Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 4011–4015, July 1980
Biochemistry

Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains

[*Escherichia coli* heat-stable (ST) toxin/infantile and travelers' diarrhea]

MAGDALENE SO* AND BRIAN J. MCCARTHY

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Communicated by Bernard D. Davis, May 2, 1980

ABSTRACT **The *Escherichia coli* heat-stable toxin (ST I) is encoded within a transposon (Tn1681) flanked by inverted repeats of insertion sequence 1 (IS1) [So, M., Heffron, F. & McCarthy, B. J. (1979) *Nature (London)* 277, 453–456]. By subcloning restriction fragments and by insertion mutagenesis, we located precisely the gene for ST I within the transposon. We determined the complete nucleotide sequence of the central portion of Tn1681 (i.e., that part flanked by IS1) and identified the coding sequence of the toxin. From the nucleotide sequence, we deduced a probable amino acid sequence for ST I. The NH_2-terminal portion of the amino acid sequence is extremely hydrophobic and bears a striking resemblance to the signal sequence of the fd phage minor coat protein. By using a subcloned restriction fragment containing the gene for ST I but no IS1 sequences, we determined (*i*) that the ST toxin with activity assayable in suckling mice (ST I) is genetically distinct from the ST toxin assayable in ligated ileal loops (ST II) and (*ii*) that ST I can be responsible for diarrheal disease in different animals.**

Enterotoxigenic *Escherichia coli* are responsible for many diarrheal syndromes, among them the devastating common diarrhea of infants and newborn animals and the milder syndrome in adults known as traveler's diarrhea (1–5).

Two types of toxin are produced by these pathogenic *E. coli* (6, 7), either alone or in combination. The heat-labile (LT) toxin is a large protein composed of one 25,500 M_r subunit and four or five 11,000 M_r subunits (8). It is immunologically similar to cholera toxin and, like cholera toxin, exerts its effects by stimulation of adenylate cyclase within the epithelial cells of the upper intestine (9, 10). The gene for LT has been cloned (11). In support of the above observation on antigenic crossreaction, restriction fragments of the insert-containing portions of the LT gene have been observed to hybridize with *Vibrio cholerae* DNA (8).

The heat-stable (ST) toxin of *E. coli* has not been as extensively studied. It is thought to be a small molecule of approximately 5000 M_r (12, 13) and has been shown to stimulate guanylate cyclase *in vitro* (14). At least two species of ST have been identified, based on solubility in methanol and activity in particular animal test systems (15): ST I is methanol soluble and can be assayed in the stomach of suckling mice or neonatal pigs, whereas ST II is methanol-insoluble and reactive only in ligated intestines of pigs. The gene for ST I has been subcloned into pBR322 as restriction fragments ranging in size from 3.4 to 0.4 kilobase (kb). Studies with these fragments indicated the ST I gene is part of a transposon flanked by inverted repeats of insertion sequence 1 (IS1) (16). In this paper we report the complete nucleotide sequence of the central portion (i.e., that part flanked by IS1) of the ST I transposon Tn1681. We also present additional cloning data that precisely define the position of the gene within the sequence. This data has allowed us to deduce the most probable amino acid sequence for the ST I toxin. By using a fragment of DNA encoding only the ST I sequence and no IS1 sequences, we show that the ST I and ST II toxins are genetically distinct; moreover, we have evidence that there may exist several classes of ST I toxins as well.

MATERIAL AND METHODS

Strains. *E. coli* K-12 strain RR1 is *pro leu thi lacY* Sm^R $r^-_k m^-_k$. *E. coli* strains 431, p16M and P16 are naturally occurring ST-producing isolates kindly provided by H. W. Smith and C. Gyles.

Methods. Transformation of plasmid DNA was carried out as described by Cohen *et al.* (17). Conditions used for digestion of DNA by restriction enzymes were according to recommendations of the supplier; *Hin*dIII was obtained from Boeringer Mannheim, and the others were obtained from New England BioLabs. Agarose and polyacrylamide gel electrophoresis was carried out as described (18). Plasmid DNA was isolated according to the method described by Kupersztock (19). Insertion of synthetic deoxyoligonucleotides into a linear DNA fragment was carried out by the method of Heffron (20). Sequence determination was done on 0.4-mm-thick polyacrylamide gels according to the method of Maxam and Gilbert (21). Filter hybridizations were performed as described by Southern (22) and ^{32}P-labeled probe was made by using the T4 polymerase method (P. O'Farrell, personal communication). ST I was assayed according to the method described by Dean *et al.* (23).

