Enhancing menaquinone-7 production in recombinant *Bacillus amyloliquefaciens* by metabolic pathway engineering

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Running title：Improving MK-7 production by bioengineering

Abbreviations: MK-n - Menaquinones; DHNA - 1,4-dihydroxy- 2-naphthoate; DAHP - 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHNA - 1,4-dihydroxy-2-naphthoate; OSB - o-succinylbenzoic acid; SEPHCH - 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid; SHCHC - 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid; EGTA - Ethylene Glycol Tetraacetic Acid; LB - Luria Bertani medium; TYG - Tryptone-Yeast extract-Glucose medium; GYS - Glycerol-Yeast extract-Soy peptone medium; SM - Spizizen minimal medium; DCW - Dry cell Weight; CCTCC - China Center for Type Culture Collection; CICIM - China Information Center of Industrial Microbial.

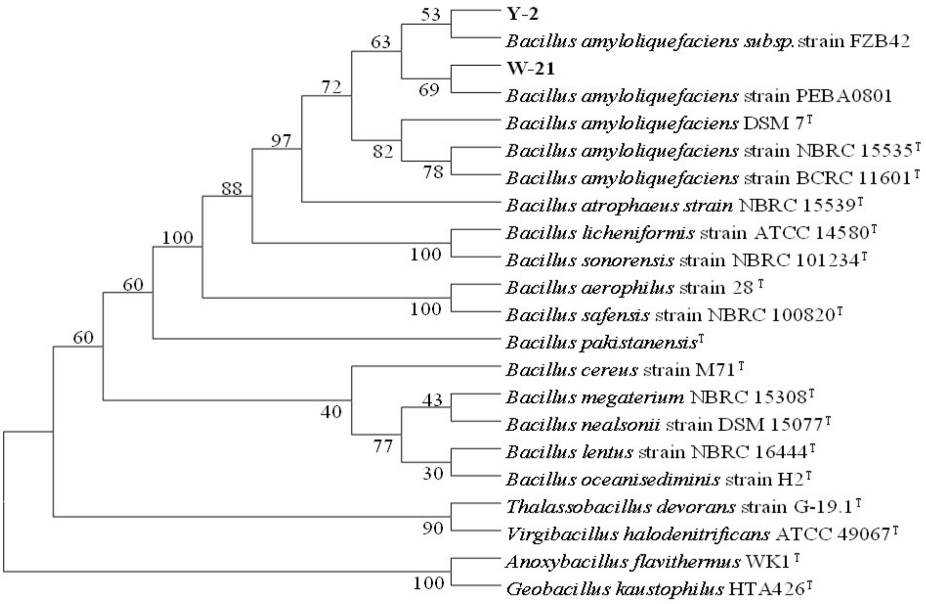


Figure S1 Phylogenetic tree inferred from 16S rRNA of strains Y-2 and W-21

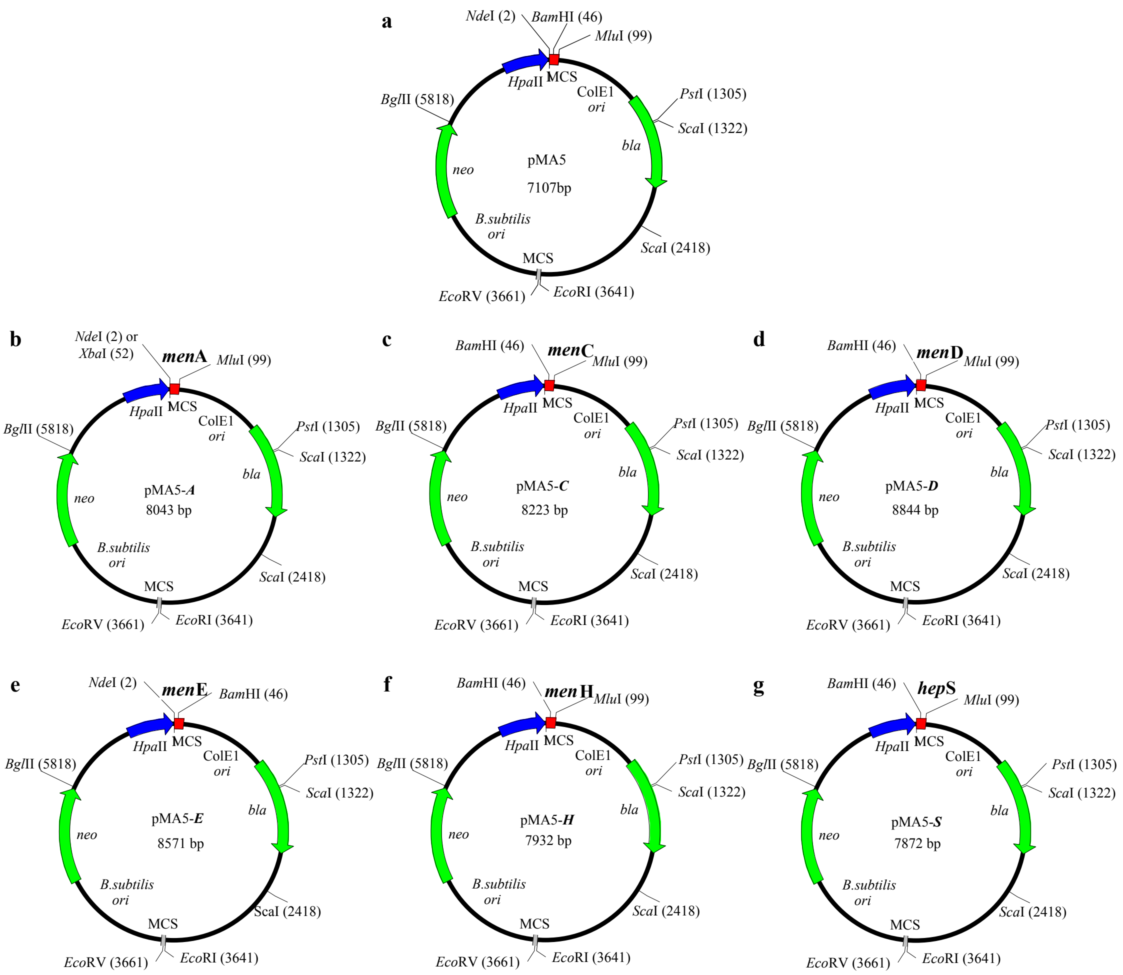
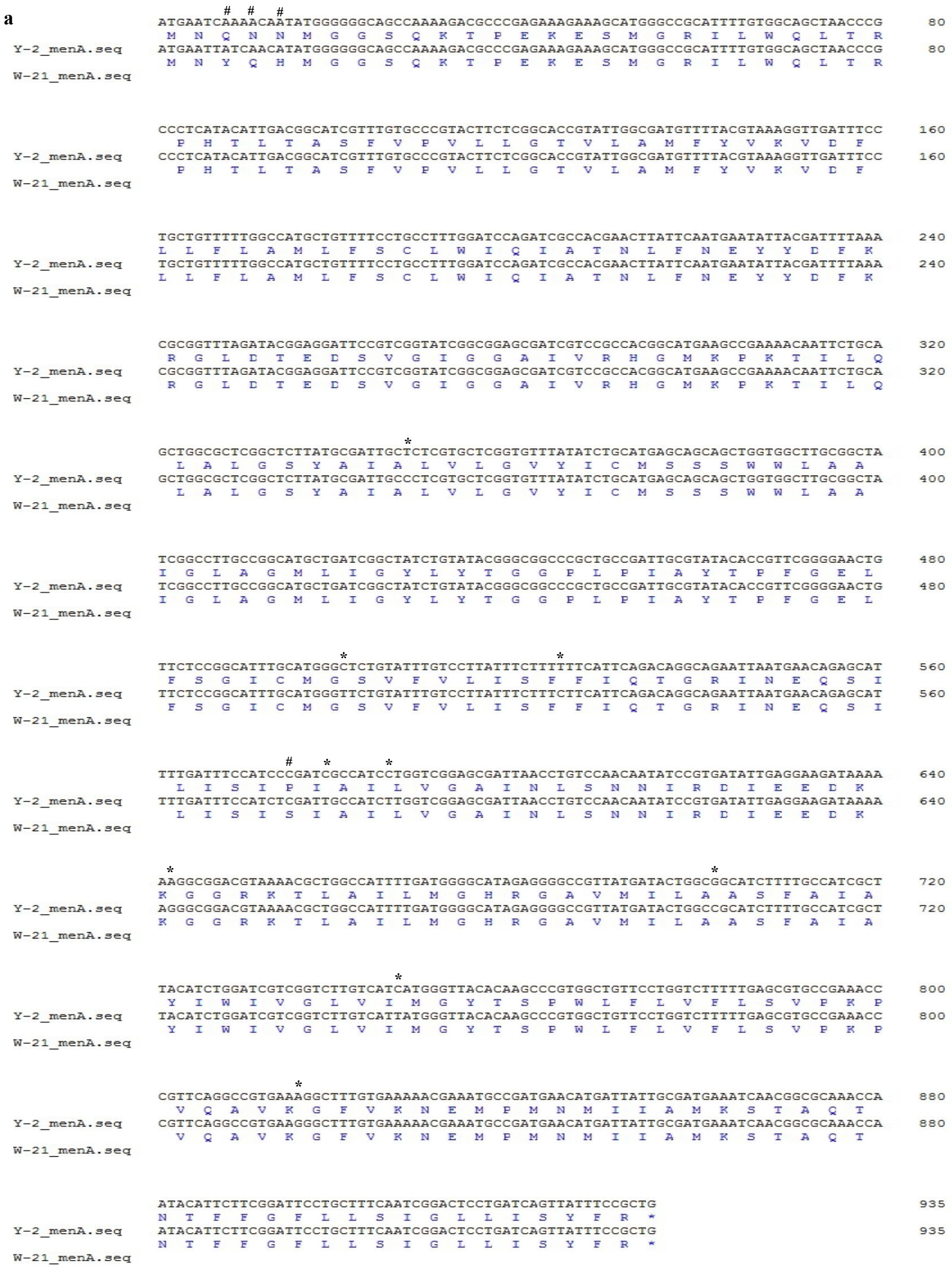


Figure S2 Plasmid maps of (**a**) pMA5, (**b**) pMA5-*A*, (**c**) pMA5-*C*, (**d**) pMA5-*D*, (**e**) pMA5-*E*, (**f**) pMA5-*H* and (**g**) pMA5-*S*. *A* represents gene *menA*; *C* represents gene *menC*; *D* represents gene *menD*; *E* represents gene *menE*; *H* represents gene *menH*; *S* represents gene *hepS*.



