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(54) KETOL-ACID REDUCTOISOMERASE USING NADH

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C07H 21/04	(2006.01)
C12N 1/00	(2006.01)
C12Q 1/26	(2006.01)
C12P 7/16	(2006.01)

(52) **U.S. Cl.** **506/9**; 435/189; 536/23.2; 435/243; 435/25; 435/160

(57) **ABSTRACT**

Methods for the evolution of NADPH specific ketol-acid reductoisomerase enzymes to acquire NADH specificity are provided. Specific mutant ketol-acid reductoisomerase enzymes isolated from *Pseudomonas* that have undergone co-factor switching to utilize NADH are described.

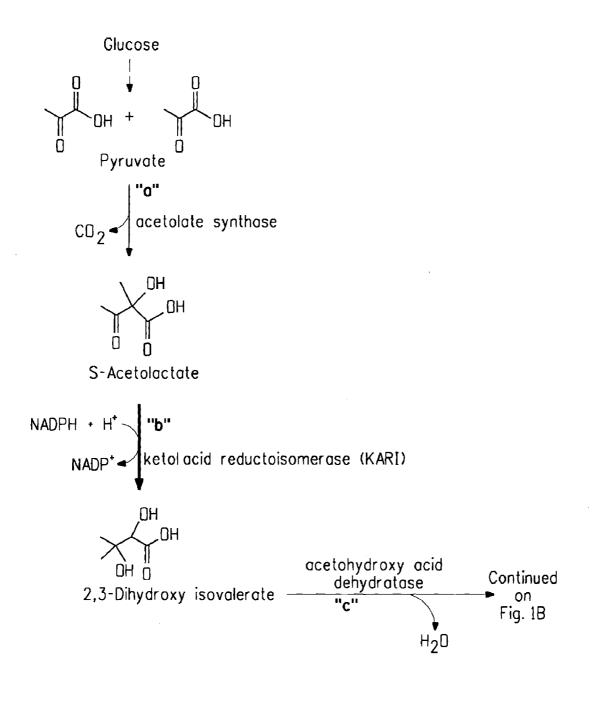
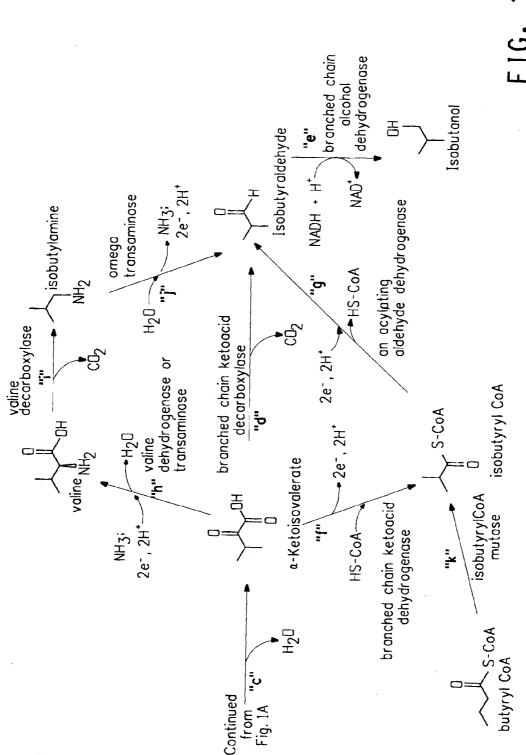


FIG. 1A





17	(44)	VGL R KG S A T VAKA
16	(44)	VGL R SG S A T VAKA
18	(162)	IGL R KG S N T FAEA

FIG. 2A

Sequence	ID	
9	(44)	VGLRKN G AS W ENAK
10	(44)	VGLRKN G AS W NNAK
11	(44)	VGLRKN G AS W ENAK
17	(44)	VGLRKGSATVAKAE
15	(44)	VGLRKN G AS W NKAV
12	(44)	IGVRKD G AS W KAAI
13	(44)	VGLERE G KS W ELAK
14	(44)	IGLRRG G KS W ELAT
Consensus	i	VGLRKN G AS W E AK