RESULTS

We have reported subcloning the ST I gene into pBR322 as a DNA fragment of approximately 600 bp (MS6) by partial *Alu* I digestion and the ligation of synthetic decameric oligodeoxynucleotides encoding a *Hin*dIII site (C-C-A-A-G-C-T-T-G-G) (*Hin*dIII decamer) to the termini of this fragment (16). Initial sequence data showed that the first and last 66 bases of the fragment were inverted repeats and identical to the last 66 bases of the right-hand end (designation of Ohtsubo, see ref. 24) of IS1. Fig. 1*b* shows the complete restriction map of MS6. Mutating the *Hin*cII site within this insert by ligating into it an EcoRI octamer or a 2.4-kb fragment of DNA encoding tetracycline resistance (Tc^R) (16) changes the phenotype of the strain carrying this plasmid from ST^+ to ST^-. Clones carrying the individual *Alu* I fragments from this insert also yielded an ST^- phenotype (Fig. 1*c*).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Ap, ampicillin; Tc, tetracycline; IS, insertion sequence; ST, *Escherichia coli* heat-stable toxin; LT, *E. coli* heat-labile toxin.

* Present address: Cold Spring Harbor Laboratories, Cold Spring Harbor, NY 11724.

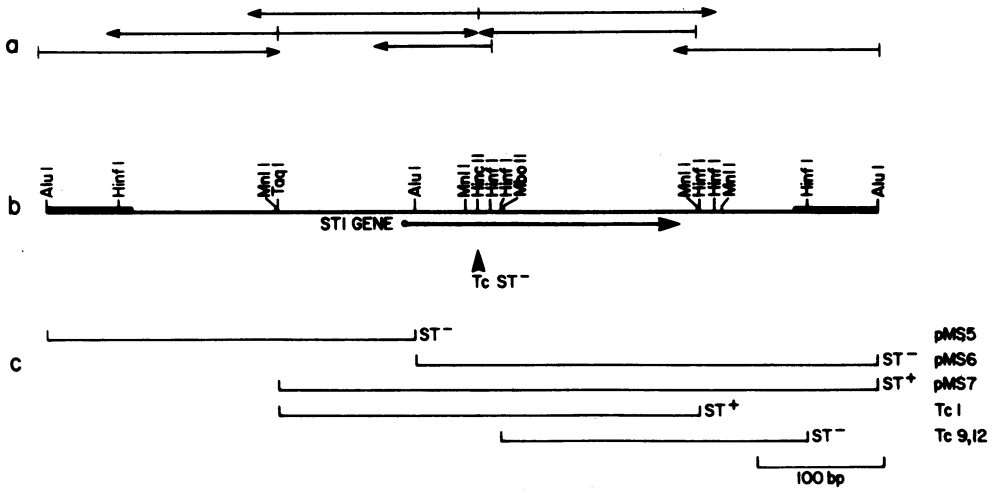

FIG. 1. (*a*) Strategy used to derive the nucleotide sequence of the pMS6 insert. The direction of the arrows indicates the direction in which the bases were read and the length of the arrows indicates the extent of the fragment sequenced. (*b*) Restriction endonuclease map of the pMS6 insert, deduced from enzyme analysis and nucleotide sequence data. Arrow indicates location and direction of transcription of the ST I gene. (*c*) Location and length of DNA fragments subcloned from the pMS6 insert into pBR322 using *Hin*dIII linkers.

We have further defined the region within this MS6 fragment in which the ST I gene lies. By using *Hin*dIII linkers, the 473 bp *Taq* I-*Hin*dIII fragment was cloned into pBR322 and transformed into RR1, selecting for resistance to ampicillin (Ap). All Ap^R clones were sensitive to tetracycline (Tc; 10 μg/ml) because insertion of a fragment into the *Hin*dIII site of pBR322 resulted in a Tc^S phenotype (25). Three of eight Tc^SAp^R clones examined contained the 473-bp insert. These clones were ST^+ and were designated pMS7-11, pMS7-13, and pMS7-17.