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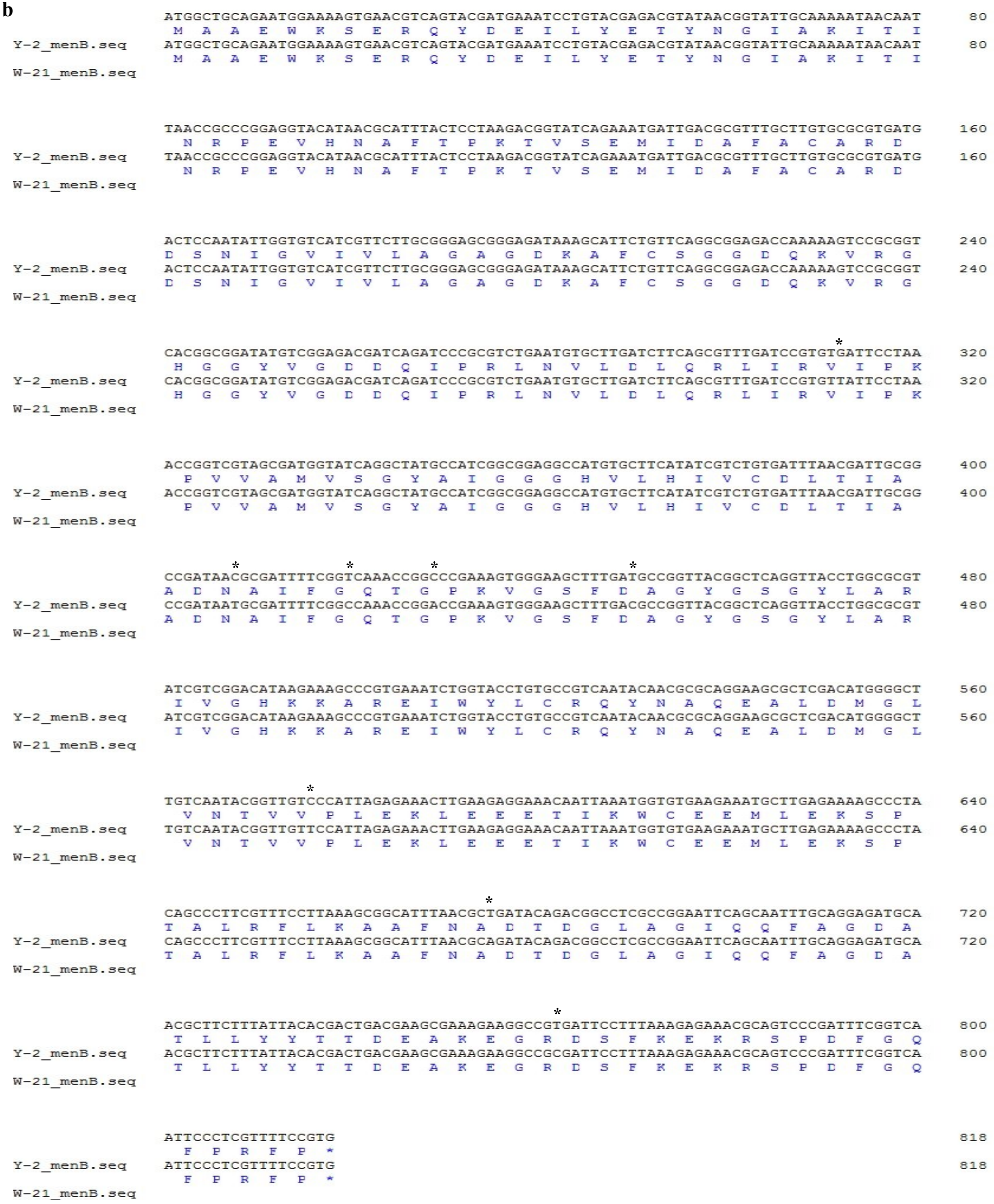

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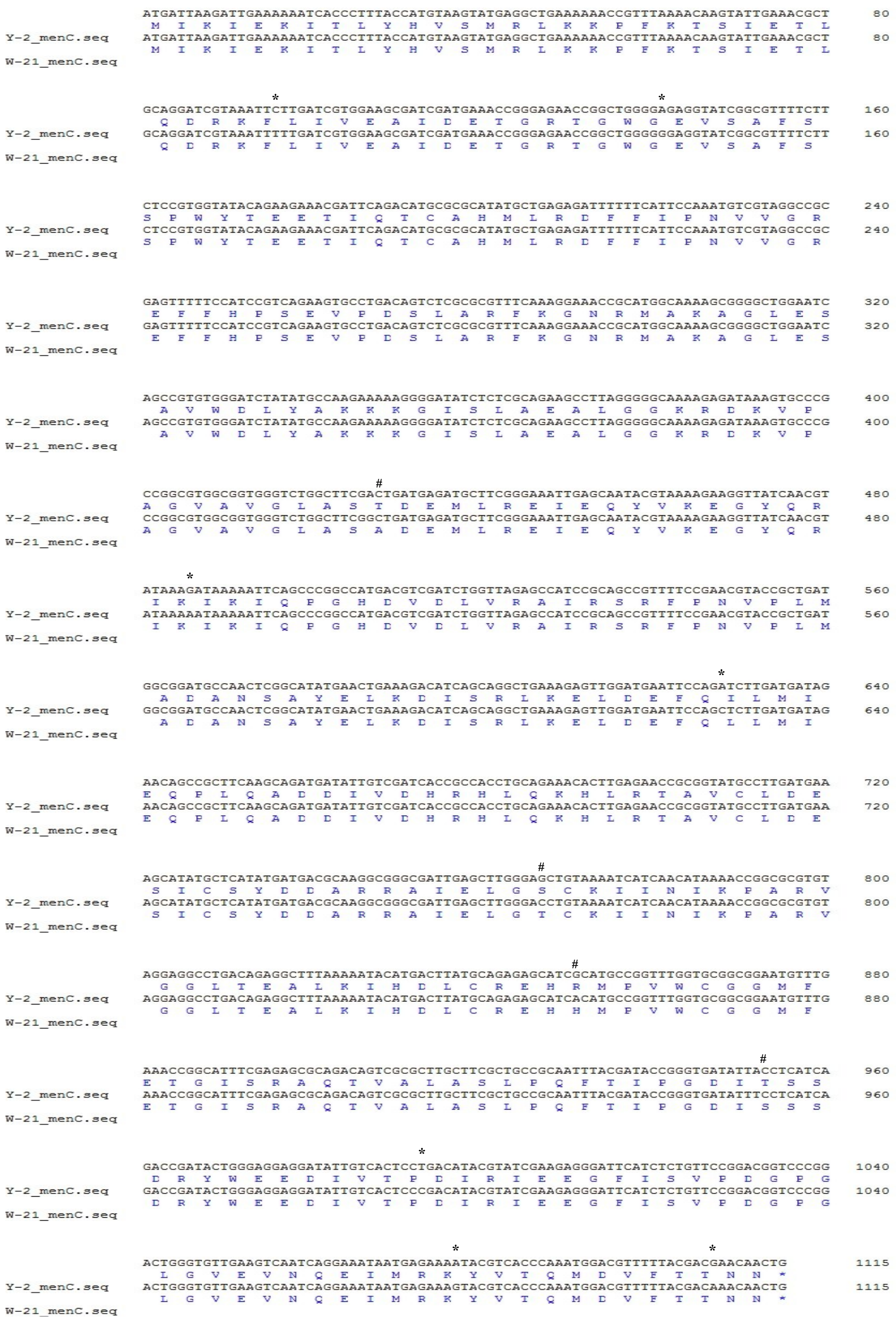

