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Molecular Cloning and Expression of *Salmonella paratyphi* A 52 kDa Specific Protein Gene

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SUMMARY **Monoclonal antibodies (MAbs) specific to *Salmonella paratyphi* A have been established by our group in 1989. These MAbs were proven to be species-specific for 52 kDa protein of *S.paratyphi* A but the nature of this protein is unknown. However, our group have proved that the 52 kDa protein which is specific to *S.typhi* was flagellin. This present study has characterized the 52 kDa protien of *S.paratyphi* A and identified its encoded gene. The plasmid containing the specific 52 kDa antigen gene was cloned from the *S.paratyphi* A genome, herein designated pSKA-4. Partial nucleotide sequences from this clone was analysed by computer program and found to be phase 1-*a* flagellin gene of *S.paratyphi* A. In addition, the nucleotide sequence analysis from such clone also showed that the structural gene for phase 1 flagellin has amino acid sequences conserved at the terminal whereas the central region is variable among *Salmonella* spp. Therefore, the central portion of flagellin which highly polymorphic in amino acid sequences would be the most specific to *S.paratyphi* A, thus, should be used as specific antigen for developing specific diagnosis of *S.paratyphi* A infection. Using the PCR technique, an expression plasmid containing the antigen gene producing only the variable region in the central portion of flagellin from *S.paratyphi* A, namely pSKA-7, has been established. The recombinant protein produced by the established plasmid has a MW 33.5 kDa as detected by immunoblotting using specific MAbs. Further study by using this specific flagellin protein for immunodiagnosis of *S.paratyphi* A infection is being carried out in our laboratory.**

Despite of the decline of enteric fever in many countries, it continues to be a problem in many developing countries such as Indonesia, Malaysia, Chile, Vietnam and Thailand. In Indonesia, where the incidence is highest in the world, it was up to 810/100,000 population/year.[1] Enteric fever can be categorized into typhoid and paratyphoid fever. *Salmonella typhi* is the causative organism of thyphoid fever. *S. paratyphi* A, *S.schottmuelleri* (formerly *S.paratyphi* B) and *S.hirschfeldii* (formerly *S.paratyphi* C) are responsible for paratyphoid,[2] among which *S.paratyphi* A is the major causative agent of paratyphoid fever. In most instances, paratyphoid is clinically indistinguishable from typhoid fever; the conventional methods for diagnosis of typhoid and paratyphoid fever are haemoculture and/or antibody detection (Widal test). These methods have some limitations, the former test takes at least 5 days for confirmative results and false negatives are often obtained if the patients have consumed antibiotics before the test. The latter requires convalescent serum which is rarely obtained and four-fold rising in antibody titre of paired sera which takes at least 10 days for absolute result. Hence, it is not rapid enough to be of clinical benefit. Therefore, an improved laboratory test for a rapid and specific diagnosis for such organisms is highly desirable, especially a test that could differentiate *S.paratyphi* A from *S.typhi*. It would be great benefit not only for early treat-

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ment but also for epidemiological control of this disease.

To date, new immunological methods for improving diagnosis of typhoid fever including antigen[3-6] and antibody[7-8] detection have been reported by many investigators. However, reports on improved diagnosis of *S.paratyphi* A infection are limited. In 1989, our group has produced monoclonal antibodies (MAbs) specific to *S.paratyphi* A. These MAbs are highly specific and recognized only 52 kDa protein of *S.paratyphi* A without cross reactivity with proteins of other bacteria causing enteric fever and enteric fever-like illness *(S.typhi, S.paratyphi* B, *S.paratyphi* C, *S.cholerae-suis, S.enteritidis, S.krefeld, S.panama, S.typhimurium, Escherichia coli, Pseudomonas pseudomallei* and *Yersinia enterocolitica).* Hence, a specific epitope of *S.paratyphi* A was thought to be located on the 52 kDa protein. We have previously reported that a specific epitope of *S.typhi,* recognized by our another MAbs, was located on protein which also has a MW 52 *kDa.*[9] *Sukosol et al.*[10] have shown that the specific 52 kDa protein of *S.typhi* is flagellin which is the monomeric form of the flagellar filament and have shown that the central portion of flagellin has potential to be used as specific antigen for immunodiagnosis of typhoid fever.

The above findings prompted us to further characterize the nature of 52 kDa protein of *S.paratyphi* A and to identify its encoded gene.

MATERIALS AND METHODS

Bacterial strains and media

Salmonella paratyphi A used in this study was isolated from a patient in Siriaj Hospital, Bangkok, Thailand. *E.coli* JM 107 (Promega Co., USA) was used as the host strain for transformation and cultured in Luria Bertani medium (LB) (Difco Laboratories, Detroit, Mich).

Monoclonal antibodies

Monoclonal antibodies specific to *S.paratyphi A* (unpublished data) were produced by immunizing mouse with Barber protein[11] prepared from *S.paratyphi* A; fusion procedure was as described earlier.[12]

DNA isolation

High-molecular-weight genomic DNA was prepared from *S. paratyphi* A using guanidine thiocyanate.[13] Large amounts of *E.coli* plasmid DNA were isolated by alkaline lysis and purified in cesium chlorid ethidium bromide gradient centrifugation. Small scale isolations of recombinant plasmid DNA were accomplished by the alkaline lysis method.[14]

Construction of genomic DNA library and screening of bacterial clone expressing 52 kDa protein antigen.