In a parallel set of experiments, DNA fragments were cloned by complete digestion of MS6 by *Taq* I, followed by partial digestion with *Hin*fI. The 5′ extensions created by the restriction enzymes were removed by digestion with T4 polymerase, and the flush-ended fragments were cloned with *Hin*dIII linkers into pBR322 and transformed into RR1, selecting for resistance to Ap (50 μg/ml). Surprisingly, 50% of the Ap^R clones were also Tc^R. These will be discussed below. Twelve Ap^RTc^R and 12 Ap^RTc^S clones were screened for the presence and size of their *Hin*dIII inserts. Clones of both phenotypes contained inserts. Although the sizes of the inserts ranged from approximately 150 to 450 bp, only those plasmids containing inserts of more than 250 bp were considered further. Three clones were examined more closely: Tc1 was Ap^RTc^R, contained an insert of approximately 350 bp and had an ST^+ phenotype; Tc9 and Tc12 were Ap^RTc^S, contained inserts of approximately 270 bp and had an ST^- phenotype. Restriction analysis showed that the Tc1 insert spanned the area from the *Taq* I site to the *Hin*fI site at base 517, whereas Tc9 and Tc12 spanned the area from the *Hin*fI site at base 352 to the *Hin*fI site at base 599 (Fig. 1). Thus the site for the ST I gene must lie within the 332-bp region between the *Taq* I site at base 185 and the *Hin*fI site at base 517, as shown in Fig. 1.

The sequence of the MS6 fragment was determined with the Maxam–Gilbert technique (21). Fig. 1*a* shows the restriction sites that were used for the sequence determinations. The direction of the arrows indicates the direction in which the bases were read, and the length of the arrows indicates the number of bases read. The sequence of each of the fragments used was determined at least twice. The heavily barred region at each end of the MS6 fragment indicates the 66-base sequence (16) homologous to the right hand end of IS1.

The nucleotide sequence of the fragment MS6 (Fig. 2) was analyzed by the PDP 11/60 computer for several features: (*i*) sites for restriction enzymes; (*ii*) stop codons (T-A-A, T-A-G, T-G-A) in three reading frames for both strands of the DNA; (*iii*) the amino acid corresponding to each triplet codon; (*iv*) directly repeated sequences and sequences with dyad symmetry equal to or greater than six bases in length.

