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Molecular Cloning and Expression of *Salmonella typhi* Flagellin : Characterization of 52 kDa Specific Antigen of *S. typhi*

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SUMMARY **We previously reported monoclonal antibodies (MAbs) specific to *S. typhi* 52 kDa antigen which do not cross react with related protein antigens from 11 bacteria causing enteric fever and enteric fever-like illness. Using the combination of these specific MAbs and recombinant DNA technology, expression plasmids containing the antigen gene producing substantial amount of the *S. typhi* protein antigen have been established. Plasmid pSKM-T7 containing the specific 52 kDa antigen gene was cloned and the antigen expressed was detectable by immunoblotting using specific mAbs. The complete nucleotide sequence of this gene was compared with other bacterial sequences and found to be highly homologous with the flagellin gene *H1-d* of *S. muenchen* except in the hypervariable region in the central portion. The specific 52 kDa antigen of *S. typhi* detected by our MAbs is thus a flagellin.**

At present, *Salmonella typhi* infection or typhoid fever continues to be a public health problem in many developing countries, but the rapid and accurate laboratory diagnosis of typhoid fever remains unsatisfactory. Laboratory diagnosis of typhoid fever is conventionally based on two methods: isolation of the causative organism, *Salmonella typhi,* from clinical specimens, mainly by hemoculture; and demonstration of rising antibody titers to the O and H antigens of *S. typhi,* mainly by the Widal test. An ideal diagnostic test for typhoid fever should be sensitive, specific and suitable for early diagnosis. It should be simple and inexpensive since typhoid fever is endemic mainly in less-developed areas of the world. Neither of the above two methods fulfill these requirements. Hence, it is necessary to develop a test that permits more rapid and accurate laboratory diagnosis. Several tests can be proposed; one of them should be directed towards detecting IgM antibodies against *S. typhi* specific antigen(s) from the body fluids of the patients.

Our group has produced and characterized monoclonal antibodies (MAbs) specific to a *S. typhi* 52 kDa antigen which do not cross-react with related protein antigens from 11 bacteria causing enteric fever and enteric fever-like illness *(S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. choleraesuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, Escherichia coli, Pseudomonas pseudomallei and Yersinia enterocolitica).* These MAbs were proven to be species-specific for 52 kDa protein of *S. typhi.*[1]

A genomic DNA library of *S. typhi* was contructed and screened for the expressed recombinant clones using the specific MAbs. The 52 kDa gene in a selected clone was sequenced and analysed by comparing to sequences deposited in the nucleotide and protein sequence databases.

MATERIALS AND METHODS

Bacterial strain

The virulent *S. typhi* SRR strain used in this study was isolated from

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a patient with typhoid fever in the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, (Komonpit P, personal communication). *S. typhi* O901 was a generous gift from Dr W Chaicumpa (Faculty of Tropical Medicine, Mahidol University). *E. coli* JM 107 was obtained from Promega Co. (USA).

Monoclonal antibodies

Clones 14B2E3, 14B4A9, 13B 1C3 and 2C1G11 were produced and characterized by our group as described previously.[1] They were highly specific to 52 kDa protein antigen with *S. typhi*.

Construction of genomic libraries

General recombinant DNA techniques used were those described by Maniatis *et al.*[2] Briefly, chromosomal DNA was partially digested with *Sau*3A I. DNA fragments ranging from 0.7 to 8 kilobases (kb) in size were isolated and cloned into the *Bam*H I site of the pIc-20R vector. Plasmid DNA was introduced into competent *E. coli* JM 107 prepared by DMSO method.[3]

Screening of recombinant clones expressing the antigen genes

The recombinant clones were transferred to nitrocellulose membranes, fixed in a chloroform saturated atmosphere for 5 minutes and lysed with lysis solution (50 mM Tris pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 40 μg/μl lysozyme, 1 μg/μl DNase I) at 37°C for 2 hr, then screened by immunoenzyme assay[4] using MAbs specific to the 52 kDa antigen and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins. Positive colonies of *E. coli* were detected with a chromogenic substrate using O-dianisidine tetrazotized (Sigma) and β-naphthyl phosphate (Sigma) which exhibited red purple color whereas negative colonies were light-brown.

Characterization of the specific or purified proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate and to determine the molecular weight of specific proteins from recombinant *E. coli*.[5] Electrophoresis was carried out using 14% acrylamide as resolving gel. The molecular weights were calculated from a standard curve of standard protein markers by the method of Weber and Osborn.[6] For Western blotting, the separated protein bands on SDS-PAGE gel were electrophoretically transferred to a nitrocellulose membrane in LKB 2005 Transphor Electroblotting unit, at 0.6A for overnight at 15°C[7] and stained by immunoenzyme assay.

Double stranded DNA template sequencing method

The DNA sequence was determined by the dideoxy nucleotide termination method of Sanger[8] using DNA sequencing kit, Sequenase Version 2.0 (United States Biochemical).

To determine the DNA sequence, unidirectional deletion of the DNA insert using Erase-a-Base system (Promega Co., USA) was performed.[9]

First, DNA was subjected to restriction enzyme digestion at the end closest to the primer annealing site, leaving a 3′ overhang which was resistant to exonuclease III digestion. The other end was digested so that there was either a 5′ overhang or blunt end which susceptible to exonuclease III. The exonuclease III digestion was performed and samples were removed at time intervals to tubes containing S1 nuclease, which removed the remaining single strand tails. Subsequently, Klenow fragment, dNTPs, ATP and DNA ligase were added to flush the ends and create circularized molecules. Plasmids were transformed and selected at appropriate intervals between deletions.

Isolation of purified *Salmonella* flagellin

S. typhi flagellin was purified according to the method of Ibrahim *et al.*[10] Briefly, highly motile isolate SRR strain of *S. typhi* was grown in the modified medium. [Solution A (grams per liter) : $(NH_4)_2SO_4$, 10.0; Na_2HPO_4,30.0; KH_2PO_4, 15.0; NaCl, 15.0; and $NaSO_4$, 0.055. Solution B: $MgCl_2$ 0.25; $CaCl_2$, 0.013; $FeCl_3.7H_2O$, 0.0006; yeast-extract, 0.125; and 0.31 of each of the amino acids DL-tryptophan, L-histidine, L-proline, L-threonine, L-arginine, glycine, DL-α-alanine, and-L-methionine; pH 7.2]. One hundred-milliliters of Solution A was added to 400 ml of Solution B and supplemented with 10 ml of 25% (w/v) sterile glucose. Flagella were detached by exposure of the bacteria to 1M HCl (final pH 2.0) then centrifuged at 100,000×g for 1 hr at 4°C. The supernatant containing flagellin was adjusted to pH 7.2 with 1M NaOH. Flagellin was precipitated with ammonium sulfate at final concentration of 2.67 M for 16 hr at 4°C. Precipitated flagellin was separated by centrifugation at 15,000× g for 15 min and dialyzed against water.

