic reagents for diarrhoeal diseases caused by enterotoxinogenic *E. coli*.

Gastrointestinal disease *Hybrid protein* *Bacterial vaccine* *Immunoenzyme technique* *Synthetic gene*

1. INTRODUCTION

Strains of *Escherichia coli* which produce enterotoxins are the main causative agents of watery diarrhoea in both humans and domestic animals [1,2]. These organisms produce either or both of two main types of enterotoxins, a heat-labile toxin (LT) and heat-stable toxins (ST) [3]. LT is very similar to cholera toxin (CT) structurally and biochemically, having an ADP-ribosylating A-subunit [4,5] (or LT_A) and five B-subunits (LT_B) that mediate binding to cell membrane receptors consisting of GM_1 ganglioside and/or a closely related glycoprotein [6,7]. The ST toxins can be subdivided into STa and STb according to their host specificity [8] and these entities have now also been found to differ substantially in protein structure [9]. The STa enterotoxins are small polypeptides of either 18 or 19 amino acids including 6 cysteine residues and have been found to stimulate guanylate cyclase activity in intestinal cells after binding to brush-border membrane receptors [10–12]. Different from the LT subunits which are good immunogens, STa is non-immunogenic unless coupled to a carrier protein, i.e. it behaves as a hapten [13,14]. The genes encoding LT and STa have been isolated and their nucleotide sequences determined with accurate location of restriction sites within the genes [15–20]. We here exploit the unique *Eco*RI site at the carboxy end of LT_A to achieve in vitro gene fusions containing almost the entire LT_A gene joined to two different lengths of the STa gene. The hybrid genes obtained were shown to direct successfully the expression of chimeric LT_A-STa proteins capable of associating with LT_B. The resulting LT-STa complex bound to GM_1 ganglioside and was recognised by monoclonal antibodies against both the LT_A and STa components.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and DNA manipulations*

The *E. coli* strains JM83 [21] and HB101 [22] were used as recipients of the different plasmids. Strains were stored frozen at −70°C in 15% glycerol until use. Isolation of plasmid DNA by CsCl/ethidium bromide gradients and transformation were performed essentially as described by Maniatis et al. [23]. Conditions used for restriction and ligation of DNA were as recommended by the supplier of the different enzymes. All enzymes were purchased from New England Biolabs. Isolation of DNA fragments from 1.8% agarose gels was done by a method based on centrifugation [24]. The synthetic linker oligonucleotides were obtained as complementary strands from SYN-TEK AB (Umeå, Sweden). Plasmid pJS004 is a derivative of pSLM004 [20] generated by cloning the *Taq*I fragment with the STa gene into the same pBR322 vector but in opposite orientation. Plasmid pTRH002 was obtained by subcloning the *Pst*I fragment harbouring the LT operon from pWD600 [25] into pACYC177 [26]. Plasmid pUC18 [27] was employed in the intermediate cloning of the STa gene with the synthetic linkers (see section 3).

2.2. *ELISA assays*

Strains were grown overnight in liquid LB broth containing the appropriate antibiotics. For sonication the cells were resuspended in 1 vol. phosphate-buffered saline (pH 7.2) and disrupted by two 30-s bursts before removing cell debris by centrifugation. GM_1 ELISA assays specific for LT_A and LT_B were done as described in [28] but using monoclonal antibodies instead of polyclonal antisera. The same assay principle was used to test for the presence of the hybrid proteins using an STa specific monoclonal antibody with the modification that the peroxidase reaction was stopped with 2.5% H_2SO_4 and read at 492 nm to improve the sensitivity of the test. A GM_1 STa inhibition ELISA for quantitative determination of STa has been reported [29].

3. RESULTS

3.1. *LTA-STa gene fusions*

The gene encoding mature STa was isolated as an *Ava*II-*Bam*HI fragment from pJS004 [20] and fitted in vitro with a linker oligonucleotide that changed the *Ava*II end to one complementary to *Eco*RI. The fragment was then ligated to *Eco*RI-*Bam*HI digested pUC18 to give plasmid pJS006 (fig.1A). Plasmid pJS007 (fig.1A) was obtained in