The sequence is very A/T-rich (>70% A+T). It contains many translational stop signals in all three reading frames on both strands of the fragment, most of them separated by no more than 80 bases (data omitted). Only one reading frame, located in the top strand, gave a sequence containing an uninterrupted translational readthrough within the confines of the 332-base area in which subcloning data indicate the ST I gene must lie. It begins at base 265 after the opal codon (T-G-A) and ends at base 502 with the ochre codon (T-A-A). The *Taq* I site (T-C-G-A) is at position 185.

```
        10         20         30         40         50         60
CTGCATGACA AAGTCATCGG GCATTATCTG AACATAAAAC ACTATCAATA AGTTGGAGTC
GACGTACTGT TTCAGTAGCC CGTAATAGAC TTGTATTTTG TGATAGTTAT TCAACCTCAG

        70         80         90        100        110        120
ATTACCCGAA CAAGAAAAGG ATAAAACCAT CACTTAATAT GATATTGATA GTAAAAAAAA
TAATGGGCTT GTTCTTTTCC TATTTTGGTA GTGAATTATA CTATAACTAT CATTTTTTTT

       130        140        150        160        170        180
CCAGATAGCC AGACAATTAA CATATATAAC ACTATGAAAA TAATATATAA AAAGCGAGTG
GGTCTATCGG TCTGTTAATT GTATATATTG TGATACTTTT ATTATATATT TTTCGCTCAC

       190        200        210        220        230        240
TACCTCGACA TATAACATGA TGCAACTCAC AAAAAAAAAT AAAAAAATTG CAAAATCCGT
ATGGAGCTGT ATATTGTACT ACGTTGAGTG TTTTTTTTTA TTTTTTTAAC GTTTTAGGCA

       250        260        270        280        290        300
TTAACTAATC TCAAATATCC GTGAAACAAC ATGACGGGAG GTAACATGAA AAAGCTAATG
AATTGATTAG AGTTTATAGG CACTTTGTTG TACTGCCCTC CATTGTACTT TTTCGATTAC

       310        320        330        340        350        360
TTGGCAATTT TTATTTCTGT ATTATCTTTC CCCTCTTTTA GTCAGTCAAC TGAATCACTT
AACCGTTAAA AATAAAGACA TAATAGAAAG GGGAGAAAAT CAGTCAGTTG ACTTAGTGAA

       370        380        390        400        410        420
GACTCTTCAA AAGAGAAAAT TACATTAGAG ACTAAAAAGT GTGATGTTGT AAAAAACAAC
CTGAGAAGTT TTCTCTTTTA ATGTAATCTC TGATTTTTCA CACTACAACA TTTTTTGTTG

       430        440        450        460        470        480
AGTGAAAAAA AATCAGAAAA TATGAACAAC ACATTTTACT GCTGTGAACT TTGTTGTAAT
TCACTTTTTT TTAGTCTTTT ATACTTGTTG TGTAAAATGA CGACACTTGA AACAACATTA

       490        500        510        520        530        540
CCTGCCTGTG CTGGATGTTA TTAAAAAGGA TAGAGGGAAT GTTTATTTTG ATTCCCTCTA
GGACGGACAC GACCTACAAT AATTTTTCGT ATCTCCCTTA GAAATAAAAC TAAGGGAGAT

       550        560        570        580        590        600
TATTATATGT ATTGGTGCTG AGAAAATCAT AATAAAAAAC AACGCTTTTA TAGGTAATGA
ATAATATACA TAACCACGAC TCTTTTAGTA TTATTTTTTG TTGCGAAAAT ATCCATTACT

       610        620        630        640        650
CTCCAACTTA TTGATAGTGT TTTATGTTCA GATAATGCCC GATGACTTTG TCATGCAG
GAGGTTGAAT AACTATCACA AAATACAAGT CTATTACGGG CTACTGAAAC AGTACGTC
```

FIG. 2. Nucleotide sequence of the pMS6 insert. The first and last 66 bases (boxed area) comprise the right hand end of IS1 as determined by Ohtsubo (24).

Other evidence further supports the contention that this region contains the coding sequence for the ST I gene. There are three in-phase translational start signals (A-T-G) at positions 271, 286, and 298. The A-T-G at position 286 is most likely the true start signal because only it is preceded by a ribosomal RNA binding site (G-G-A-G-G) (26). This sequence is at position 277 and is separated from the A-T-G by four bases. A possible −10 homology sequence (27) occurs at position 191. This sequence (T-A-T-A-A-C-A) differs from the "classic" −10 sequence (T-A-T-A-A-T-A) in only one position. The coding sequence terminates at the ochre signal (T-A-A) at position 502. After the translational stop signal is an A-rich area followed by a T-rich area. There is also a 12-bp region of dyad symmetry within this region that starts at position 510, ends at 541, and encompasses the T-rich region. An RNA sequence transcribed from this area could fold into a stem-and-loop structure as shown in Fig. 3. Such stem-and-loop structures near the 3′ terminus of the RNA are thought to act as transcriptional termination signals and in some cases also to regulate the transcription of genes downstream (28). They occur at the termination point of the lambda *cro* (29) and 6S (30) transcripts and the *trp* attenuator (31). There is also a region of dyad symmetry in the β-lactamase structural gene just distal to its translational stop signal (32); in this case, the region of symmetry is composed of a 10-bp sequence.