Analysis of DNA sequence

DNA sequence was analysed using Mac Vector Program Version 4.0 (IBI). Comparison of sequence was carried out with sequences published in GenBank Database Release 71.0 on CD-ROM (NCBI, Washington DC).

RESULTS

Cloning of 52 kDa antigen gene of *S. typhi*

Eight positive expressed recombinant clones with sizes of inserted

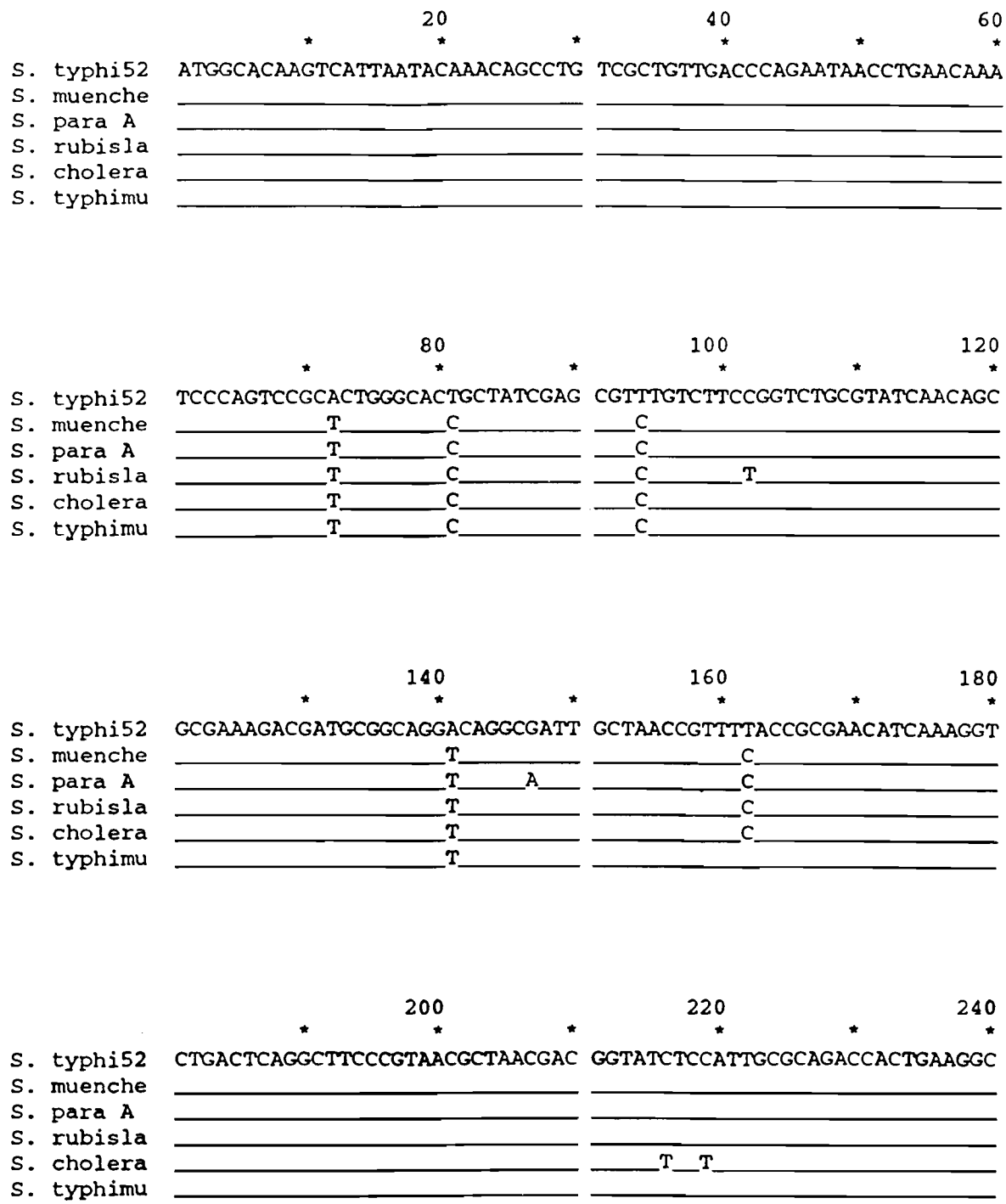
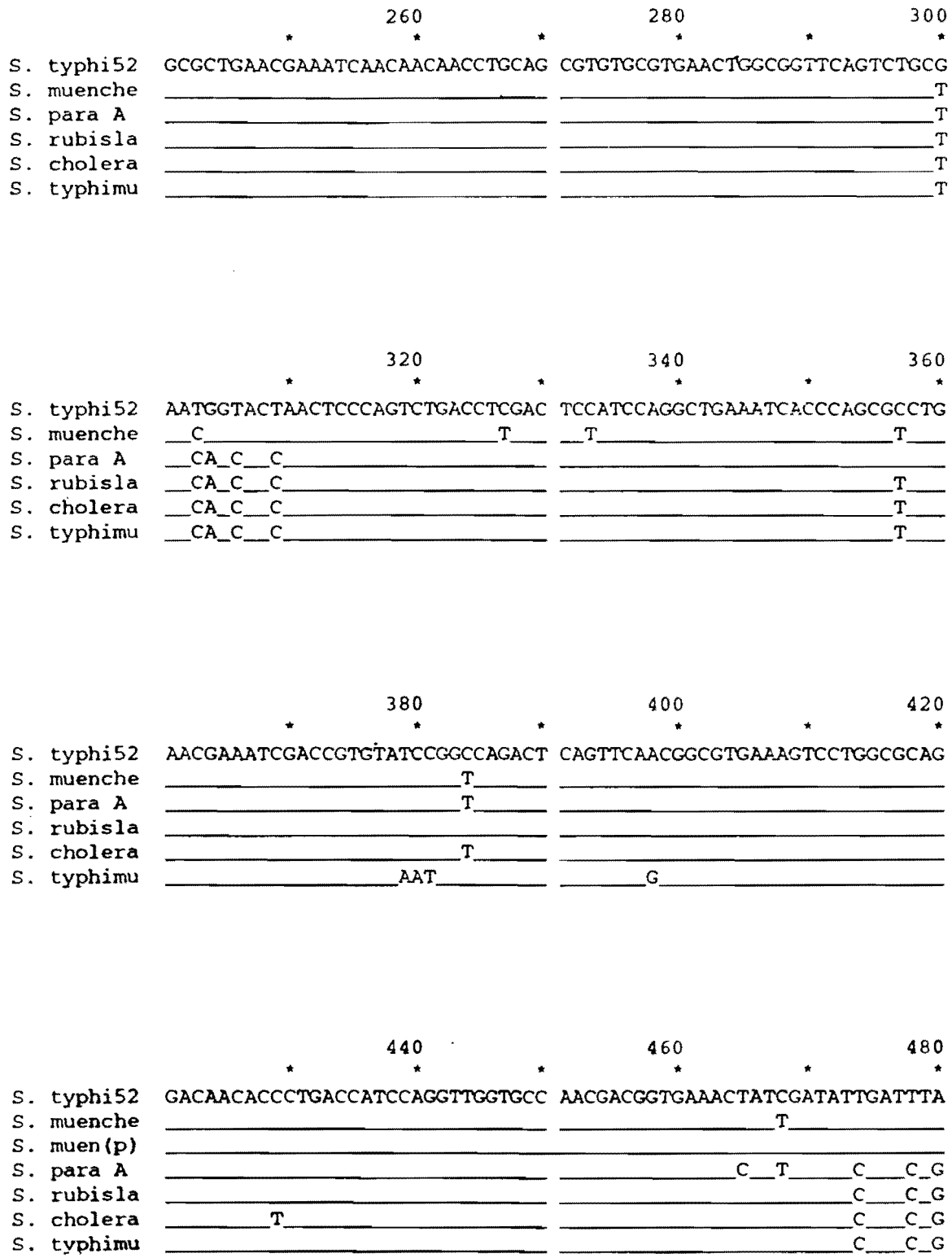