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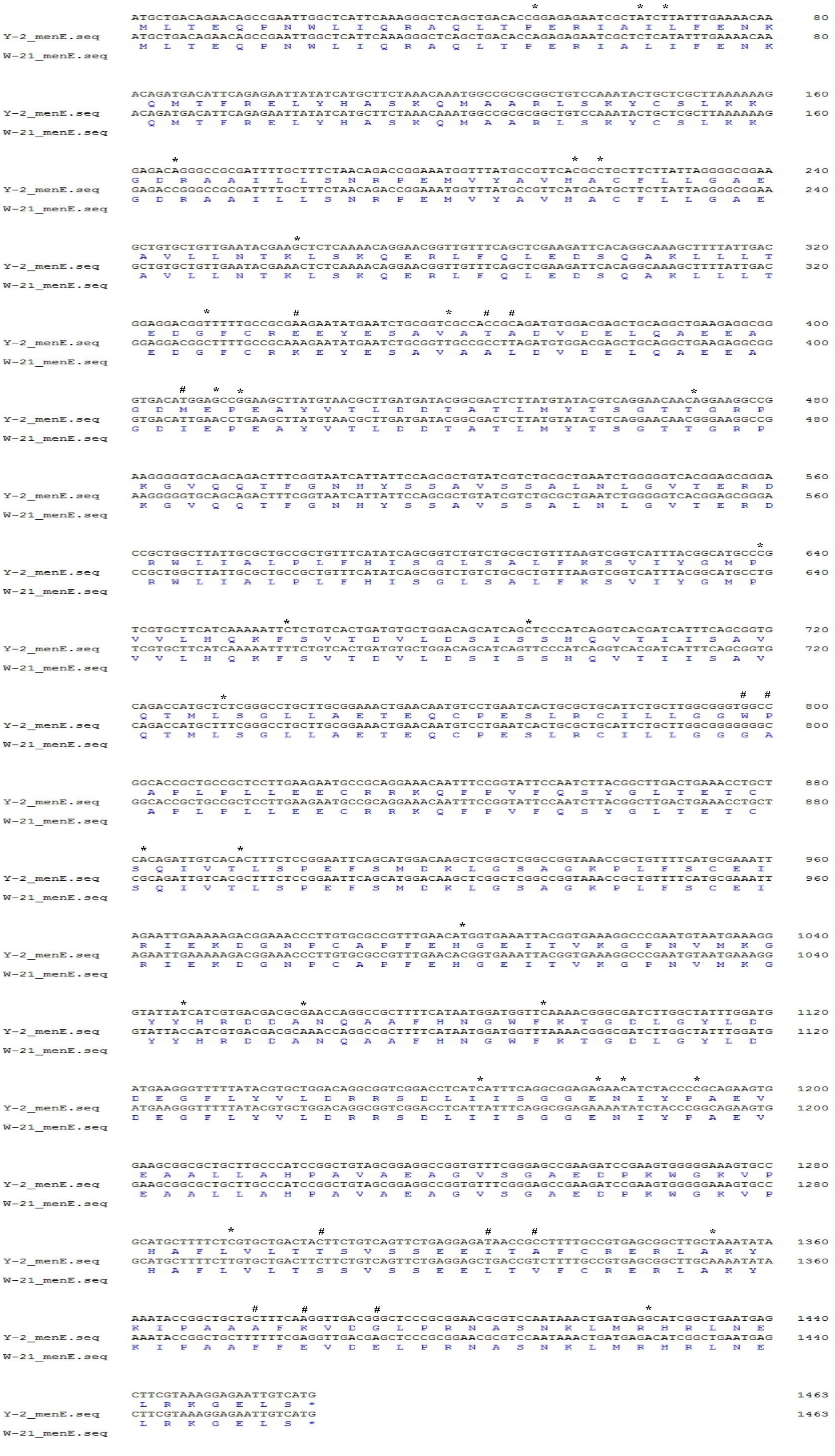