S.paratyphi A genomic DNA was partially cleaved by restriction endonuclease *Sau* 3AI. DNA fragments ranging from 1.5-6.0 kb were size-fractionated by electrophoresis on 0.7% agarose gel. After electroelution, the DNA was purified using an Elutip-d column according to the manufacturer's instructions (Schleicher & Schuell) and ligated to the *Bam* HI site of the plasmid pIC20R.[15] The ligation was carried out overnight at 4°C in the presence of T_4 ligase, using the reaction buffer provided by Bethesda Research Laboratories, Gaithersburg, Md. The plasmids were then transformed into competent *E.coli* JM 107.[14] For detection of bacterial clones expressing the 52 kDa protein antigen specific to *S.paratyphi* A, the genomic DNA library was screened with MAbs. In brief, the transformed colonies were replicated onto nitrocellulose membrane (Schleicher & Schuell), immersed in a chloroform-atmosphere tank for 5 minutes and incubated in lysis solution (Tris-HCl 50 mM, NaCl 50 mM, $MgCl_2$ 10 mM, lysozyme 40 μg/μl and DNaseI 1 μg/μl) at 37°C for 2 hours. Subsequently, the filter was incubated in phosphate-buffered saline containing 0.1% tween 20 (PBST) supplemented with 3% bovine serum albumin (PBST-BSA) at room temperature for 1 hour, and washed three times with PBST. The filter was then incubated for 1 hour with MAbs specific to *S.paratyphi* A (diluted 1:250 in PBST-BSA), washed again, and further incubated with goat anti-mouse immunoglobulins-alkaline phosphatase conjugated (Dakopatts), diluted 1:250 with PBST-BSA. After 1 hour of incubation, non reactive conjugate was washed away, and o-dianizidine tetrazotised (60 mg/ml) and beta-naphthyl acid phosphate (10 mg/ml) (Sigma) in substrate buffer (0.1M carbonate buffer containing 1 mM $MgCl_2$) were added. Positive colonies of *E.coli* exhibited a red purple color while negative colonies were light-brown.

SDS-PAGE and immunoblotting

Purified flagellin protein from *S.paratyphi* A, protein extracted from whole cell of *S.paratyphi* A, recombinant *E.coli* containing pSKA-4 or pSKA-7 were subjected to SDS-PAGE in 12% acrylamide gel containing 0.1% SDS and 0.5 M urea[16] and was run at 40 mA constant current per gel slab at 25°C for 3 hours. Thereafter, proteins were stained with Coomassie brilliant blue and the molecular weights were calculated from a standard curve of standard protein markers by the method of Weber and Osborn.[17] For Western blotting, the separated protein bands on SDS-PAGE gel were electroblotted onto nitrocellulose paper in methanol-tris-glycine buffer at 6 V/cm for 16 hours as described by Towbin *et al.*[18] After electrotransfer, the nitrocellulose paper was stained with MAbs according to bacterial colony screening.

DNA sequencing and analysis of sequence data

DNA fragment from plasmid clone pSKA-4 were subcloned into the plasmid Bluescript KS (Stratagene) for nucleotide sequencing. The sequencing was performed by the dideoxy chain termination method of Sanger[19] using a modified T7 DNA polymerase ("Sequenase" US Biochemicals). DNA sequence data was analysed using Mac Vector program version 4.0 (IBI). Comparisons of sequence were carried out with sequences published in GenBank database release 71.0 on CD-ROM (NCBI, Washington DC).

Isolation of *S.paratyphi* A flagellin

Salmonella paratyphi A flagellin was purified according to the method of Ibrahim *et al.*[20] In brief, flagella were detached by exposure of bacteria to pH $\leqslant$ 2 with 1 N hydrochloric acid, then centrifuged at 100,000 g for 1 hour at 4°C. The supernatant containing flagellin was adjusted to pH 7.2 with 1 N sodium hydroxide, precipitated with ammonium sulfate at final concentration of 2.67 M, and left for 16 hours at 4°C. Precipitated flagellin was separated by centrifugation at 15,000 × g for 15 minutes and dialyzed against water for 18 hours at 4°C. The dialyzed flagellin preparations were then lyophilized and stored at 4°C in the dark over dried silica gel.

Primer used for amplification

For the amplification of the variable region in the phase 1 flagellin gene, the following two primers were used: 5'GAGGATCCAGGTTGGTGCCA3' (primer I) and 5'GTGAATTCGGAGTTGAAACG3' (primer 2) according to nucleotide positions 452-463 and 1314-1303 of *S.paratyphi* A *fliC* gene.[21] To facilitate gene cloning, nucleotide sequences specific for restriction enzyme *Bam* HI and *Eco* RI was included in primers 1 and 2, respectively.

Amplification and cloning of variable region of *fliC* gene

DNA was amplified by the polymerase chain reaction (PCR)[22], A reaction 100 µl (10-40 ng) of pSKA-4 plasmid DNA, 50-100 pM(each) primer (primers 1 and 2), 200 µM nucleotide mixture (ultrapure deoxynucleoside triphosphate set; Pharmacia Biotechnology LKB, Uppsala, Sweden), 10 µl of 10 × *Taq* polymerase buffer (Amersham, United Kingdom) and 2.5 µl of *Taq* polymerase (Amersham). The reaction mixture was covered with 75 µl of mineral oil (Sigma Chemical Co., St. Louis, Mo.), and the PCR was performed with a Perkin-Elmer Cetus DNA Thermal Cycler 480 (Perkin-Elmer Corporation, Norwalk, Conn.). After an initial denaturation step of 5 minutes at 94°C, 35 cycles were performed. Each cycle comprised the following steps: 1 minute at 94°C (denaturation), 1 minute at 50°C (primer annealing), and 2 minutes at 72°C (elongation). PCR product were analyzed on 1.5% agarose gel. The amplified DNA fragment was isolated from agarose gel[14] digested with *Bam* HI and *Eco* RI before ligated to the *Bam* HI-*Eco* RI site of plasmid pGEX-3X.[23] The recombinant plasmid was transformed into the *E.coli* JM 107. Positive clone was identified by immunostaining of bacterial colony with MAbs, using the same screening method for identifying bacterial clone expressing the 52 kDa protein.

RESULTS

Cloning of the 52 kDa protein antigen gene of *S.paratyphi* A

When genomic DNA library of *S.paratyphi* A was screened with MAbs which specific to 52 kDa protein of *S.paratyphi* A, one positive clone was identified and was named, pSKA-4. This recombinant *E.coli* contained 2.7 kb of DNA insert. The restriction enzyme mapping was illustrated in Fig. 1. Analysis of expression protein from clone pSKA-4 by immunoblotting revealed that this clone can express a full length of the 52 kDa protein recognized by the MAbs similar to the whole protein antigen of *S. paratyphi* A (Fig. 2).