Fig. 1 Alignment of phase-1 flagellin gene. The S. *typhi* 52 kDa antigenic gene was aligned and compared with the sequences of phase-1 flagellin gene from *S. muenchen* (complete) (S. muenche), *S. muenchen* antigen-determining region (S. muen(p)), *S. paratyphi* A (S. para A). *S. rubislaw* (S. rubisla), *S. choleraesuis* (S. cholera), *S. typhimurium* (S. typhimu) and the partial sequence of *S. typhi* flagellin gene *(S. typhi (p))*.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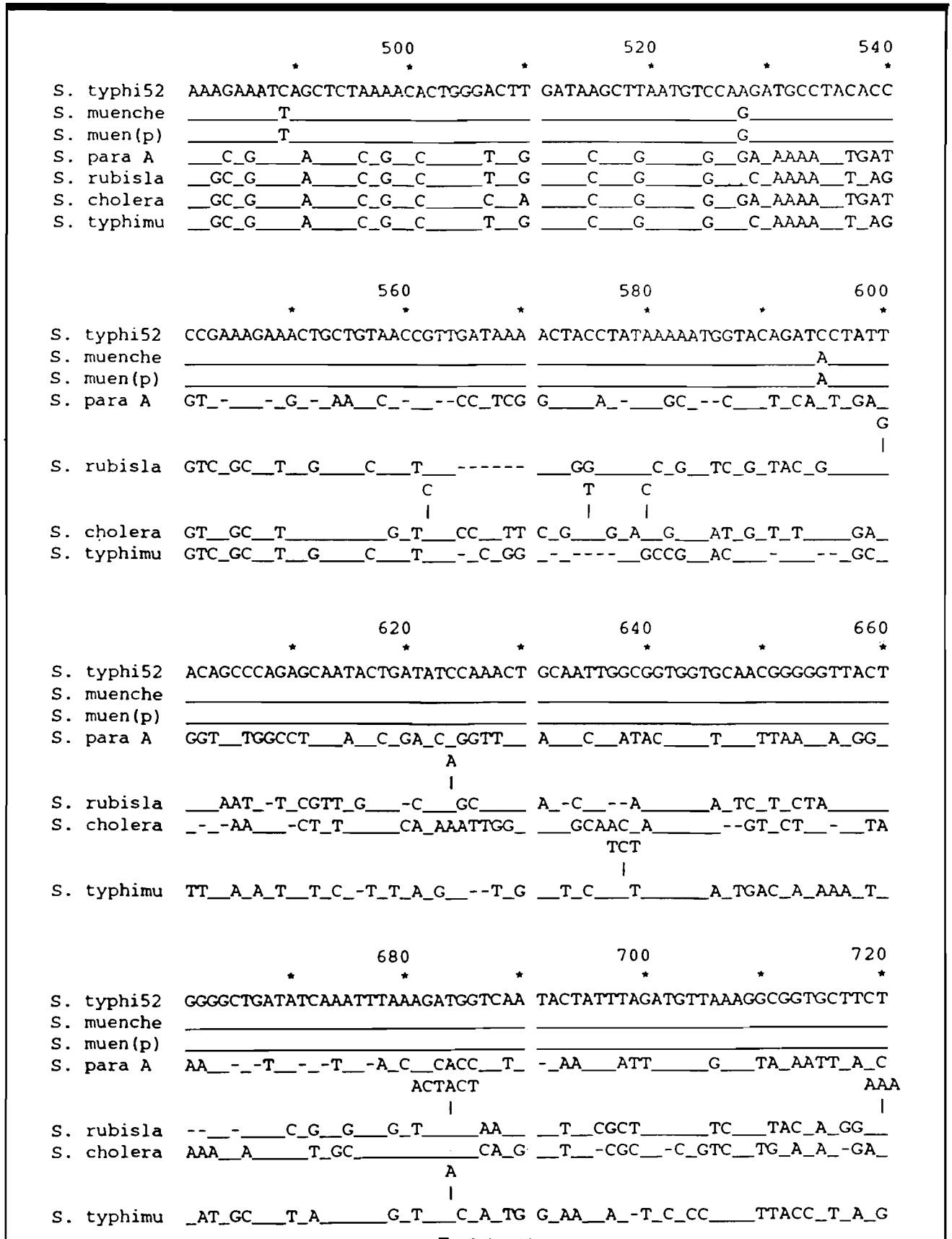
S. typhi52 GCGCTGAACGAAATCAACAACAACCTGCAG CGTGTGCGTGAACTGGCGGTTCAGTCTGCG
S. muenche T T
S. para A T T
S. rubisla T T
S. cholera T T
S. typhimu T T
320 340 360
S. typhi52 AATGGTACTAACTCCCAGTCTGACCTCGAC TCCATCCAGGCTGAAATCACCCAGCGCCTG
S. muenche C T T T
S. para A CA C C
S. rubisla CA C C T
S. cholera CA C C T
S. typhimu CA C C T
380 400 420
S. typhi52 AACGAAATCGACCGTGTATCCGGCCAGACT CAGTTCAACGGCGTGAAAGTCCTGGCGCAG
S. muenche T
S. para A T
S. rubisla
S. cholera T
S. typhimu AAT G
440 460 480
S. typhi52 GACAACACCCTGACCATCCAGGTTGGTGCC AACGACGGTGAAACTATCGATATTGATTTA
S. muenche T
S. muen(p)
S. para A C T C C G
S. rubisla C C G
S. cholera T C C G
S. typhimu C C G