FIG. 2B

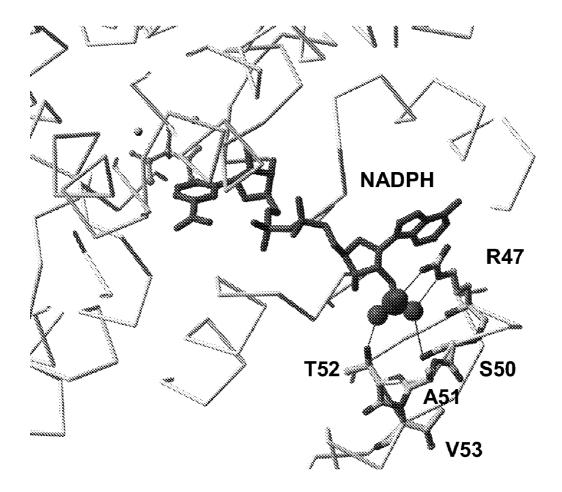
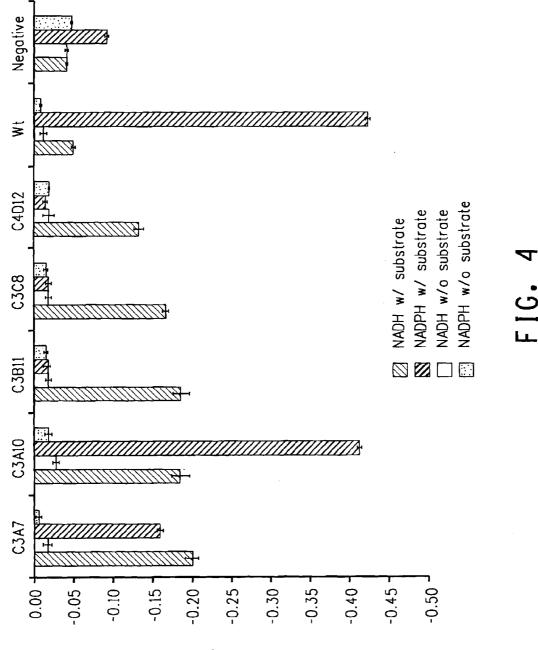


FIG. 3



Cotactor consumption (340)