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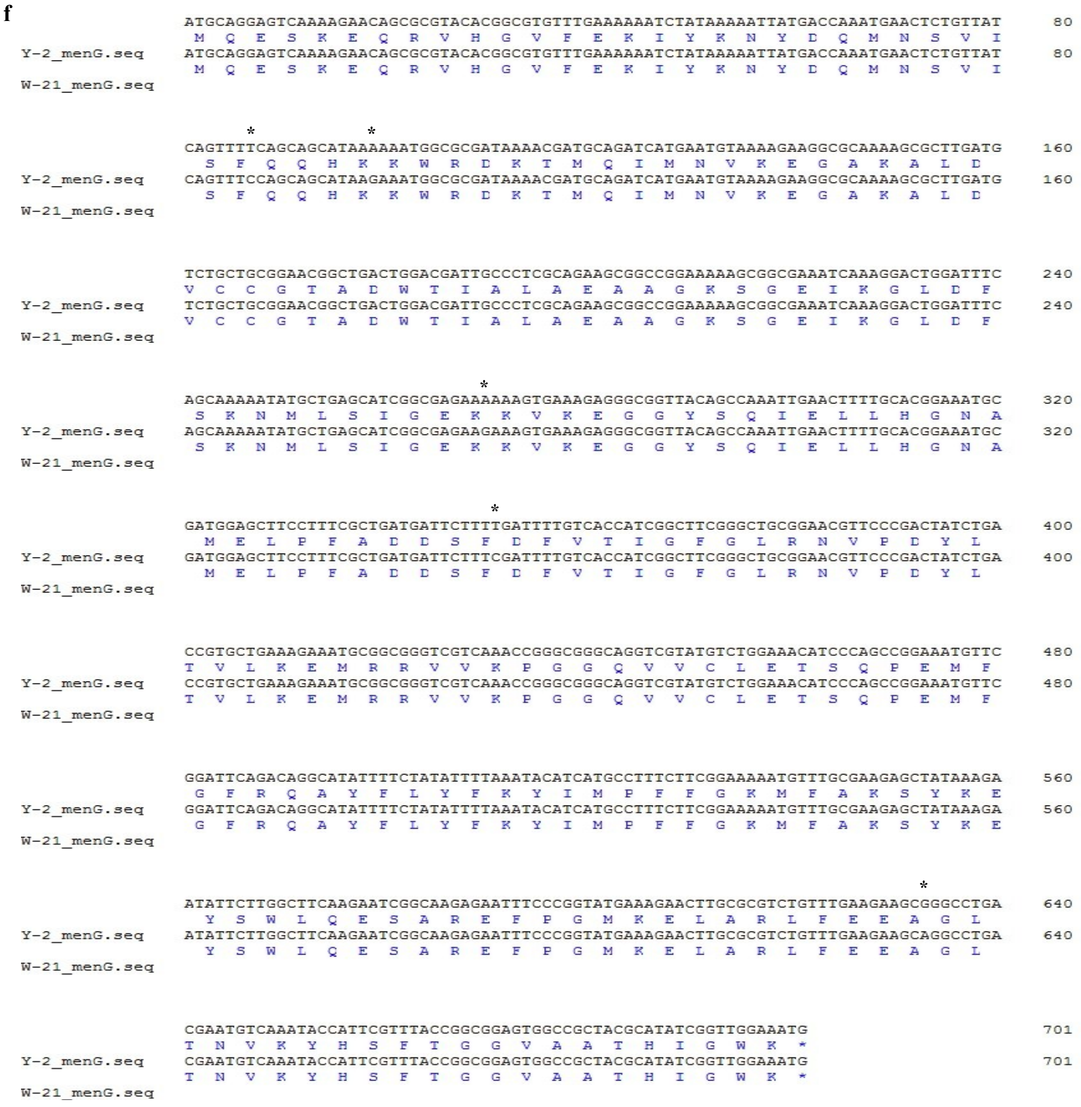

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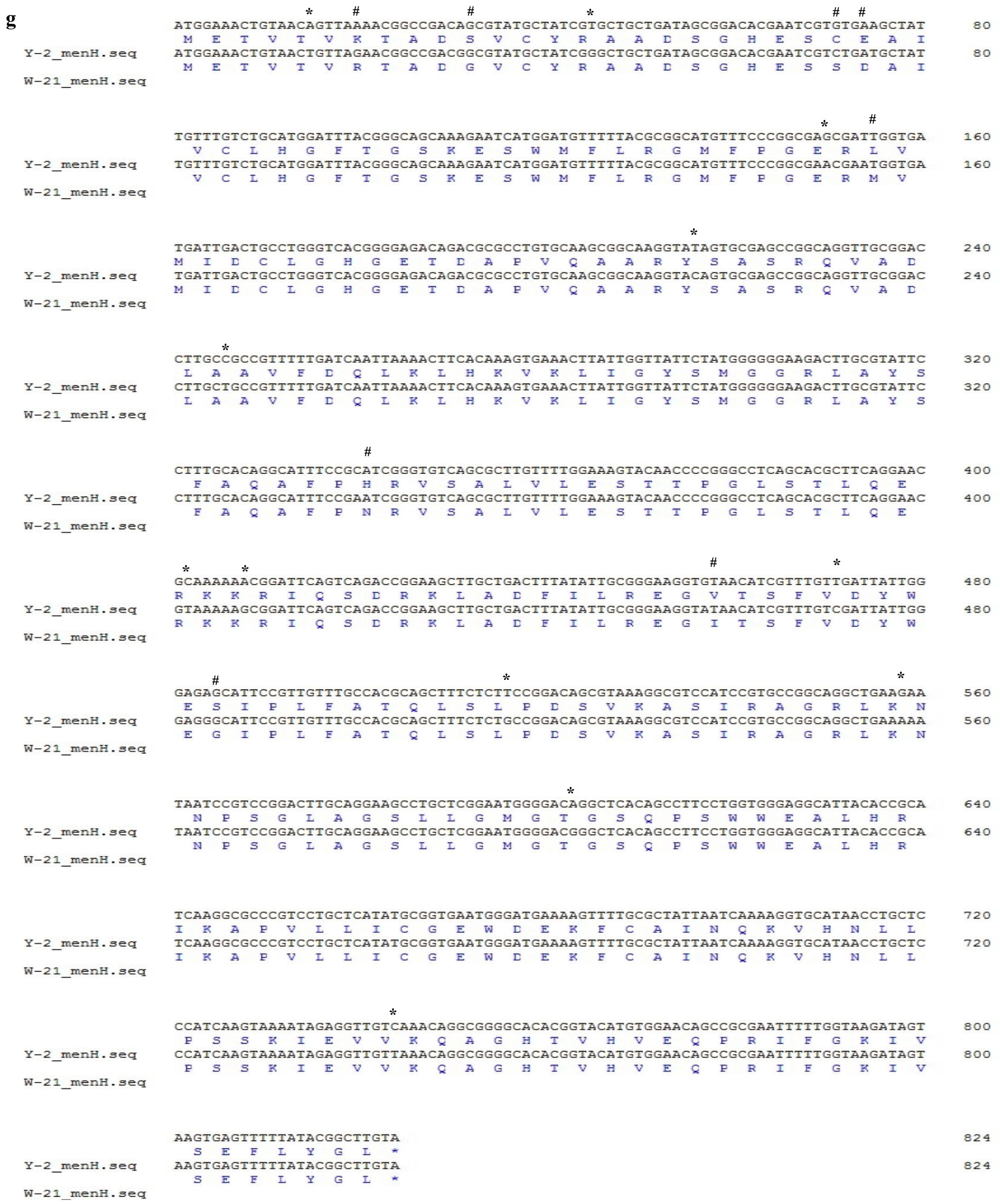