Partial DNA sequences of *S.paratyphi* A 52 kDa protein gene

The nucleotide sequences of 300 bp obtained from clone pSKA-4 were compared with nucleotide sequence data deposited in GenBank database. From comparison, the result (data not shown) indicated that the gene encoding 52 kDa protein is phase 1 flagellin gene *(fliC)* of *S.paratyphi* A that have been previously deposited by Wei and Joys in 1985.[21] The entire coding sequence of *filC (a)* gene is 1497 bp.

In order to confirm that the 52 kDa protein expressed from clone pSKA-4 is the flagellin protein, *S. paratyphi* A flagellin was prepared. After SDS-PAGE and Coomassie

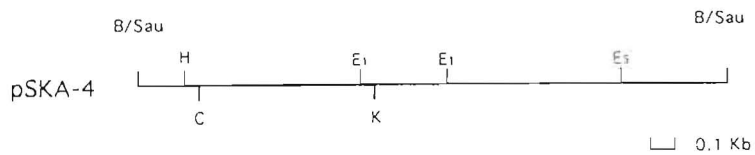


Fig. 1 Restriction map of insert DNA in pSKA-4. Restriction enzymes: B, *Bam* HI; Sau, *Sau* 3AI; H, *Hind* III; E1, *Eco* RI; E5, *Eco* RV; K, *Kpn* I and C, *Cla* I.

brilliant blue staining, only one protein band was observed, with estimated molecular weight of 52 kDa (Fig. 2A). The flagellin from SDS-PAGE gel was transferred to nitrocellulose membrane and stained by immunoenzyme assay using our established MAbs. The result showed that *S.paratyphi* A flagellin at 52 kDa reacted with specific MAbs identical to the extract from whole cells of *S.paratyphi* A and the protein extracted from clone pSKA-4 (Fig. 2). Several minor bands of smaller sizes were also detected in Fig. 2B lane 3, possibly due to the degradation product of flagellin. In addition, the recombinant protein from clone pSKA-4 also showed positive staining with anti-serum against H antigen from *S.paratyphi* A (a gift from National Institute of Health, Thailand) at MW 52 kDa (data not shown). These findings confirmed that the specific MAbs recognized the phase 1-*a* flagellin of *S.paratyphi* A.

Analysis of sequence data from phase 1-*a* of *S.paratyphi* A with 5 other flagellin sequences including *S.typhi*,[10] *S.cholerae-suis*, *S.muenchen*,[21] *S.typhimurium*[24] and *E.coli*[25] showed highly homology at both ends among *Salmonella* spp.; 470 bp of the 5′ ends and 200 bp of 3′ ends were almost identical (Fig. 3). This conserved sequence, 300 bp from the 5′ end, is also conserved in *E.coli*. However, 200 bp at the 3′ end of *fliC* gene from *Salmonella* spp. showed lesser degree of nucleotide sequence conservation comparing with *E.coli*

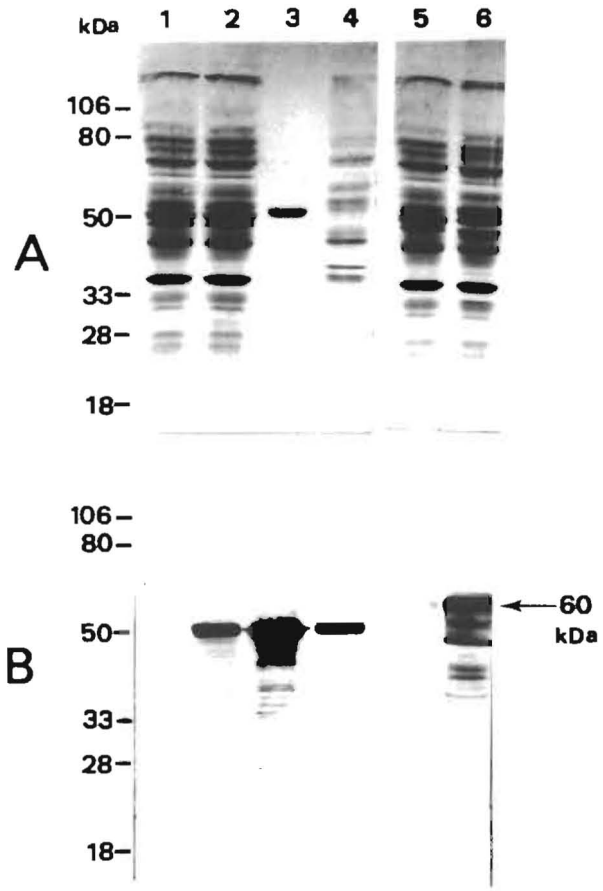


Fig. 2 SDS-PAGE of proteins extracted from *E.coli* containing pIC20R (lane 1), *E.coli* containing pSKA-4 (lane 2), *S.paratyphi* A (lane 4), *E.coli* containing pGEX-3X (lane 5), *E.coli* containing pSKA-7 (lane 6) and purified *S.paratyphi* A flagellin (lane 3) are subjected to the following procedures

A) Coomassie brilliant blue staining.

B) Immunostaining of Western blot. The proteins were transferred to nitrocellulose membrane and then detected by MAbs specific to *S.paratyphi* A.