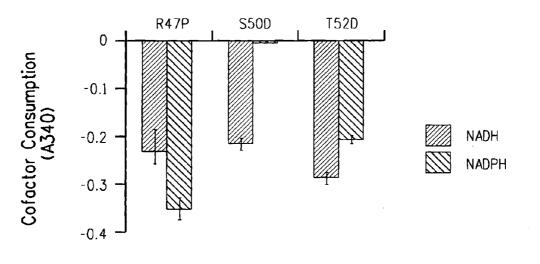


FIG. 5A

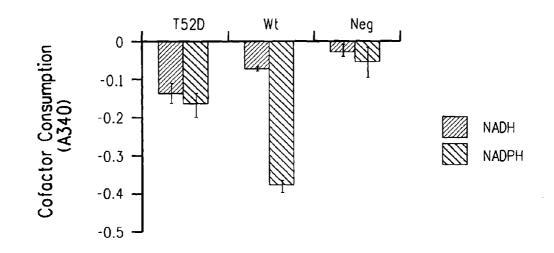
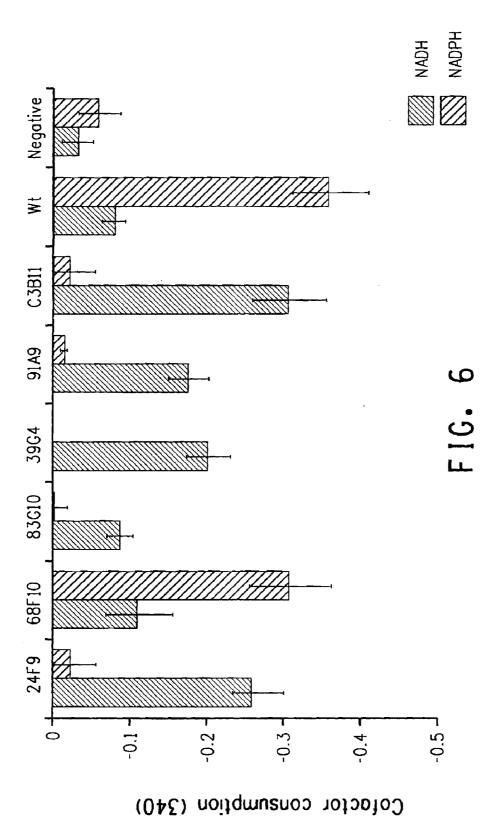
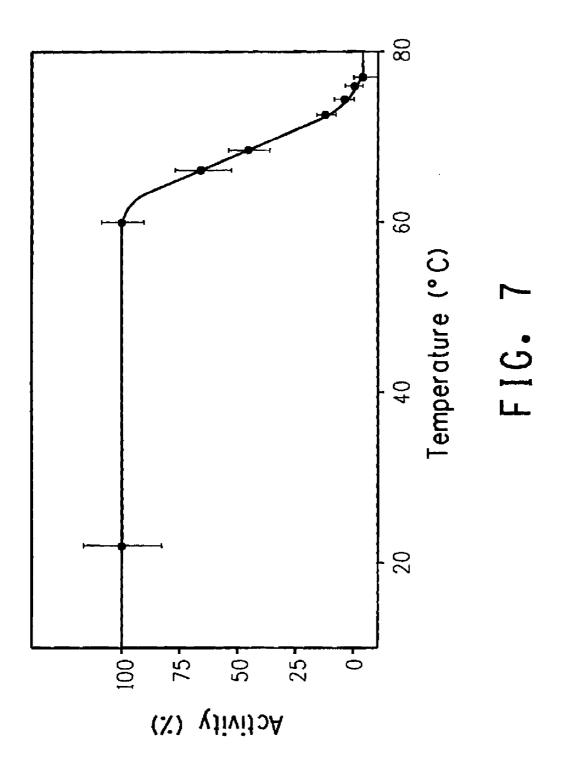


FIG. 5B





100

Sequence ID

51

47	(18)KKVAIIG Y GSQGHAHAQNLRDNGFDVVVGLRKG~KSWDKA	
48	(17)KTVAVIG Y PSQGHAQAQNLRDSGVEVVVGVRPG-KSFEVA	
18	(51) FKGIKQIGVIGWGSQAPAQAQNLKDSLTEAKSDVVVKIGLRKGSNSFAEA	
16	(17)KKVAIIGYSQGHAHACNLKDSGVDVTVGLRSGSATVAKA	
17	(17)KKVAIIGYGSQGHAQACNLKDSGVDVTVGLRKGSATVAKA	
		150
47 48	(57) KEDGFSVYTVAEAAKOADVVMILLPDELQPEVYEAEIAPNLQAGN (56) KTDGFEVMSVSEAVRFAQVVQMLLPDEQQAHVYKAGVEENLREGO	
48 18	(56) KTDGFEVMSVSEAVRFAQVVQMLLPDEQQAHVYKAGVEENLREGQ (101) RAAGFSEENGTLGDMWETISGSDLVLLLESDSAQADNYEKVFSHMK-PNS	
16	(57) EAHGLK~~~~~VADVKTAVAAADVVMIIITPDEFQGRLYKEEIEPNLKKGA	
17	(57) EAHGLKVTDVAAAVAGADLVMILTPDEFQSQLYKNEIEPNIKKGA	
	151	200
47	(102) SLVFAHGENVHFDQVKEPANVDVFLVAPKGPGHLVRRTFSEG	
48	(101) MLLFSHGFNIHFGQINFPSYVDVAMVAPKSPGHLVRRVFQEG	
18	(150) ILGLSHGFLLGHLQSLGQDFPKNISVIAVCPKGMGPSVRRLYVQGKEVNG	
16	(102) TLAFAHGFSIHYNQVV-~~FRADLDVIMIAPKAPGHTVRSEFVKG	
17	(102) TLAFSHGFAIHYNQVVERADLDVIMIAPKAPGHTVRSEFVKG	
	201	250
47	(144) GAVPALFAVYQD \mathbf{A} TGVATEKALSYAD \mathbf{G} IGATRAGVLETTFKEETETDLFG	
48	(143) NGVPALVAVHQD A TGTALHVALAYAK G VGCTRAGVIETTFQEETETDLFG	
18	(200) AGINSSFAVHQD V DGRATDVALGWSI A LGSPFTFATTLEQEYKSDIFG	
16	(144) GGIPDLIAIYQDASGNAKNVALSYACGVGGGRTGIIETTFKDETETDLFG	
17	(144) GGIPDLIAIYQD A SGNAKNVALSYAA G VGGGRTGIIETTFKDETETDLFG	