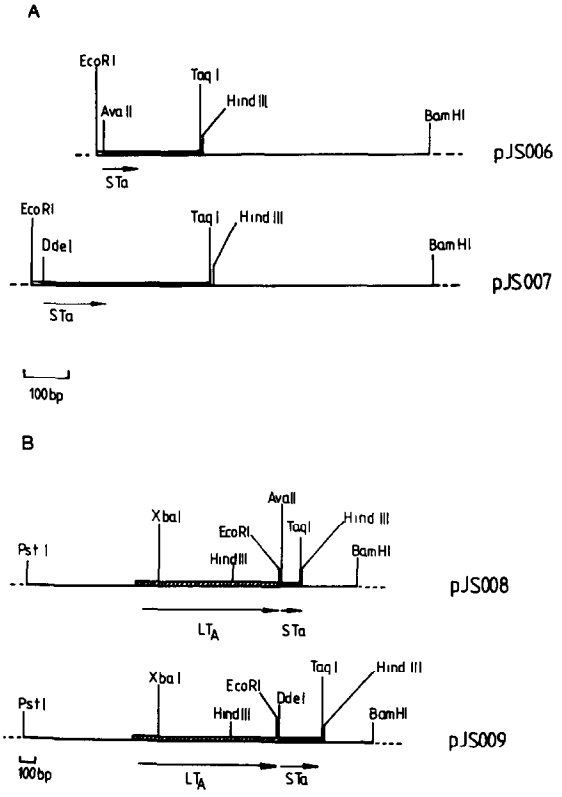

Fig.1. Inserts carrying the synthetic linkers at the *Ava*II and *Dde*I ends of the restricted STa gene (A) and after their fusion to the *Eco*RI end of the LT_A gene (B). (A) Plasmid pJS006 carries the *Ava*II-*Bam*HI STa insert after addition of the *Ava*II-*Eco*RI linker while pJS007 has the *Dde*I-*Bam*HI STa segment after ligation of the *Dde*I-*Eco*RI synthetic linker. Fusion to the *Eco*RI-*Bam*HI LT_A fragment from plasmid pTRH002 (see section 2) of the *Eco*RI-*Bam*HI insert from pJS006 gives plasmid pJS008 while fusion of the *Eco*RI-*Bam*HI fragment from pJS007 generated pJS009. In (B) not all the plasmid is shown but the *Pst*I site is given for orientation. The thick line in (A) and (B) shows the DNA from the original pSLM004 [20]. The white box in (A) represents the adapters and is not to scale. The hatched line in (B) indicates the LT_A gene [15]. The arrows indicate the direction of transcription.

a similar manner and contained a stretch of the STa gene encompassing the *Dde*I site located further upstream [20]. In this construction a synthetic linker was joined to the *Dde*I end to convert it into an *Eco*RI complementary terminus. Plasmids pJS006 and pJS007 were subsequently restricted with *Eco*RI and *Bam*HI to reisolate the modified STa gene fragments from agarose gels (see section 2). The purified fragments were then ligated to the *Eco*RI-*Bam*HI segment from plasmid pTRH002 that now contained the LT_A and the kanamycin resistance genes [25,26], thus allowing positive selection of extransformants. The *Eco*RI site in this segment lies at the carboxy end of the LT_A structural gene [17].

The linker oligonucleotides were designed in such a way that the reading frame of both the LT_A and STa genes was maintained. This procedure (a detailed description will be published elsewhere) ensured translation of the fused genes into linked LT_A-STa peptide chains. Fusion of the modified *Ava*II-*Bam*HI fragment to the LT_A gene gave plasmid pJS008 (fig.1B), while fusion of the modified *Dde*I-*Bam*HI resulted in plasmid pJS009 (fig.1B). Plasmid pJS008 directed expression of a protein containing LT_A up to amino acid 236 [17], the mature STa, and the five amino acids immediately upstream to it. In addition, the linker itself encoded arginine and proline which lay between the two proteins. Plasmid pJS009 coded for a protein that had the same 236 LT_A amino acids but joined to amino acid 35 upstream the start of STa, arginine, proline and glycine as a three amino acid extension encoded by the linker, and the mature STa itself.

3.2. *Immunoreactivity of the hybrid proteins*

Plasmids pJS008 and pJS009 were transformed into HB101 strains containing the tetracycline resistance plasmid pWD615 which encodes LT_B [25] and the transformed strains were tested for the expression of LT_B/LT_A-STa protein complexes which bound to GM_1 and could be detected in GM_1 ELISA using STa monoclonal antibody. After overnight growth of cells in liquid medium, cell sonicates of HB101 (pJS008, pWD615) and, even more strongly, HB101 (pJS009, pWD615) reacted with the STa monoclonal antibody in GM_1-coated ELISA plates (fig.2). In contrast, the HB101 strain