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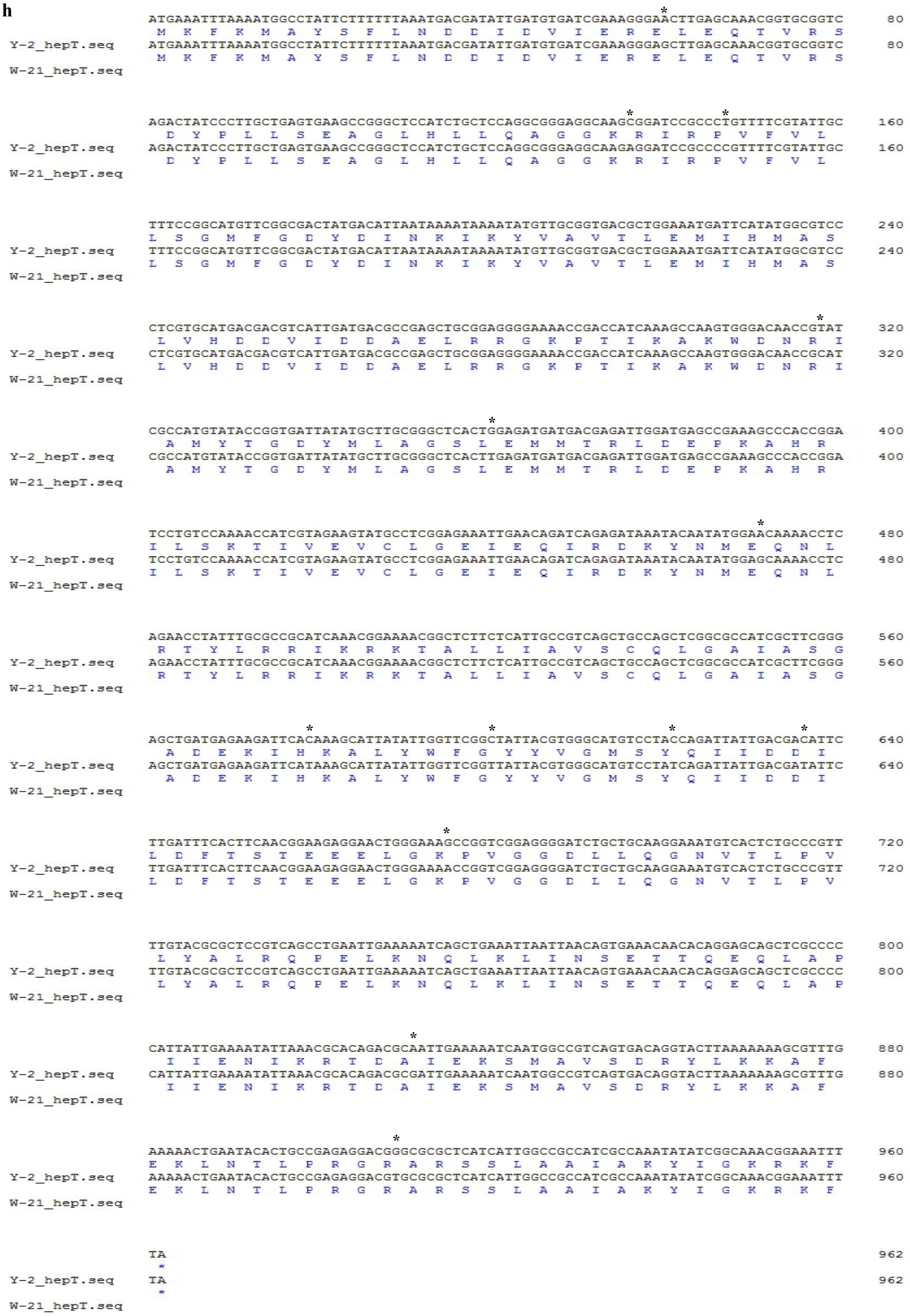

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Y-2_hepT.seq   CATTATTGAAAATATTAAACGCACAGACGCGATTGAAAAATCAATGGCCGTCAGTGACAGGTACTTAAAAAAAGCGTTTG   880
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W-21_hepT.seq

                                           *
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               E  K  L  N  T  L  P  R  G  R  A  R  S  S  L  A  A  I  A  K  Y  I  G  K  R  K  F
Y-2_hepT.seq   AAAAACTGAATACACTGCCGAGAGGACGTGCGCGCTCATCATTGGCCGCCATCGCCAAATATATCGGCAAACGGAAATTT   960
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W-21_hepT.seq