FIG. 8

Sequence ID

FIG. 9A

	70	80	90	100	110	120
			(
17	~~~~~ <u>M</u>		KVFYI	KDCDLS	IIQG	
43			KVFYI	OKDCDLS	~~IIQG~	
44	M		KVFYC	OKDCDLS	IIQG	
16	/M/M		RV FYI	OKDCDLS	~~IIQG~	
35	M		QVYYI	OKDADLS	IIQG	
39			QVYY[OKDCDLS	IIQG	
41	/M		KVYYI	OKDCDLS	IIQS	
38	M		NVYY6	DKDCDLS	~~IVQG	
15			- KV FY I	DKDADLS	LIKG	
40	M		K VYYI	DKDADLS	LIKQ	
42	M		KVFYI	DKDCDLS-~-	IIQG	
37	M		QVYY	DKDCDLS~~-	IIQG	
46	M		KV FY I	DKDCDLS	IIQG	
34	M		KVYYI	DSDADLG-~-	LIKS	
36	M		-AVSIYY	DKDCDIN	LIKS	
33	M		RVYY	DRDADVN	LIKS	
13	M		-KCTSKIYT	DNDANLD	LIKG	
30	M		-TD-ATIYY)	DDDAEST	VLDD	
14	/M		-AKIYT	DREASLE	PLKG	
32	M		-AIELLY	DADADLS	LIQG	
31	M		-VKVYY	NGDIKEN	VLAG	
47	M		-AKVYY	EKDVTVN	VLKE	~
48	M		KTYY	EKDANVE	LLKG	
18	ANGGGSALSAQMVS!	APSINTPSATT	FDFDSSVFK	KEKVTLSGHD	EYIVRGGRNL	FPLLPD