PCR amplification of *S.paratyphi* A *fliC* gene

Since data from nucleotide sequence analysis indicated that 5′ and 3′ ends of flagellin gene are highly conserved whereas the central region is polymorphism. To express specific protein for immunodiagnosis, the PCR technique was used to generate sequences about 900 bp from the central region of the *fliC* gene (phase 1 flagellin) of *S.paratyphi* A. PCR primers were chosen from the conserved N-terminal (primer 1) and C-terminal (primer 2) regions of *S.paratyphi* A *fliC* gene. After 35 cycles of PCR amplification, a fragment of the expected size was cloned into the expression vector, pGEX-3X.[23] Recombinant *E.coli* expressed variable region of flagellin protein from *S.paratyphi* A was identified by colony staining with specific MAbs. Chimeric protein comprised of *S.paratyphi* A flagellin and GST proteins at MW 60 kDa was detected from clone pSKA-7 (Fig. 2), minor degradation product bands were also observed. The MW 60 kDa of this chimeric protein resulted from 26.5 kDa of GST and 33.5 kDa of the central region of *S.paratyphi* A phase 1 fla-

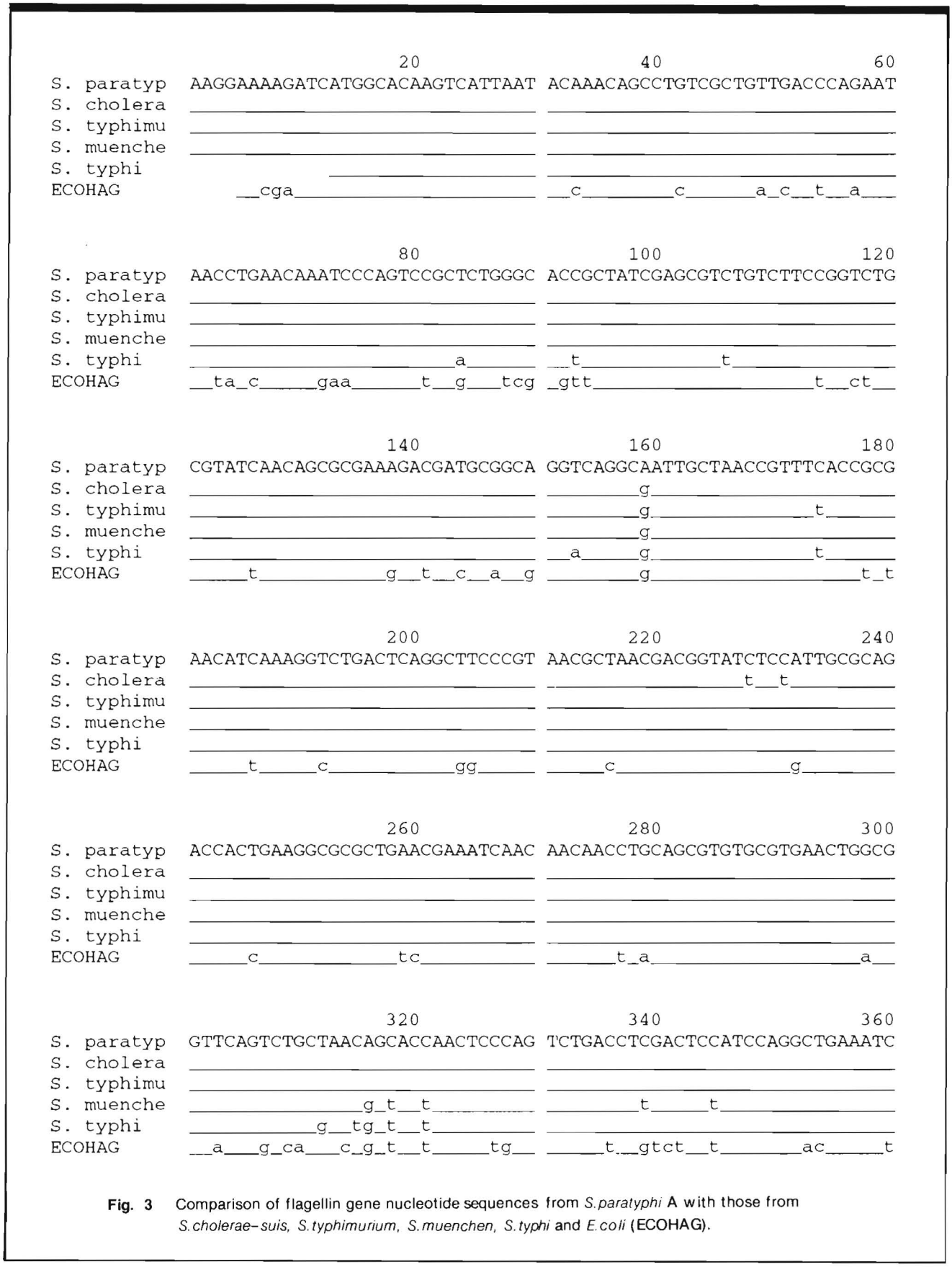
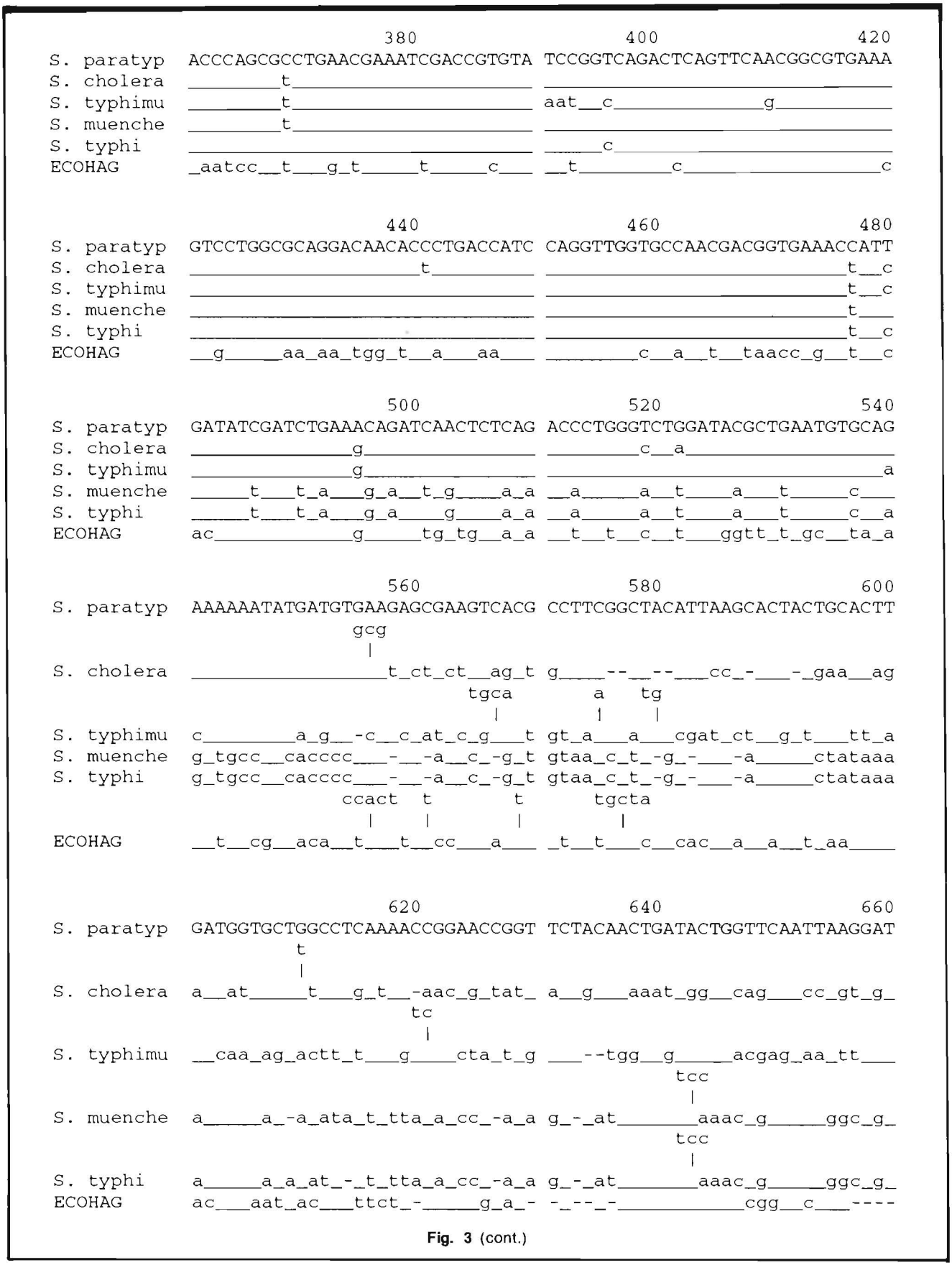