We can offer further evidence indicating that the stem-and-loop structure is important in the termination of transcription of ST I. Restriction mapping of the Tc1 and pMS7-17 plasmids indicates the Tc1 and pMS7-17 inserts are cloned into the *Hin*dIII site of pBR322 in the orientation in which the direction of transcription of both ST I and Tc^R is the same. The DNA sequence of the right-hand portion of the Tc1 insert (Fig. 1) shows that it terminates at base 521 within the stem (Fig. 3). Thus the ST I transcripts of Tc1, unlike pMS7-17, would not be able to form the secondary stem-and-loop structure, and transcriptional termination might be expected to be less efficient. This, in turn, should affect the expression of the tetracycline resistance gene downstream, because the *Hin*dIII site of pBR322, into which the Tc1 and pMS7-17 inserts were cloned, lies within the Tc promoter (32; K. Bachman and H. W. Boyer, personal communication). Strains RR1 (pMS7-17), RR1 (Tc1), RR1 (pBR322) and RR1 were checked for their level of resistance to tetracycline. Cultures were grown to a density of 5 × 10^7 cells per ml in the presence of Ap(20 μg/ml), if they contained the pBR322 plasmid. Approximately 100 cells were plated on freshly poured Mueller–Hinton agar (Difco) and Mueller–Hinton agar + Tc. The percentage of Tc^R cells was plotted against the concentration of Tc in the plates (Fig. 4). Whereas RR1 and pMS7-17 are sensitive to 2.5 μg of Tc per ml, RR1(Tc1) is resistant to over 7.5 μg of Tc per ml. We conclude that nucleotides 510–541 are essential to transcriptional termination. A similar phenomenon involving the regulation of the Tc gene by transcription of the trpE and trypD genes cloned into the *Hin*dIII site has been observed (27).

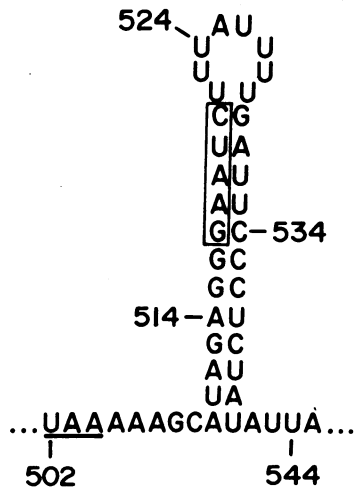

FIG. 3. A stem-and-loop structure potentially formed by the 3′-COOH terminus of the ST I transcript. (U-A-A) at position 502 is the ST I translational stop signal. Boxed sequence (G-A-A-T-C) is the *Hin*fI site comprising the right-hand end of the Tc1 insert.

The 350-bp Tc1 insert contains the entire ST I gene and is free of IS1 sequences. We used this insert as a probe to determine whether the ST I and ST II toxins are related. We have studied two ST-producing strains in detail: P16M and P16. P16M, responsible for an outbreak of diarrheal disease in pigs, is both pig-active and baby-mouse-active while P16, a derivative of P16M, has lost its toxicity for baby mice (C. Gyles, personal communication). Fig. 5*a* shows plasmid DNA isolated from 1.5 ml of culture with a quick screening procedure. Because the procedure does not involve a CsCl-equilibrium-density purification step, some sheared chromosomal as well as plasmid DNA can still be seen. P16M contains five plasmids, labeled 1–5, while P16 has lost plasmids 2 and 4. The plasmid DNA was transferred to a nitrocellulose filter by the method of Southern (22). The Tc1 insert was labeled with [α-^{32}P]dCTP using T4 polymerase (P. O'Farrell, personal communication) and the probe was hybridized to the filter-bound DNA under fairly stringent conditions (50% formamide/0.75 M NaCl/0.075 *M* sodium citrate, pH 7, 37°C) and under more relaxed conditions (30% formamide/0.75 M NaCl/0.075 M sodium citrate, pH 7, 37°C). Only one band in the P16M lane hybridized to the Tc1 probe, corresponding to plasmid 2 in both sets of conditions. Thus the ST I gene in P16M must lie in this plasmid. The Tc1 probe did not crossreact with DNA from P16, the strain which has lost the mouse-active component but which still retains the pig-active component. Therefore it appears the genetic information for the baby-mouse-active (ST I) component is distinctly different from that for the (ST II) pig-active component.