Fig. 1 (cont.)


500 520 540
S. typhi52 AAAGAAATCAGCTCTAAAACACTGGGACTT GATAAGCTTAATGTCCAAGATGCCTACACC
S. muenche
S. muen(p)
S. para A
S. rubisla
S. cholera
S. typhimu
560 580 600
S. typhi52 CCGAAAGAAACTGCTGTAACCGTTGATAAA ACTACCTATAAAAATGGTACAGATCCTATT
S. muenche
S. muen(p)
S. para A
S. rubisla
S. cholera
S. typhimu
620 640 660
S. typhi52 ACAGCCCAGAGCAATACTGATATCCAAACT GCAATTGGCGGTGGTGCAACGGGGGTTACT
S. muenche
S. muen(p)
S. para A
S. rubisla
S. cholera
S. typhimu
680 700 720
S. typhi52 GGGGCTGATATCAAATTTAAAGATGGTCAA TACTATTTAGATGTTAAAGGCGGTGCTTCT
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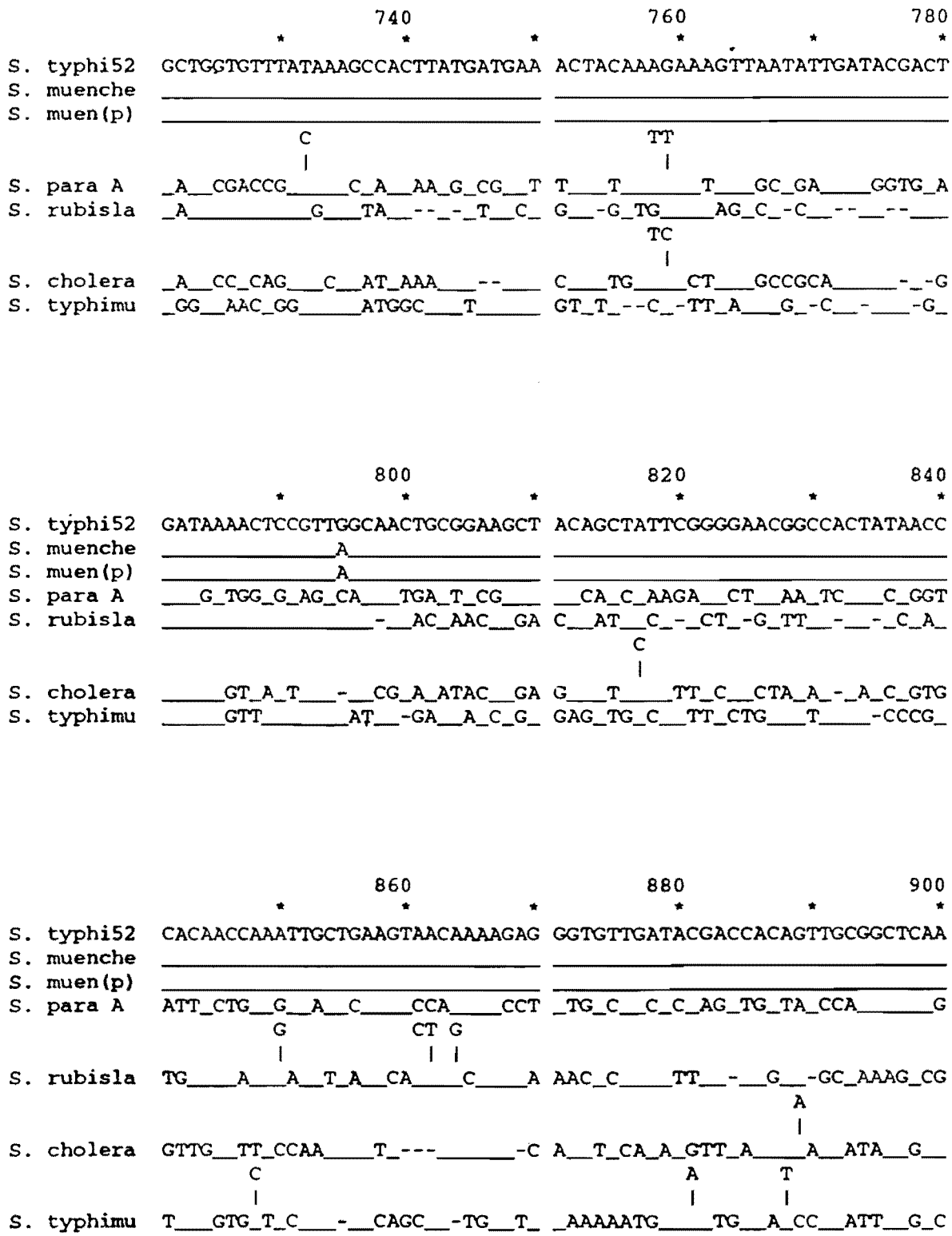
S. para A
S. rubisla
S. cholera
S. typhimu

Fig. 1 (cont.)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760
780
S. typhi52 GCTGGTGTTTATAAAGCCACTTATGATGAA ACTACAAAGAAAGTTAATATTGATACGACT
S. muenche
S. muen(p)
S. para A
S. rubisla
S. cholera
S. typhimu
800
820
840
S. typhi52 GATAAAACTCCGTTGGCAACTGCGGAAGCT ACAGCTATTCGGGGAACGGCCACTATAACC
S. muenche
S. muen(p)
S. para A
S. rubisla
S. cholera
S. typhimu
860
880
900
S. typhi52 CACAACCAAATTGCTGAAGTAACAAAAGAG GGTGTTGATACGACCACAGTTGCGGCTCAA
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S. para A
S. rubisla
S. cholera
S. typhimu

Fig. 1 (cont.)