Fig. 3 Comparison of flagellin gene nucleotide sequences from *S.paratyphi* A with those from *S.cholerae-suis*, *S.typhimurium*, *S.muenchen*, *S.typhi* and *E.coli* (ECOHAG).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S. cholera _____t_____ _____
S. typhimu _____t_____ aat__c_____g_____
S. muenche _____t_____ _____
S. typhi _____ _____c_____
ECOHAG _aatcc__t___g_t_____t_____c___ __t_____c_____c
440 460 480
S. paratyp GTCCTGGCGCAGGACAACACCCTGACCATC CAGGTTGGTGCCAACGACGGTGAAACCATT
S. cholera _____t_____ _____t__c
S. typhimu _____ _____t__c
S. muenche _____ _____t____
S. typhi _____ _____t__c
ECOHAG __g_____aa_aa_tgg_t__a___aa___ _____c__a__t__taacc_g__t__c
500 520 540
S. paratyp GATATCGATCTGAAACAGATCAACTCTCAG ACCCTGGGTCTGGATACGCTGAATGTGCAG
S. cholera _____g_____ _____c__a_____
S. typhimu _____g_____ _____a
S. muenche _____t___t_a___g_a__t_g____a_a __a_____a__t____a___t_____c___
S. typhi _____t___t_a___g_a____g____a_a __a_____a__t____a___t_____c__a
ECOHAG ac_____g_____tg_tg__a_a __t__t__c__t___ggtt_t_gc__ta_a
560 580 600
S. paratyp AAAAAATATGATGTGAAGAGCGAAGTCACG CCTTCGGCTACATTAAGCACTACTGCACTT
gcg
S. cholera _____t_ct_ct__ag_t g____--__--___cc_-___-_gaa__ag
tgca a tg
S. typhimu c_____a_g__-c__c_at_c_g___t gt_a___a___cgat_ct__g_t___tt_a