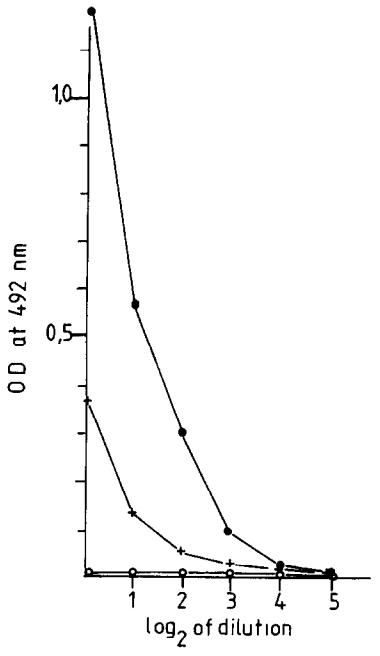

Fig.2. Detection of the STa antigen with specific monoclonal antibodies in the LT_B/LT_A-STa complexes after binding to GM_1-coated plates. The vertical axis represents the absorbance values obtained from the ELISA reader (Titertek). The horizontal axis indicates a logarithmic expression of each 2-fold dilution of the sonicates tested. Sonicates were obtained from the LT_B-only HB101 strain carrying pWD615 [15] after transformation with pJS008 (+) or with pJS009 (●). A negative control HB101 carrying both pTRH002 (LT plasmid) and pJS004 (STa plasmid) is shown (○).

carrying only pJS008 or pJS009 or pWD615 or both the LT plasmid pTRH002 and the STa plasmid pJS004 (see section 2) completely failed to give a positive reaction in this assay (fig.2).

Semiquantitative detection of LT_A and LT_B antigens was also performed by GM_1-ELISA using monoclonal antibodies, and in addition STa was measured by the GM_1-STa inhibition ELISA with the same STa monoclonal antibody as used in the direct STa GM_1-ELISA. The results of these analyses are presented in table 1, and provide evidence that not only the STa but also the LT_A portion of the fused gene product directed by pJS008 and pJS009 in these various strains retained antigen activity.

Table 1

Detection of LT and STa antigens

Plasmids carried by the HB101 host	Antigens encoded	μg/ml of culture[a]			STa by direct GM_1-ELISA
		LT_B	LT_A	STa by GM_1 inhibition ELISA	
pJS008	LT_A-STa hybrid	ND[b]	ND	0.40	–
pJS009	LT_A-STa hybrid	ND	ND	0.35	–
pJS008, pWD615	LT_A-STa hybrid, LT_B	7.34	10.13	NA[c]	+[e]
pJS009, pWD615	LT_A-STa hybrid, LT_B	2.03	4.01	NA	+
pTRH002, pJS004	LT (holotoxin), STa	1.47	–[d]	4.4	–[e]
pWD615	LT_B	3.9	ND	–[d]	–
pJS004	STa	ND	ND	6.7	–

[a] Data on LT_A and LT_B were obtained by GM_1-ELISA and are provided for cell sonicates while the STa values are for culture supernatants. Where values are given they are expressed as equivalents of pure CT or STa antigens

[b] ND, not detectable (values below 0.005 μg/ml are considered negative). Either antigen is absent or binding to GM_1 does not occur

[c] NA, not applicable. Interference by the positive effect of the STa bearing hybrid

[d] Data not available

[e] See fig.2

4. DISCUSSION

The strategy here taken of manipulating the LT_A subunit to be part of a holotoxin-like quaternary structure was decided upon on the assumption that the addition of the relatively small STa peptides at the carboxy end of LT_A would not prevent it from associating with LT_B. The fusions we have constructed indeed result in LT_A-STa hybrid molecules able to form a complex with LT_B as shown by their binding to GM_1. Additionally, the complexes generated are recognised by LT and STa specific monoclonals which suggests that folding of the engineered peptide chains is close to their native structure. There was, however, consistently lower reactivity towards the STa antibody by the fusion product encoded by plasmid pJS008 as compared to that from pJS009. This suggests that the shorter spacing peptide between LT_A and mature STa in pJS008 may prevent the latter from adequately exposing the STa epitope. Evidence that folding of the LT_A portion (and LT_B) is correct is found in the level of this antigen(s) indicated in the ELISA (table 1). Accordingly, the presence of a 35 plus amino acid extension in pJS009 seems to allow appropriate folding of both LT_A and STa and also to give the STa epitope ample opportunity to react with antibody by letting it extend out of the protein complex. The final outcome was an efficient recognition of the hybrid encoded by pJS009 by anti-STa monoclonal antibody. This together with the capacity to associate with LT_B make these hybrids attractive reagents for STa immunodiagnostic tests. Thus, it is possible that molecules containing LT_B and the fusion products described here could be used to replace chemically prepared coating conjugates containing the cholera toxin B-subunit and STa used in a recently described, efficient STa-diagnostic GM_1 inhibition ELISA [29]. Studies are also in progress in animals to test these genetically constructed chimeric proteins as immunoprophylactic agents against *E. coli* diarrhoeal disease; the presentation of LT and STa antigens on the same molecule may give rise to an

immune response against both toxins and thereby possibly to protection against diarrhoeal disease caused by *E. coli* organisms irrespective of their enterotoxin profile.

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