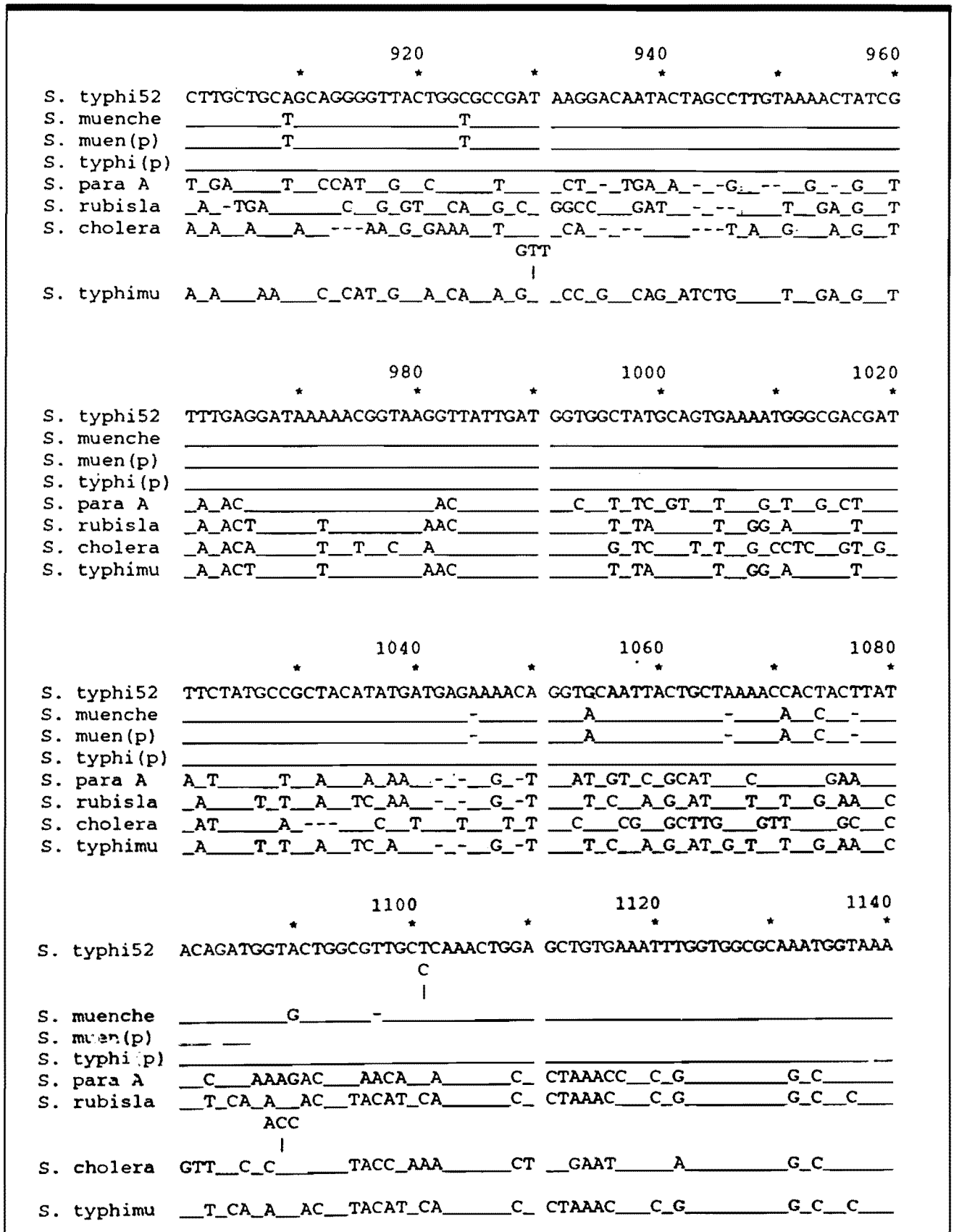
920 940 960
S. typhi52 CTTGCTGCAGCAGGGGTTACTGGCGCCGAT AAGGACAATACTAGCCTTGTAAAACTATCG
S. muenche
S. muen(p)
S. typhi(p)
S. para A
S. rubisla
S. cholera
GTT
S. typhimu
980 1000 1020
S. typhi52 TTTGAGGATAAAAACGGTAAGGTTATTGAT GGTGGCTATGCAGTGAAAATGGGCGACGAT
S. muenche
S. muen(p)
S. typhi(p)
S. para A
S. rubisla
S. cholera
S. typhimu
1040 1060 1080
S. typhi52 TTCTATGCCGCTACATATGATGAGAAAACA GGTGCAATTACTGCTAAAACCACTACTTAT
S. muenche
S. muen(p)
S. typhi(p)
S. para A
S. rubisla
S. cholera
S. typhimu
1100 1120 1140
S. typhi52 ACAGATGGTACTGGCGTTGCTCAAACTGGA GCTGTGAAATTTGGTGGCGCAAATGGTAAA
C
S. muenche
S. muen(p)
S. typhi(p)
S. para A
S. rubisla
ACC
S. cholera
S. typhimu

Fig. 1 (cont.)