S. muenche g_tgcc__cacccc___-__-a__c_-g_t gtaa_c_t_-g_-____-a_____ctataaa
S. typhi g_tgcc__cacccc___-__-a__c_-g_t gtaa_c_t_-g_-____-a_____ctataaa
ccact t t tgcta
ECOHAG __t__cg__aca__t___t__cc___a___ _t__t___c__cac__a__a__t_aa____
620 640 660
S. paratyp GATGGTGCTGGCCTCAAAACCGGAACCGGT TCTACAACTGATACTGGTTCAATTAAGGAT
t
S. cholera a__at_____t___g_t__-aac_g_tat_ a__g___aaat_gg__cag___cc_gt_g_
tc
S. typhimu __caa_ag_actt_t___g____cta_t_g ___--tgg__g_____acgag_aa_tt___
tcc
S. muenche a_____a_-a_ata_t_tta_a_cc_-a_a g_-_at_____aaac_g_____ggc_g_
tcc
S. typhi a_____a_a_at_-_t_tta_a_cc_-a_a g_-_at_____aaac_g_____ggc_g_
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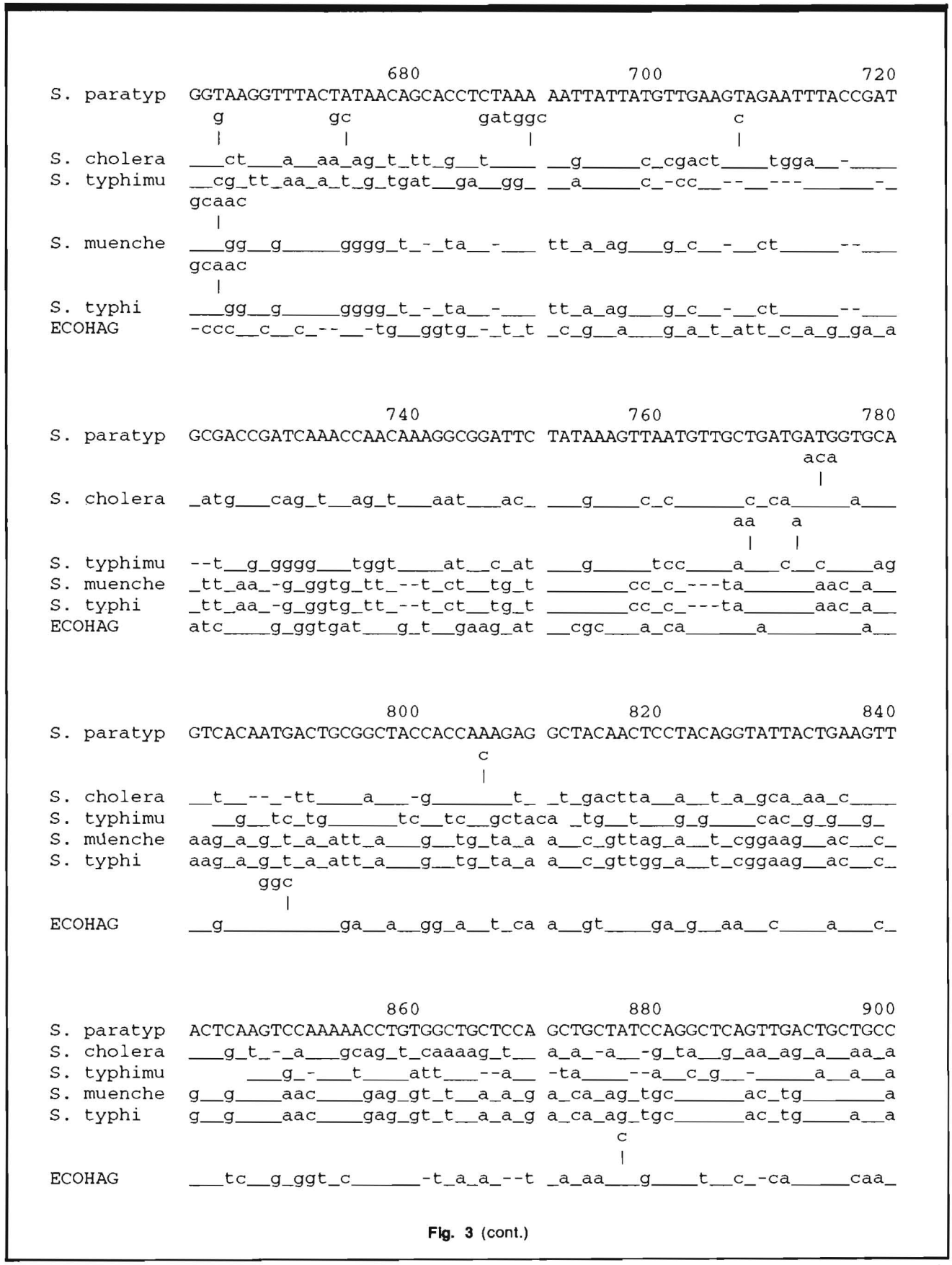
Fig. 3 (cont.)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S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
740 760 780
S. paratyp GCGACCGATCAAACCAACAAAGGCGGATTC TATAAAGTTAATGTTGCTGATGATGGTGCA
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
800 820 840
S. paratyp GTCACAATGACTGCGGCTACCACCAAAGAG GCTACAACTCCTACAGGTATTACTGAAGTT
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
860 880 900
S. paratyp ACTCAAGTCCAAAAACCTGTGGCTGCTCCA GCTGCTATCCAGGCTCAGTTGACTGCTGCC
S. cholera
S. typhimu
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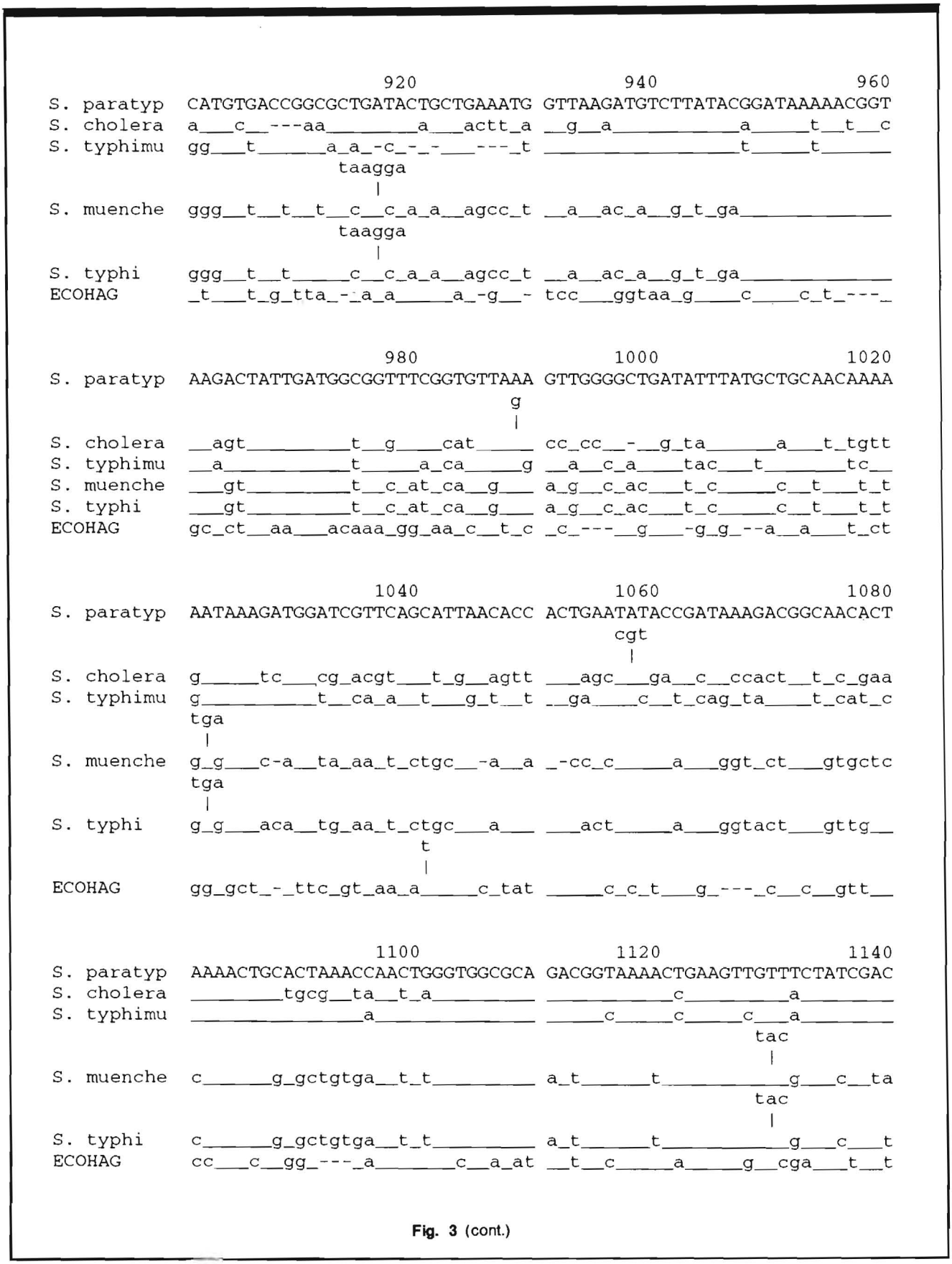
ECOHAG