FIG. 9B

	130	140	150	160	170	180
		· · · · ! · · · · · · · · · · ·				
17	KKVAIIGKGBC	GHADACNIKDS	GV-D	VFVGLRKGSA	ATVAKAEAHGLE	K
43	KKVAIIGYG50	GHAQACNUKDS	GV-D	VIVGLRKGSF	TVARAEAHGL	K
44	KKVAIIGKG50	GHADACNUKDS	GV-D	vrvglrkgs <i>f</i>	ATVAKAEAHGLI	K
16	KKVAIIGKG60	GHAHACNIKDS	GV-D	VIVGLRSGSF	ATVAKAEAHGL	< - -
35	KKVAVIGYG50	GHAHANNIKES	GV-D	VVVGLREGSS	SAARAOKAGL	
39	KKVAIIGYG50	GHAHANNIKDS	GV-D	vbvglrkgsg	SWAKAENAGLA	
41	KKVAI IGYGSC	GHAHACNUKDS	GÝ-D	VYVGLRAGSA	SVARAEAHGL	 [
38	KKVAI IGYGSC	GHAHALNIQDS	NV-D	VIVGLRADSC	SWKKAENAGL	<- -
15	KNVTIIGYGBC	GHAHAININDS	GV-K	VEVGLRKNGA	ASWNHAVNAGLO)
40	RKVAIVGYG50	GHAHANNIKDS	GV-D	VTVALRPGSA	SAKKAENAGL	~ F
42	KKVAI IGYGSC	1 1 6 4 4			TVARAEAHGL	
37	KKVAILGEGBC	GHAHACNIKDS	GV-D	VVVGLRAGSS	SIANAEAYGL	<
46	KKVAI IGYGSC	GHADACNIKDS	GV-D	VIIGLRKGSA	TVARAEAHGLE	<
34	KKIAILGYGBC	GHAHAQNIRDS	GVAE	VAIALRPDSA	SVKRADDAGF	<
36	KKVAIIGFGSC					
33	KKVAVIGYGSC					
13	KRIAVLGYGBC		1		SWELAKSDGI	
30	KTVAVIGYGSC	GHAHAQNIDDS	GV-D	VVVGLREDSS	SRSAAEADGLI)
14	KTIAVIGKGK	GRADALNIRDS	GL-E	VIIGLRRGGK	SWEILATSEGFE	3
32	RKVAIVGYGSC	GHAHSONLADS	GV-E	VVIGLREGSF	SAENAKEAGFE	5
31	KTVAVIGYGSC	GHAHAINIKES	GV-D	VIVGVRQGK-	SFTCADEDGH	<
47	KKVAIIGYGSC	CHAHAQNIRDN		. –	SWORAKEDGES	
48	KTVAVIGYGSC	GHADAQNIRDS	GV-E	vvvgvrpgk-	SFEVARTDGFE	
18	AFKGIKQIGVIĞWĞS	ŽAPAQAQNIKDS	LTEAKSDV-V	VKIGLRKGSN	ISFAEARAAGES	SEE
	* *		* * * *	*		

GXGXX(G/A)