DISCUSSION

The gene for the ST I toxin has been located within the transposon Tn1681 by means of insertion mutagenesis and by subcloning various restriction endonuclease fragments. From this information we were able to pinpoint the nucleotide sequence for the ST I gene. This gene has all the properties necessary for autonomous expression in bacteria: a −10 homology

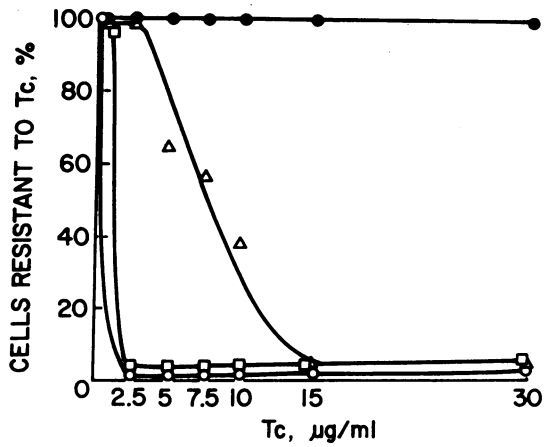

FIG. 4. Resistance to tetracycline of several strains containing various subclones of the pMS6 insert. ○, RR1; ●, RR1(pBR322); △, RR1(Tc1); □, RR1(pMS7-17).

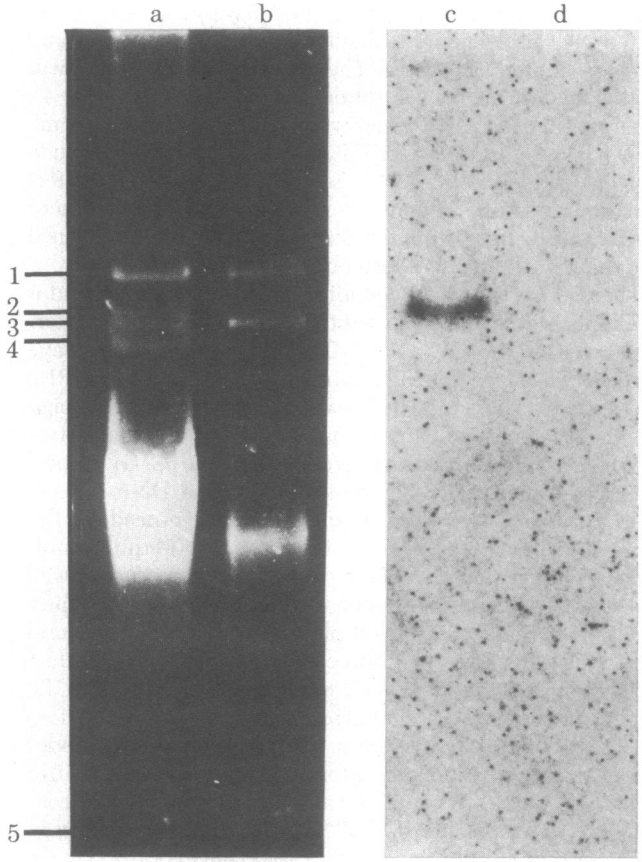

FIG. 5. Plasmid DNA from the cleared lysate of strains (*a*) P16M, which produces both baby-mouse- and ileal-loop-assayable ST toxins, and (*b*) P16, a derivative of P16M that has lost the baby-mouse-assayable ST component. Southern hybridizations using the Tc1 probe against plasmid DNA from (*c*) P16M and (*d*) P16.

sequence, a 16S ribosomal RNA binding site, translational start and stop signals, and a transcriptional stop signal. Only one uninterrupted sequence of codons falls within the confines of the 332 bases known to harbor the ST I gene.

The availability of the nucleotide sequence for the ST I gene allows us to deduce a probable amino acid sequence of its product. Fig. 6 shows the amino acid sequence that corresponds to the nucleotide sequence of the ST I gene, starting at the second translational start signal (A-T-G) at base 286. The gene product is 72 amino acids in length, with a molecular weight of 8076, which is considerably larger than the previously estimated size of 5000. However, this discrepancy can be explained if ST I is viewed as a transported protein—a reasonable assumption because ST-producing *E. coli* are noninvasive. The majority of the first 18 amino acids at the NH_2-terminus are hydrophobic (boxed area), suggesting that this region of the polypeptide acts as a signal sequence for the ST I toxin. One common feature of many bacterial signal sequences is the presence of basic amino acids, such as lysine, immediately adjacent to the NH_2-terminal methionine, followed by a series of hydrophobic amino acids. This is true for the signal sequences of the maltose-binding protein (33), the λ receptor (34), the fd major and minor coat proteins, and the lipoprotein of *E. coli* (35). In the ST I "signal sequence," the first four amino acids, Met, Lys, Lys, Leu, are identical to those of the fd minor coat protein signal sequence. Further comparison shows that there is a significant degree of homology between the two sequences (Fig. 6).