Fig. 3 (cont.)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S. cholera a___c__---aa_____a___actt_a __g__a_____a_____t__t__c
S. typhimu gg___t_____a_a_-c_-_-___---_t _____t_____t_____
taagga
S. muenche ggg__t__t__t__c__c_a_a__agcc_t __a__ac_a__g_t_ga_____
taagga
S. typhi ggg__t__t_____c__c_a_a__agcc_t __a__ac_a__g_t_ga_____
ECOHAG _t___t_g_tta_-_a_a_____a_-g__- tcc___ggtaa_g____c____c_t_---_
980 1000 1020
S. paratyp AAGACTATTGATGGCGGTTTCGGTGTTAAA GTTGGGGCTGATATTTATGCTGCAACAAAA
g
S. cholera __agt_____t__g____cat_____ cc_cc__-__g_ta_____a___t_tgtt
S. typhimu __a_____t_____a_ca_____g __a__c_a____tac___t_____tc__
S. muenche ___gt_____t__c_at_ca__g___ a_g__c_ac___t_c_____c__t___t_t
S. typhi ___gt_____t__c_at_ca__g___ a_g__c_ac___t_c_____c__t___t_t
ECOHAG gc_ct__aa___acaaa_gg_aa_c__t_c _c_---__g____-g_g_--a__a___t_ct
1040 1060 1080
S. paratyp AATAAAGATGGATCGTTCAGCATTAACACC ACTGAATATACCGATAAAGACGGCAACACT
cgt
S. cholera g_____tc___cg_acgt___t_g__agtt ___agc___ga__c__ccact__t_c_gaa
S. typhimu g_____t__ca_a__t___g_t__t __ga____c__t_cag_ta____t_cat_c
tga
S. muenche g_g___c-a__ta_aa_t_ctgc__-a__a _-cc_c_____a___ggt_ct___gtgctc
tga
S. typhi g_g___aca__tg_aa_t_ctgc___a___ ___act_____a___ggtact___gttg__
t
ECOHAG gg_gct_-_ttc_gt_aa_a_____c_tat _____c_c_t___g_---_c__c__gtt__
1100 1120 1140
S. paratyp AAAACTGCACTAAACCAACTGGGTGGCGCA GACGGTAAAACTGAAGTTGTTTCTATCGAC
S. cholera _____tgcg__ta__t_a_____ _____c_____a_____
S. typhimu _____a_____ _____c_____c_____c___a_____
tac
S. muenche c_____g_gctgtga__t_t_____ a_t_____t_____g___c__ta
tac
S. typhi c_____g_gctgtga__t_t_____ a_t_____t_____g___c___t
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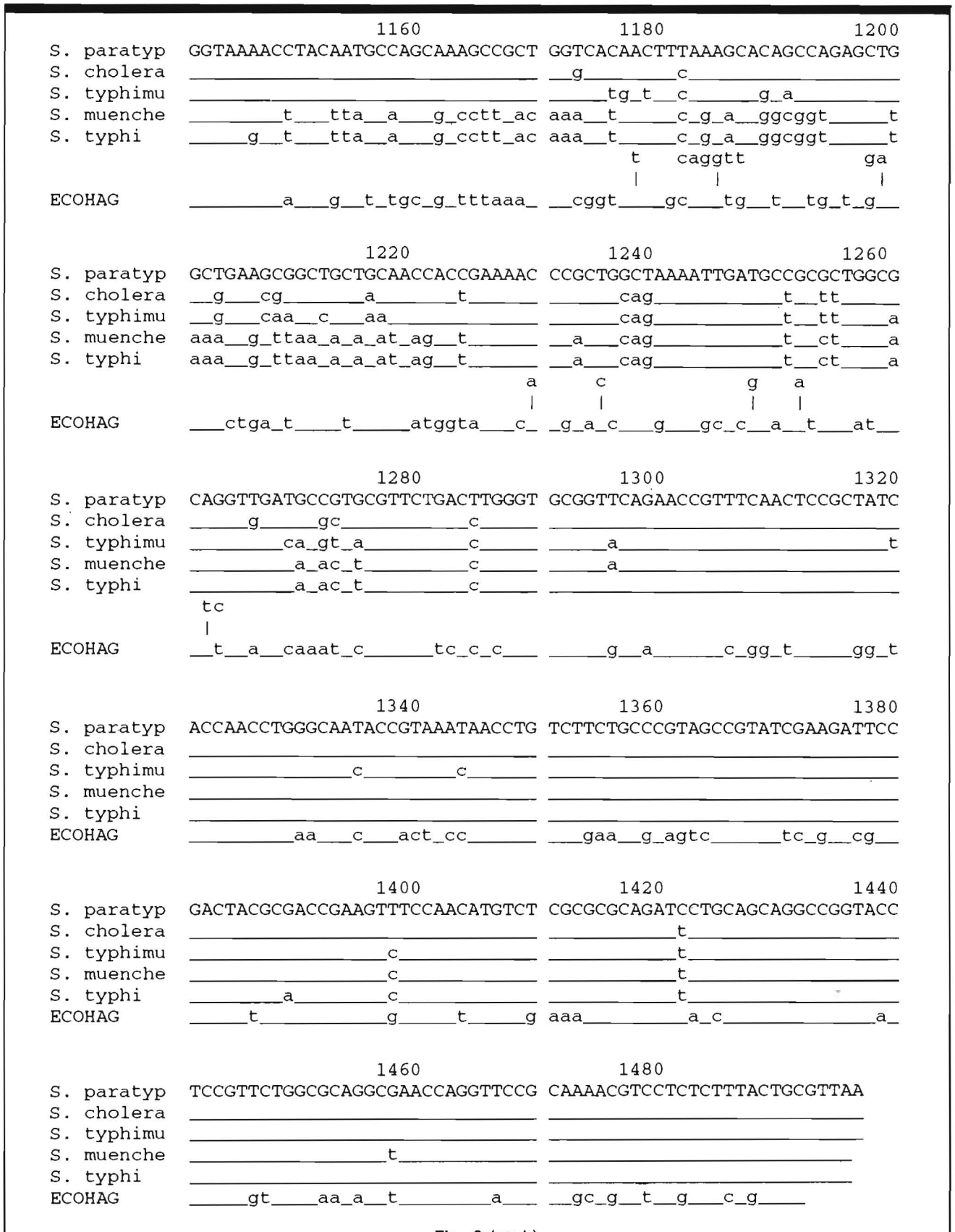
Fig. 3 (cont.)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S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
1220 1240 1260
S. paratyp GCTGAAGCGGCTGCTGCAACCACCGAAAAC CCGCTGGCTAAAATTGATGCCGCGCTGGCG
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
1280 1300 1320
S. paratyp CAGGTTGATGCCGTGCGTTCTGACTTGGGT GCGGTTCAGAACCGTTTCAACTCCGCTATC
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
1340 1360 1380
S. paratyp ACCAACCTGGGCAATACCGTAAATAACCTG TCTTCTGCCCGTAGCCGTATCGAAGATTCC
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
1400 1420 1440
S. paratyp GACTACGCGACCGAAGTTTCCAACATGTCT CGCGCGCAGATCCTGCAGCAGGCCGGTACC
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG
1460 1480
S. paratyp TCCGTTCTGGCGCAGGCGAACCAGGTTCCG CAAAACGTCCTCTCTTTACTGCGTTAA
S. cholera
S. typhimu
S. muenche
S. typhi
ECOHAG