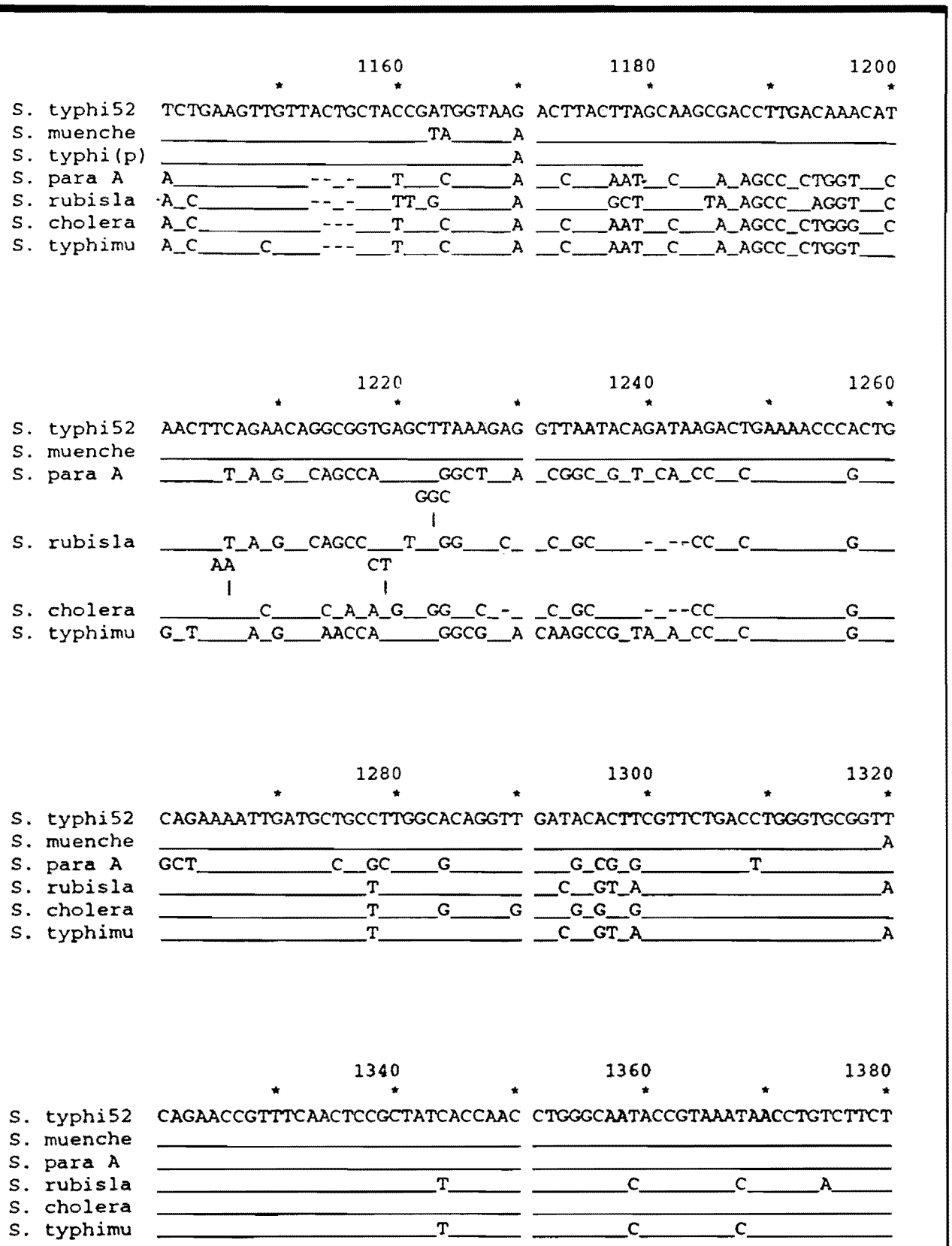
1160 1180 1200
S. typhi52 TCTGAAGTTGTTACTGCTACCGATGGTAAG ACTTACTTAGCAAGCGACCTTGACAAACAT
S. muenche TA A
S. typhi(p) A
S. para A A T C A C AAT C A AGCC CTGGT C
S. rubisla A C TT G A GCT TA AGCC AGGT C
S. cholera A C T C A C AAT C A AGCC CTGGG C
S. typhimu A C C T C A C AAT C A AGCC CTGGT
1220 1240 1260
S. typhi52 AACTTCAGAACAGGCGGTGAGCTTAAAGAG GTTAATACAGATAAGACTGAAAACCCACTG
S. muenche
S. para A T A G CAGCCA GGCT A CGGC G T CA CC C G
GGC
S. rubisla T A G CAGCC T GG C AA CT C GC - -- CC C G
S. cholera C C A A G GG C - C GC - -- CC G
S. typhimu G T A G AACCA GGCG A CAAGCCG TA A CC C G
1280 1300 1320
S. typhi52 CAGAAAATTGATGCTGCCTTGGCACAGGTT GATACACTTCGTTCTGACCTGGGTGCGGTT
S. muenche A
S. para A GCT C GC G G CG G T
S. rubisla T C GT A A
S. cholera T G G G G G
S. typhimu T C GT A A
1340 1360 1380
S. typhi52 CAGAACCGTTTCAACTCCGCTATCACCAAC CTGGGCAATACCGTAAATAACCTGTCTTCT
S. muenche
S. para A
S. rubisla T C C A
S. cholera
S. typhimu T C C

Fig. 1 (cont.)

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                          1400                1420                1440
                     *         *         *         *         *         *
S. typhi52   GCCCGTAGCCGTATCGAAGATTCCGACTAC GCAACCGAAGTCTCCAACATGTCTCGCGCG
S. muenche   _____________________________ __G_____________________________
S. para A    _____________________________ __G________T_____________________
S. rubisla   _____________________________ __G_____G__T_____________________
S. cholera   _____________________________ __G_______ T_____________________
S. typhimu   _____________________________ __G_____________________________

                          1460                1480                1500
                     *         *         *         *         *         *
S. typhi52   CAGATTCTGCAGCAGGCCGGTACCTCCGTT CTGGCGCAGGCGAACCAGGTTCCGCAAAAC
S. muenche   _____________________________ ___________T_____________________
S. para A    _____C_______________________ _____________________________
S. rubisla   _____________________________ ___________T_____________________
S. cholera   _____________________________ _____________________________
S. typhimu   _____________________________ _____________________________

                          1520
                     *         *
S. typhi52   GTCCTCTCTTTACTGCGTTAA
S. muenche   ____________________
S. para A    ____________________
S. rubisla   ___________________
S. cholera   ____________________
S. typhimu   ____________________
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Fig. 1 (cont.)