A striking feature of the amino acid sequence for the ST I toxin is the high proportion of cysteines in the molecule (7 cysteines per molecule). If the first 18 amino acids are excluded from consideration, then cysteines comprise approximately 18% of the total amino acids of the protein. The abundance of cysteines in the toxin may account for its relative heat stability. Their participation in intramolecular bonding would contribute to a more compact structure and make the molecule more readily renaturable.

Recently, Robertson (13) reported the amino acid composition of a heat-stable toxin isolated from *E. coli* strain 431. Its NH_2-terminal amino acid was glycine and the molecular weight was 4700. This data and the general amino acid composition differ markedly from the data we have obtained for our ST I toxin, whether or not we take into consideration the hydrophobic region. Because of the presence of the signal sequence in ST I we cannot assign an NH_2-terminal amino acid to our sequence; however there is only one glycine, at position 69, in the molecule. The above discrepancy most likely is due to the differences in strains used in the two studies. 431 produces both the baby-mouse-active component and the pig-active component. It is quite possible that ST II has copurified with ST I in Robertson's preparations, and the amino acid composition he reported may be a reflection of the composition of ST II or a mixture of ST II and ST I. Indeed we find that the ST I gene is present in 431 by DNA homology studies (unpublished observations).

The presence in enterotoxigenic *E. coli* of two chemically and genetically distinct heat-stable toxins is intriguing. That one can be assayed in suckling mice and the other in weaned pigs suggests a possible difference in toxin receptor sites on the target cell surface in the gastrointestinal tract of these test animals during development. This difference in assayability of the ST toxins also suggests that the clinical syndromes of trav-

```
                                                                  met  lys
TTT AAT CTC AAA TAT CCG TGA AAC AAC ATG ACG GGA GGT AAC ATG AAA
                                                        •    •
lys leu met leu ala ile phe ile ser val leu ser phe pro ser phe
AAG CTA ATG TTG GCA ATT TTT ATT TCT GTA TTA TCT TTC CCC TCT TTT
 •   •       •   •       •       •
ser gln ser thr glu ser leu asp ser ser lys glu lys ile thr leu
AGT CAG TCA ACT GAA TCA CTT GAC TCT TCA AAA GAG AAA ATT ACA TTA

glu thr lys lys cys asp val val lys asn asn ser glu lys lys ser
GAG ACT AAA AAG TGT GAT GTT GTA AAA AAC AAC AGT GAA AAA AAA TCA

glu asn met asn asn thr phe tyr cys cys glu leu cys cys asn pro
GAA AAT ATG AAC AAC ACA TTT TAC TGC TGT GAA CTT TGT TGT AAT CCT

ala cys ala gly cys tyr OC
GCC TGT GCT GGA TGT TAT TAA AAA GCA TAG AGG GAA TCT TTA TTT TGA
```

FIG. 6. Amino acid sequence corresponding to the nucleotide sequence of the ST I gene. Boxed areas indicate hydrophobic amino acids. Dots underneath the amino acids indicate homology with the signal sequence of the fd minor coat protein.

eler's diarrhea and infantile diarrhea may be totally different diseases caused by two chemically distinct types of heat-stable toxins. Only a more detailed study of the molecular nature of ST toxins of the mechanism of action of ST, and of target-cell toxin interactions will clarify this situation.

The genetic situation already promises to be more complicated. Again by using Tcl as a probe, we have observed that not all baby-mouse-reactive strains crossreact with the probe, which indicates that there may be still another genetically distinct mouse-assayable toxin (unpublished observation). From the epidemiological standpoint, it would be useful and interesting to monitor over a long period the distribution and frequencies of occurrence of these *E. coli* toxins, both heat-stable and heat-labile, in various epidemics on a global scale. The Tcl insert, free of IS 1 sequences, is a step in that direction. However, only the acquisition of a complete set of these ST genes will make such studies possible. The Southern hybridization studies reported here reveal an important finding, namely that the same gene is responsible for diarrheal outbreaks in two different suckling animals (pigs and cows).