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                *
Y-2_hepT.seq   TA                                                                                 962
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W-21_hepT.seq
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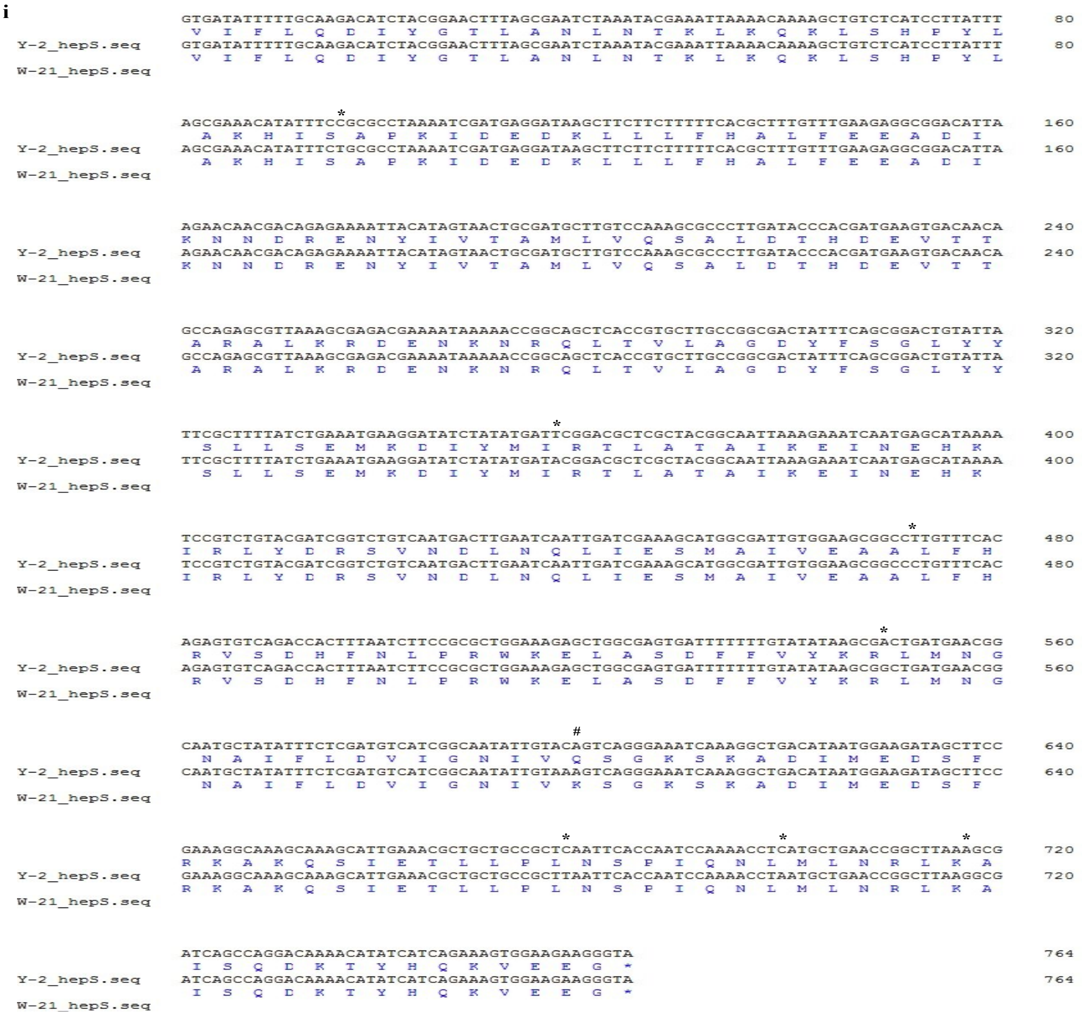

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W-21_hepS.seq

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Y-2_hepS.seq     AGCGAAACATATTTCTGCGCCTAAAATCGATGAGGATAAGCTTCTTCTTTTTCACGCTTTGTTTGAAGAGGCGGACATTA    160
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W-21_hepS.seq

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Y-2_hepS.seq     AGAACAACGACAGAGAAAATTACATAGTAACTGCGATGCTTGTCCAAAGCGCCCTTGATACCCACGATGAAGTGACAACA    240
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W-21_hepS.seq

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W-21_hepS.seq

                                                                                        *
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Y-2_hepS.seq     TCCGTCTGTACGATCGGTCTGTCAATGACTTGAATCAATTGATCGAAAGCATGGCGATTGTGGAAGCGGCCCTGTTTCAC    480
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W-21_hepS.seq

                                                                                     *
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Y-2_hepS.seq     AGAGTGTCAGACCACTTTAATCTTCCGCGCTGGAAAGAGCTGGCGAGTGATTTTTTTGTATATAAGCGGCTGATGAACGG    560
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W-21_hepS.seq

                                                       #
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Y-2_hepS.seq     CAATGCTATATTTCTCGATGTCATCGGCAATATTGTAAAGTCAGGGAAATCAAAGGCTGACATAATGGAAGATAGCTTCC    640
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W-21_hepS.seq

                                                      *                     *                 *
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Y-2_hepS.seq     GAAAGGCAAAGCAAAGCATTGAAACGCTGCTGCCGCTTAATTCACCAATCCAAAACCTAATGCTGAACCGGCTTAAGGCG    720
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W-21_hepS.seq