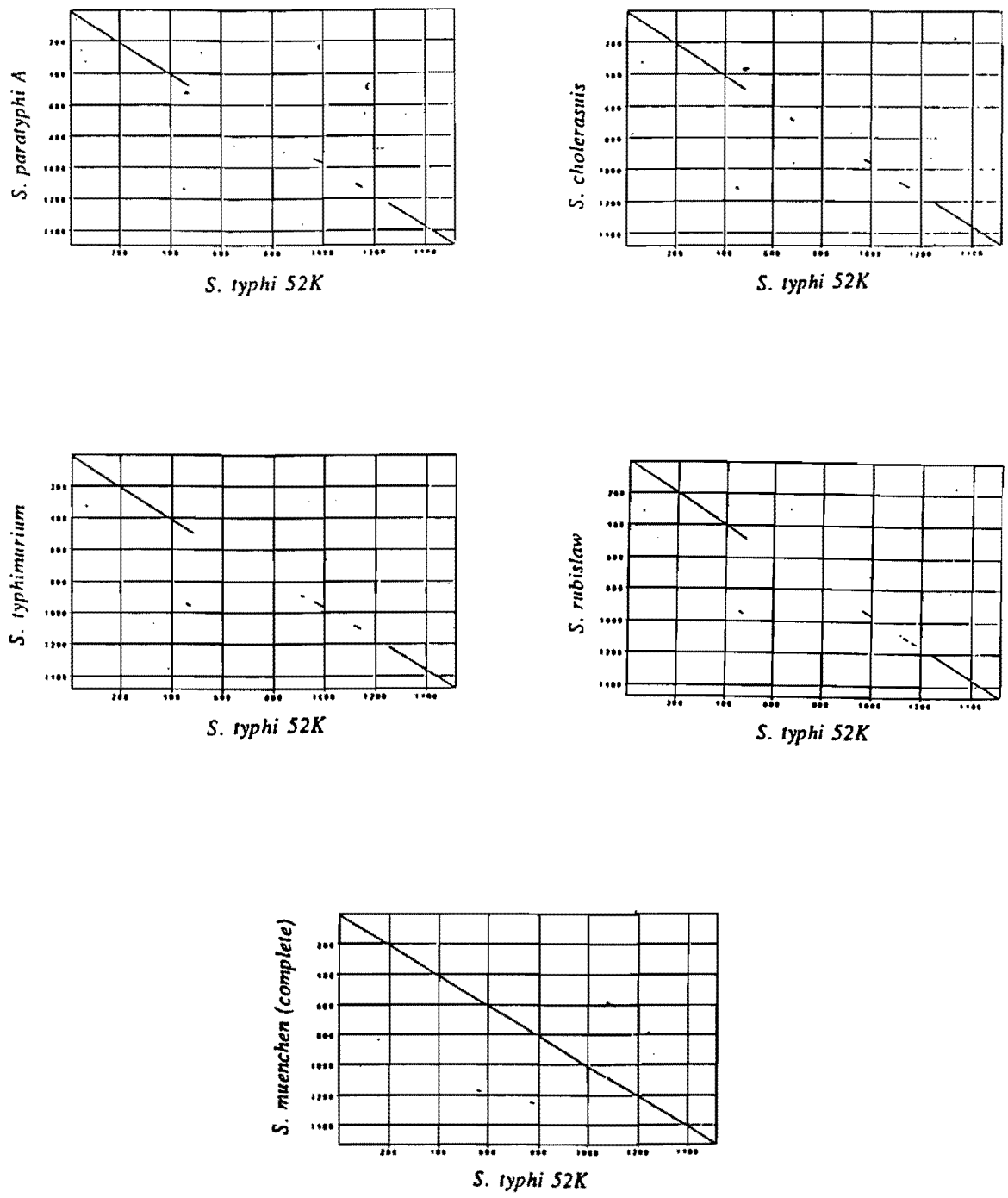

Fig. 2 Comparison of the *S. typhi* 52 kDa antigen gene and other phase-1 flagellin sequences by dot-matrix plots

DNA fragments ranging from 2 to 5 kb from the *S. typhi* genomic library were obtained. All positive clones were induced to express the proteins and subjected to immunoblotting using MAbs. The specific band at 52 kDa similar to the whole protein antigen of *S. typhi* was observed in 2 clones; one clone was selected for further study and was named pSKM-T7. This plasmid contained the insert of 2.0 kb and expressed a full length 52 kDa protein.

DNA sequence of *S. typhi* 52 kDa antigen gene

The complete nucleotide sequence of *S. typhi* 52 kDa antigen gene was deposited in GenBank (accession number L 21912) and shown in Fig. 1. The sequence was compared with Bacterial Division published in the GenBank Release 71.0 containing 6,583 entries. Seven published sequences[11-15] phase 1 flagellin genes from five bacteria of genus *Salmonella* were most similar. The 1,521 basepairs (bp) open reading frame of *S. typhi* 52 kDa antigen gene was aligned and compared with these seven sequences (Fig. 1). Comparison of the *S. typhi* 52 kDa antigen gene and five phase 1 flagellin sequences by dot-matrix plots (Fig. 2) showed highly homology at both ends; 400 bp of the 5′ ends and 200 bp of 3′ ends were almost identical. In particular, the phase 1-*d* flagellin gene of *S. muenchen* has extensive homology except in the portion of 1,054-1,170 nucleotides in the middle portion (Figs. 1, 2). Comparison of the nucleotide sequences within the same region indicated a similarity of 98%. There was a published 227 bp sequences of *S. typhi* flagellin *(H1-d)* gene[15] which was identical to the newly cloned 52 kDa antigen gene between nucleotides (nt) 952 to 1,179; except at nt 1,170 of our 52 kDa antigen gene which was G instead of A from published *S. typhi (H1-d)* sequence.

Characterization of the 52 kDa antigen gene of *S. typhi*

In order to prove that the 52 kDa antigen gene of *S. typhi* was phase 1-*d* flagellin gene, the bacteria with or without phase 1-*d* flagellin were compared. *S. muenchen,* a *Salmonella* group C with phase 1-*d* flagellin; *S. typhi* mutant strain without flagella(O901); *S. typhi* and *E. coli* negative control were grown on nitrocellulose-filter, fixed, lysed and stained by immunoenzyme assay using MAbs specific to the 52 kDa *S. typhi* proteins. The results showed positive red purple color with *S. muenchen* and *S. typhi* while *S. typhi* O901 and negative control were negative.