We wish to thank Fred Heffron for his careful reading of the manuscript and for his support during the course of these experiments and to Carlton Gyles and H. W. Smith for sending us their strains. We especially thank Hugo Martinez for developing the computer programs that were used to analyze our DNA sequence.

1. Smith, H. W. & Linggood, M. A. (1971) *J. Med. Microbiol.* **4,** 464–485.
2. Smith, H. W. & Linggood, M. A. (1972) *J. Med. Microbiol.* **5,** 243–250.
3. Sack, R. B. (1975) *Annu. Rev. Microbiol.* **29,** 333–353.
4. Ryder, R. W. xx, xx & xx (1976) *N. Engl. J. Med.* **295,** 849–853.
5. Gorbach, S. L. & Khurana, C. M. (1972) *N. Engl. J. Med.* **287,** 791–795.
6. Smith, H. W. & Gyles, C. L. (1970) *J. Med. Microbiol.* **3,** 419–426.
7. Gyles, C. L. (1971) *Ann. N.Y. Acad. Sci.* **176,** 314–322.
8. Dallas, W. D. & Falkow, S. (1979) *Nature (London)* **277,** 406–407.
9. Gyles, C. L. (1974) *J. Infect. Dis.* **129,** 277–288.
10. Guerrant, R. L., Brinton, L. L., Schnartman, T., Rebhun, L. I. & Gilman, A. G. (1974) *Infect. Immun.* **10,** 320–327.
11. So, M., Dallas, W. D. & Falkow, S. (1972) *Infect. Immun.* **5,** 622–624.
12. Jacks, T. M. & Wu, B. J. (1974) *Infect. Immun.* **9,** 342–347.
13. Aldorete, J. F. & Robertson, D. C. (1978) *Infect. Immun.* **19,** 1021–1030.
14. Field, M., Graf, L. H., Laird, W. J. & Smith, P. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2800–2804.
15. Burgess, M. N., Bywater, R. J., Cowley, C. M., Millan, N. A. & Newsome, P. M. (1978) *Infect. Immun.* **21,** 526–531.
16. So, M., Heffron, F. & McCarthy, B. J. (1979) *Nature (London)* **277,** 453–456.
17. Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2110–2114.
18. Greene, P. J., Betlach, M., Goodman, H. M. & Boyer, H. W. (1974) in *Methods in Molecular Biology,* ed. Wickner, R. B. (Marcel Dekker, New York), Vol. 9, pp. 87–111.
19. Kupersztock, Y. M. & Helinski, D. (1973) *Biochem. Biophys. Res. Commun.* **54,** 1451–1459.
20. Heffron, F., So, M. & McCarthy, B. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6012–6016.
21. Maxam, A. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 560–564.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
23. Dean, A. G., Ching, Y., Williams, R. G. & Handen, L. B. (1972) *J. Infect. Dis.* **125,** 407–411.
24. Ohtsubo, H. & Ohtsubo, E. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 615–619.
25. Bolivar, F., Rodriguez, R., Betlach, M. & Boyer, H. W. (1977) *Gene* **2,** 95–113.
26. Shine, J. & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1342–1346.
27. Pribnow, D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 784–788.
28. Adhya, S. & Gottesman, M. (1978) *Annu. Rev. Biochem.* **47,** 967–996.
29. Rosenberg, M., Court, D., Wulff, D. L., Shimatake, H. & Brady, C. (1978) *Nature (London)* **272,** 414–423.
30. Lebowitz, P., Weissman, S. M. & Radding, C. M. (1971) *J. Biol. Chem.* **246,** 5120–5139.
31. Lee, F. & Yanofsky, C. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4365–4369.
32. Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77–90.
33. Bedouelle, H., Bassford, P. J., Jr., Fowler, A. V., Zabin, I., Beckwith, J. & Hofnung, M. (1980) *Nature (London)* **285,** 78–81.
34. Emr, S. D., Hedgpeth, J., Clément, J.-M., Silhavy, T. J. & Hofnung, M. (1980) *Nature (London)* **285,** 82–85.
35. Halegoua, S. & Inouye, M. (1979) in *Bacterial Outer Membranes: Biogenesis and Functions,* ed. Inouye, M. (Wiley & Sons, New York), pp. 68–113.