FIG. 9C

Sequence ID	190	200	210	220	230	240
	· · · · · · [· · · ·] · · · ·]		[
17	VTDVAAAVAGADI	LVMILTEDEF	QSQLYKNEIE	PNIKKGATLA	FSHGFAIHYNG	QVVPR
43	~VTDVASAVAAADI	LVMILIPDEF	QSQLYKNEVE	PNLKKGATLA	FSHGFAIHYN	2VVPR
44	VADVATAVAAADI	LVMILTPDEF	QGALYKNEIE	PNIKKGATLA	FSHGESIHYN	2VVPR
16	VADVKTAVAAAD	VMILTEDEF	QGRLYKEEIE	PNLKKGATLA	FANGESINYN	QVVPR
35	VASIEDAAAQAD	VMILAPDEH	QAVIYHNQIA	APNVKPGAAIA	FAHGENIHFG	QIQPA
39	VKEVAEAVAGAD	VMILTEDEF	QAQLYKSEIE	PNLKSGATLA	FANGESINYN	QIVPR
41	VKSVKDAVAAAD	VMILTPDEF	QGRLYKDEIE	PNLKKGATLA	FANGESINYNG	QVVPR
38	VAEVEEAVKAAD	IMILTPOEF	QKELYNDVIE	EPNIKQGATLA	FAHGFAIHYN	QVIPR
15	VKEVAEAVKDAD	VMILLEDEQ	IADVYKNEV	IGNIKQGAALA	FAHGENVHYG	AVIPR
40	VKSVPEAVAGAD	LVMILIPDEF	QSRLYRDEIE	EPNIKQGATLA	FANGESINYN	QVVPR
42	VTDVASAVAAAD	LVMILTPDEF	QSQLYKNEVE	EPNLKKGATLA	FSHGFAIHYN	OVVPR
37	TSDVASAVASAD	vvmvlipdef	QAQLYREEIE	EPNLKQGATLA	FANGFAINYN	QIVPR
46	VTDVATAVAAAD	LVMILTPDEF	QGQLYKQEI	EPNIKKGATLA	FSHGFAIHYN	QVVPR
34	VLTNAEAAKWAD	ігмігафрен	QAAIYAEDLE	CONLRPGSAIA	FAHGLNIHFGI	LIEPR
36	VKSVKEATKEAD	LIMILAPDEI	QSEIFNEEIH	<pre><pre><pre><pre><pre><pre><pre><pre></pre></pre></pre></pre></pre></pre></pre></pre>	FAHGENIHYG	QIVAP
33	VLTPAEAAAWAD	vvmilippdel	QADLYKSELA	ANLKPGAALV	FAHGLAIHFKI	LIEAR
13	PLHTKDAVKDAD	IIIFLVEDMV	QRTLWLESV) PYMKKGADLV	FAHGFNIHYKI	LIDPP
30	VATPRGAAEQAD	lvsvlvpdrv	QPAVYE-QIE	EDVLQPGDTLQ	FANGENINYG	QIEPS
14	VYEIGEAVRKAD	VI LVLI POME	QPKVWQEQI <i>i</i>	APNLKEGVVVD	FAHGENVHEGI	LIKPP
32	VKTTAEAAAWAD	VIMILAPDIS	QAEIFTNDI	EPNLNAGDALL	FGHGLNIHFD	LIKPA
31	VFSVKEAAAQAE	IIMVLLPDEQ	QQKVYEAEIH	KDELŤAGKSLV	FAHGENVHFH	QIVPP
47	VYTVAEAAKQAD	VVMILLPDEL	QPEVYEAE1	APNLQAGNSLV	FAHGENVHED	QVKPP
48	VMSVSEAVRTAQ	VVQMLLPDEQ	QAHVYKAGVI	EENLREGOMLL	FSHGENIHEG	QINPP
18	NGTLGDMWETISGSD *	LVLLIISDSA	QADNYE-KVI	FSHMKPNSILG	LSHGFLLGHL	QSLGQ