In another approach, *S. typhi* flagellin protein was prepared. Only one protein band was observed after SDS - PAGE and Coomassie blue staining with estimated molecular weight of 52 kDa. The flagellin was transferred to a nitrocellulose membrane and stained by immunoenzyme assay using specific MAbs. The result showed that *S. typhi* flagellin at 52 kDa reacted with specific MAbs identical to the extract from whole cells of *S. typhi* as well as the recombinant protein (Fig. 3). These findings confirmed that the specific MAbs recognized the phase 1-*d* flagellin of *Salmonella* including *S. typhi.*

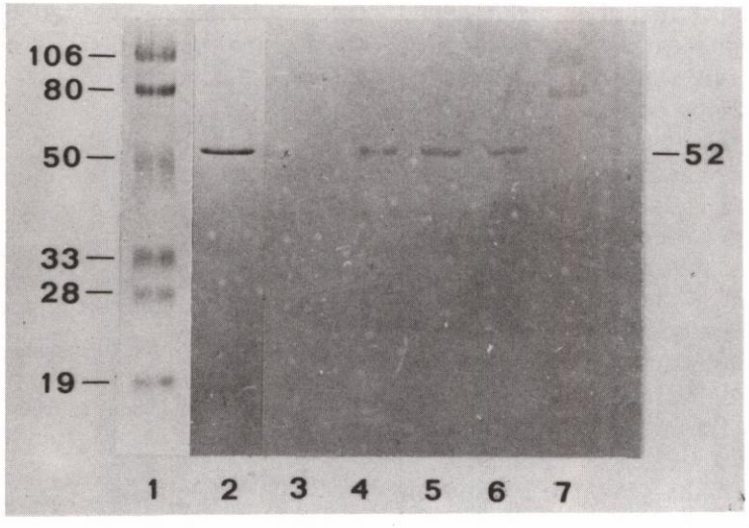

Fig. 3 a. *S. typhi* flagellin was subjected to SDS-PAGE and Coomassie blue staining
Lane 1 = Standard protein markers
Lane 2 = *S. typhi* flagellin
b. *S. typhi* flagellin was detected by immunoblotting technique using specific monoclonal antibodies to 52 kDa *S. typhi* protein.
Lane 3 = *E. coli* with pIC-20R (negative control)
Lane 4 = *S. typhi* whole cells (positive control)
Lane 5 = *S. typhi* flagellin
Lane 6 = *E. coli* with pSKM-T7 plasmid
Lane 7 = Standard protein markers

DISCUSSION

The major problem of diagnosis of typhoid fever is the differentiation between *S. typhi* infection and typhoid fever-like illness caused by members of the family *Enterobac-*

teriaceae. The most dependable way to establish a definitive diagnosis is by hemoculture which, however, has certain disadvantages. Many cultures gave false negative owing to prior antibiotic therapy. Even when appropriate hemocultures are taken, the presumptive bacteriological results require at least 48 hours for confirmative results.

Serological diagnosis of typhoid fever by the Widal test has been found to be unreliable[16,17] and confirmation requires both acute and convalescent phase serum samples, which are rarely collected. In endemic areas such as Thailand, the titer of the Widal test is quite high even in the normal population. The result of a single specimen is thus unable to be used for diagnosis. Therefore, many researchers are attempting to develop a better laboratory diagnosis of typhoid fever.

Because of their medical importance, bacteria of the genus *Salmonella* have been subjected to intensive serological analysis, and more than 60 types of flagella have been identified based upon the antigenic determinants present on the filaments. Individual *Salmonella* serotypes usually alternated between the production of the antigenic forms of flagella, termed "phase-1" and "phase-2".[18] Some species, including *S. typhi* are monophasic, with only a phase-1 antigen.[19] The flagella antigen, *d*, of *S. typhi* is present as a phase-1 antigen similar to the case for some members of *Salmonella* genus such as *S. muenchen* and *S. stanley*.[20]

To date, there is no single immunodiagnostic test that can be recommended for accurate and rapid diagnosis of typhoid fever. Such a test should be directed towards detecting IgM antibodies highly specific to *S. typhi* from sera of patients. We have established MAbs specific to an epitope of the 52 kDa protein of *S. typhi*. When acute sera from patients with typhoid fever were allowed to react with whole cell antigens in the imunoblot assay, specific IgM antibodies to this 52 kDa protein were detected. It appeared that these specific components were highly immunogenic and specific.[1]

Thus, using recombinant DNA technology, we cloned and expressed the 52 kDa gene from *S. typhi* in *E. coli*. Eight positive expressed recombinant clones were established. Among them, pSKM-T7 contained the smallest DNA insert of 2.0 kb and expressed the complete 52 kDa protein when subjected to SDS-PAGE and detected by immunoblotting using immunoenzyme assay. In order to determine the entire sequence of the *S. typhi* 52 kDa antigen gene, we subcloned by unidirectional deletions using Erase-a-Base system with exonuclease III. The sequence data were computer analysed and compared with those of the corresponding genes of other bacteria published in GenBank. It showed highly homology with the phase 1-*d* flagellin gene of *S. muenchen* (98% similarity) and 227 bp sequence of part of *S. typhi* flagellin gene (99.56% similarity). When the *S. typhi* sequence was compared with that of *S. muenchen*, 16 silent (third base) differences and 4 substitution mutations were identified. One extensive region of difference was found: a sequence of 9 codons, 350 to 358 of *S. muenchen* encoding 9 amino acids, all of which differed from the 10 amino acids specified by the corresponding sequence in the *S. typhi* gene. Analysis of the nucleotide sequence showed that insertion of an additional A (nt 1,048) in the sequence of the *S. muenchen* gene producing a reading frame shift, together with insertion of an A (nt 1,069) and T (nt 1,077) would change the *S. muenchen* sequence to one differing from the *S. typhi* sequence in only 30 positions.

The five phase-1 flagellin genes from *Salmonella* which have been cloned and sequenced[11-13] were highly homologous at their ends: the 300 bp of the 5′ ends were 99% identical, as were the 200 bp of their 3′ ends. However, the genes showed decreasing homology towards the middle portion. From the genetic analysis, Lino and co-workers[21,22] suggested that both ends of the *Salmonella* flagellin molecule are highly conserved and necessary for function, whereas the central region is variable.

The sequence in the central region of the *S. typhi* flagellin gene can be used to design primers for polymerase chain reaction (PCR) technique. The high sensitivity of this method may allow detection of *S. typhi* for diagnosis and epidemiological study of typhoid fever.

ACKNOWLEDGEMENTS

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