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Y-2_hepS.seq     ATCAGCCAGGACAAAACATATCATCAGAAAGTGGAAGAAGGGTA                                        764
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W-21_hepS.seq
```

Figure S3 The comparison of nucleotide sequence and deduced amino acid sequence between strains Y-2 and W-21. **a** *menA*, encoding DHNA octaprenyltranse, GenBank No. is KX243543 for strain Y-2 or KX243533 for strain W-21; **b** *menB*, encoding DHNA synthase, GenBank No. is KX243546 for strain Y-2 or KX243546 for strain W-21; **c** *menC*, encoding OSB synthase, GenBank No. is KX243538 for strain Y-2 or KX243548 for strain W-21; **d** *menD*, encoding SEPHCHC synthase, GenBank No. is KX243542 for strain Y-2 or KX243532 for strain W-21; **e** *menE*, encoding OSB-CoA synthase, GenBank No. is KX243540 for strain Y-2 or KX243550 for strain W-21; **f** *menG*, encoding demethylmenaquinone methyltransferase, GenBank No. is KX243539 for strain Y-2 or KX243549 for strain W-21; **g** *menH*, encoding SHCHC synthase, GenBank No. is KX243541 for strain Y-2 or KX243551 for strain W-21; **h** *hepT*, encoding heptaprenylpyrophosphate synthase II, GenBank No. is KX243544 for strain Y-2 or KX243534 for strain W-21; **i** *hepS*, encoding heptaprenylpyrophosphate synthase I, GenBank No. is KX243545 for strain Y-2 or KX243536 for strain W-21. * represents the nonsense mutation; # represents the missense mutation.

Table S1 The fibrinogenase activity and MK-7 production of every isolated strain from Chinese fermented bean "*douchi*"

Strains	T-26	S-33	R-5	Q-11	Y-2	W-21
Fibrinogenase activity (IU·mL^{-1}) [a]	1472 ± 25	1484 ± 55	1466 ± 36	1438 ± 17	1481 ± 33	1459 ± 28
MK-7 production (mg·L^{-1})	3.9 ± 0.23	3.1 ± 0.16	5.5 ± 0.40	5.6 ± 0.15	7.1 ± 0.21	≤ 0.1

[a] The fibrinogenase activity was determined as described by Tage and Sten (A. Tage, and M. Sten, *Arch. Biochem. Biophys*., 1952, **40**, 346-351).

All data are meaning values of three determinations of three independent experiments with errors (± SD).

Table S2 The oligonucleotides used in this study

Oligonucleotide [a]	5'→3' sequence [b]	Cloning site	Purposes
menA$_{\text{CS}}$-F	AAGCCTTTAATAGAAAGGAAGAGG	-	Used for sequencing
menA$_{\text{CS}}$-R	TCATTTTTCCAATCTTCCAT	-	*menA* gene
menB$_{\text{CS}}$-F	TTGGTAAGATAGTAAGTGAGTTTTT	-	Used for sequencing
menB$_{\text{CS}}$-R	AGCATGTTATCATCTCCGAGGTAAAA	-	*menB* gene
menC$_{\text{CS}}$-F	CCGCCGGAACGCGTCCAATAA	-	Used for sequencing
menC$_{\text{CS}}$-R	TAAAAAACCGGCTGGGTCA	-	*menA* gene
menD$_{\text{CS}}$-F	GAATATGAAGAAACACAAATTAAA	-	Used for sequencing
menD$_{\text{CS}}$-R	GTAAACCCGTGCAGACAAAC	-	*menD* gene
menE$_{\text{CS}}$-F	GGTCCAAGCTGTTTTCTTTTCAA	-	Used for sequencing
menE$_{\text{CS}}$-R	GCTTCCACGATCAAAAATTTACG	-	*menE* gene
menG$_{\text{CS}}$-F	TATCATCAGAAAGTGGAAGAAGGGT	-	Used for sequencing
menG$_{\text{CS}}$-R	TAGGCCATTTTAAATTTCATATCAT	-	*menG* gene
menH$_{\text{CS}}$-F	GCCGCAGGCGGACAAGCCCGACT	-	Used for sequencing
menH$_{\text{CS}}$-R	TTTTCCATTTCTGCAGCCATT	-	*menH* gene
hepS$_{\text{CS}}$-F	GTGATATTTTTGCAAGACATCTACG	-	Used for sequencing
hepS$_{\text{CS}}$-R	TTACCCTTCTTCCACTTTCTGATGATA	-	*hepS* gene
hepT$_{\text{CS}}$-F	ATGTCAAATACCATTCGTTTACCG	-	Used for sequencing
hepT$_{\text{CS}}$-R	CGAAATCGTAACGTCTTTCTGA	-	*hepT* gene
MenA$_{\text{Y-2}}$-F	GGAATTCCATATGATGAATCAAAACAATATG	*Nde*I	Cloning for *menA*
menA$_{\text{Y-2}}$-R	CGACGCGTTCAGCGGAAATAACTGATCAGGA	*Mlu*I	gene from Y-2
MenA$_{\text{W-21}}$-F	GCTCTAGAATGAATTATCAACATATG	*Xba*I	Cloning for *menA*
menA$_{\text{W-21}}$-R	CGACGCGTTCAGCGGAAATAACTGATCAGGA	*MluI*	gene from W-21
menC$_{\text{Y-2}}$-F	CGGGATCCATGATTAAGATTGAAAAAATC	*Bam*HI	Cloning for *menC*
menC$_{\text{Y-2}}$-R	CGACGCGTTCAGTTCTTCGTAAAAAC	*Mlu*I	gene from Y-2
menC$_{\text{W-21}}$-F	CGGGATCCATGATTAAGATTGAAAAAATC	*Bam*HI	Cloning for *menC*
menC$_{\text{W-21}}$-R	CGACGCGTTCAGTTCTTTGTAAAAAC	*Mlu*I	gene from W-21
menD-F	CGGGATCCTTGACAAACCCGATTACACAT	*Bam*HI	Cloning for *menD*
menD-R	CGACGCGTTTACAGTTTCCATTCTTTTTC	*Mlu*I	gene
menE-F	GGAATTCCATATGATGCTGACAGAACAGCCG	*Nde*I	Cloning for *menE*
menE-R	CGGGATCCTCATGACAATTCTCCTTTACGAAG	*Bam*HI	gene
MenH$_{\text{Y-2}}$-F	CGGGATCCATGGAAACTGTAACAGTTAAAAC	*Bam*HI	Cloning for *menH*
menH$_{\text{Y-2}}$-R	CGACGCGTTTACAAGGCCGTATAAAAACTCAC	*Mlu*I	gene from Y-2
menH$_{\text{W-21}}$-F	CGGGATCCATGGAAACTGTAACTGTTAG	*Bam*HI	Cloning for *menH*
menH$_{\text{W-21}}$-R	CGACGCGTTTACAAGGCCGTATAAAAACTCAC	*Mlu*I	gene from W-21
hepS-F	CGGGATCCGTGATATTTTTGCAAGACATC	*Bam*HI	Cloning for *hepS*
hepS-R	CGACGCATTTACCCTTCTTCCACTTTCTGATG	*Mlu*I	gene

[a] $_{\text{CS}}$: Conserved sequence.

[b] Underlined nucleotide represents restriction site.

Table S3 The difference of amino acid variations between strains Y-2 and W-21

Gene	Different loci in amino acid sequence	Strains	
		Y-2	W-21
menA	3	Gln	Tyr
	4	Asn	Gln
	5	Asn	His
	192	Pro	Ser
menC	143	Thr	Ala
	256	Ser	Thr
	284	Arg	His
	318	Thr	Ser
menD	144	Val	Ile
	191	Lys	Arg
	280	Ala	Val
	314	Ser	Trp
menE	113	Glu	Lys
	121	Thr	Ala
	122	Ala	Leu
	135	Met	Ile
	299	Trp	Gly
	300	Pro	Ala
	435	Thr	Ser
	442	Ile	Leu
	444	Ala	Val
	459	Ala	Phe
	461	Lys	Glu
	464	Gly	Glu
menH	7	Lys	Arg
	11	Ser	Gly
	24	Cys	Ser
	25	Glu	Asp
	51	Cys	Met
	117	His	Asn
	153	Val	Ile
	162	Ser	Gly
hepS	200	Gln	Lys