Fig. 3 (cont.)

gellin (corresponding to nucleotide numbers 452-1314 of *S.paratyphi* A *fliC* gene). The fusion of flagellin to GST would allow convenient purification of recombinant flagellin from *E.coli*.

DISCUSSION

A rapid, specific and sensitive method is needed for the dignosis of *S.paratyphi* A, the major causative agent of paratyphoid fever. The problem of laboratory diagnosis of paratyphoid fever is mainly caused by the delay of 4-7 days using the conventional culture technique succeeds in isolating around 60-70% of the clinically diagnosed *S.paratyphi* A infection.[1] The used of Widal agglutination test is unreliable[26] and confirmation requires both acute and convalescent phase serum samples, which are rarely collected.

To date, there is no single immunodiagnostic test that can be recommended for accurate and rapid diagnosis of paratyphoid fever. Such a test should be directed towards detecting IgM antibodies specific to *S.paratyphi* A from sera of patients.[10] We have established MAbs directed against a specific epitope on the 52 kDa protein of *S.paratyphi* A. The nature of this protein is unknown. However, when sera which contain IgM from patients with acute *S. paratyphi* A infections were allowed to react with whole cell antigens in the immunoblot assay, IgM band at 52 kDa protein was detected only in patients with positive Widal test (unpublished data). Therefore, the component of this 52 kDa protein has potential to be used as specific antigen for detection of IgM antibody from *S.paratyphi* A infected patients.

Thus, using recombinant DNA technology, we cloned and expressed the 52 kDa gene from *S.paratyphi* A in *E.coli*. One positive recombinant clone was established, namely pSKA-4. This clone contained DNA insert of 2.7 kb and expressed 52 kDa protein as detected by immunostaining of Western blot with MAbs specific to *S.paratyphi* A. When 300 nucleotides obtained from clone pSKA-4 was analysed and compared with gene sequence data published in GenBank database. It showed highly homology with the phase 1-*a* flagellin gene of *S. paratyphi* A that has been previously deposited by Wei and Joys in 1985.[21] Apart from nucleotide suquence analysis, result from immunostaining of flagellin protein purified from *S.paratyphi* A interacted with the specific MAbs strongly indicated that the 52 kDa protein which contained specific epitope of *S.paratyphi* A is flagellin. This result is similar to our previous report that MAbs specific to *S.typhi* also recognized flagellin.[10]

Flagellin proteins is monomeric form of bacterial flagellum. Most *Salmonella* strains possess two structural genes *(fliC* and *fljB)* coding for flagellins. No more than one of these structural genes is expressed at a time in one bacterial cell. Non motile strains generally possess the structural genes but are unable to build up a functional flagellum.[27] Several sequences of the gene coding for phase 1 flagellin *(fliC)* have been published. Comparison between *fliC* gene sequences from *S.paratyphi* A and other *Salmonella* spp. showed conservation of nucleotide sequences at both ends whereas the central region is variable. Therefore, specific epitope of MAbs on flagellin would localize within the central region which might be useful as specific antigen to detect IgM antibody from serum.

Using the PCR technique, a clone containing pSKA-7 expressed fusion protein between the central region of flagellin and GST proteins was successfully isolated. This fusion protein has a total MW of 60 kDa which is composed of 33.5 kDa contained only variable region of flagellin protein fused to a 26.5 kDa GST protein. Further study by using this specific flagellin protein for immunodiagnosis of *S. paratyphi* A infection is being carried out in our laboratory.

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