FIG. 9D

ence ID						
	250	260	270	280	290	300
	<u>.</u>	· _ · · <u> ·</u> · · · · ·		· · · · · · <u> </u> · · <u>·</u> · ·	· • <u></u> · · - <u>-</u>	
17	ADLDVIMIAPK	PGHTVRSEFVKO	;C	GIPDLIATYODAS	SNAKNVAL	SYAAGV
43	ADLOVIMIAPKA	PGHTVRTEFVKG	5(GIPDLIAVYQDAS	JNAKNVAL	SYASGV
44	ADLDVIMIAPKA	APGHTVRSEFVKO	3(GIPDLIAIYQDAS	SNAKNVAL	SYASGV
16	adldvimiapka	LEGHTVRSEFVKG	30	GIPDLIAIYQDAS	SNAKNVAL	SYACGV
35	ADLDVIMVAPKO	FGHLVRSTYVEG	3C	GVPSLIAIHODAT	SKAKDIAL	SYASAN
39	ADLDVIMIAPK	PGHTVRSEFVKO	;(GIPDLIAIFQDAS	JSAKDLAL	SYASGV
41	ADLOVIMIAPK	RGHTVRSEFVRG	5 - (GIPDLIAVYQDAS	SNAKNLAL	SYACGV
38	SDLDVIMVAPK	PGHTVRSEFAKO	G -(GIPDLIAIYQDAS	ggakqlal	SYAAGV
15	ADLDVILMVAPK	APGHTVRGTYAQO	3(GVPHLIAVHODKS	JSARDIAL	SYATAN
40	ADLDVIMIAPR	APGHTVRSEFVKC	6(GIPDLIAIYQDAS	3KAKETAL	SYASAI
42	ADLDVIMIAPK	APGHTVRTE FVKC	5(GIPDLIAVYQDAS	3NAKNVAL	SYASGV
37	KDLDVIMVAPK	APGHIVRTEFTKC	5(GGI POLIAI FODAS	3NAKNVAL	SYASGI
46	ADLDVIMIAPK	APGHTVRSEFVKC	; - (GIPDLIAIYQDAS	gnaknval	SYASGV
34	KDIDVEMIAPK	PGHTVRSEYVRC	G(3GVPCLVAVDQDAS	JNAHDIAL	AYASGI
36	KGIDVIMIAPK	APGHTVRHEFSIC	3(GGTPCLIAIHQDES	KNAKNLAL	SYASAI
33	ADLOVEMVAPK	FIGHTVRGEYLK	3(GGVPCLMAVAQNPT	GNALELAL	SYASAI
13	KDSDVYMIAPK	FIGPTVREYYKAG	3 (GVPALVAVHODVS	STALHKAL	AIAKGI
3,0	EDVNVIMVAPKS	SPGHLVRRNYENI)	egtpglijavyodps	SEAHDLGL	AYAKAI
14	KNIDVIMVAPK	AFGKAVREEYLAG	5]	RGVPALVAVYQDYS	gsalkyal	ALAKGI
32	DDIIVGMVAPK	FEGHLVRRQFVDC	3	KGVPCLIAVIQDPT	STAQALTL	SYAAAI
31	ADVDVFLVAPK	FGHLVRRTYEQC	3 <i>i</i>	AGVPALFAIYQDVT	SEARDKAL	AYAKGI
47	ANVCVFLVAPK	GPGHLVRRTFSEC	G (GAVPALFAVYQDAT	GVATEKAL	SYADGI
48	SYVDVAMVAPK	SEGHLVERVEQE	33	NGVPALVAVHODAT	gt[a]lhva]l	AYAKGV
18	DFPKNISVIAVCPK	GMGPSVRRLYVQ	SKEVNG.	AGINSSFAVHQDVD	GRATDVAL	GWSIAL
	*	*				

FIG. 9E

	310	320	330	340	350	360
	— П К. Т	ETTAL ECENAUT		ריין		
IDIX	TFKD	ETDLFGEQAVI	CCCTVELVE	GGTVELVKAGFETLVEAGKAPEMAK	SYAPEMAYFECLHEL	нег НЕГ
GGGRTGIIE	TTFKDET	ETTDLFGEQAVL		dgbтvецvкадгетцvеаgkаремауг	зкаремак ғесінеі	HEL
GGGRTGIIE	TTFKDE	片	debever.vk	CGGCVELVKAGFETLVEAGYAPEMAYF	[L]	CLHEL
GGGRAGVIE	TSFREE	TETDLFGEQAVI	ddg i tsrid	dgeitsliqagfetlveagyapemax	ЗУАРЕМАУ ГЕССИЕТ	НЕТ
RTGI	TFFKDE		dggavelv ^k	dggavelvkagfetlveagkapemakf	зУАРЕМАУ ГЕССНЕГ	HEL
GGGRTGIIE	TTFKD	<u>ы</u> Ц	dddcvervr	<pre>ddpcvelvkagfetlvedgkapemakf</pre>	зү аремаү ғестнег	HEL
GCGRSGIIE	FKD	ETETDLFGEQAVI	dggavelvr	dggavelvkmgfetlteagkapemakf	з <u>каремак</u> гесцнец	HEL
GGGRAGIIETN	FREE	т Е трр и гја е ја у ц	dggtvelik	dggtvelikagfetlveagkapenjak	акаремак ғесьнет	HEL
GGGRTGIIE	TTFKDE	ITETID LEGEQAVI	dggavelv ^F	EQAVIQG5AVELVKAGFDTLVEAGKAPEMAKF	SKAPEMAKFECLHEL	HEL
GGGRTGIIE	TLFKDE	TETTDLEGEQAVI	dGGTVELVF	FGEQAVLQGGTVELVKAGFETLVEAGKAPEMAKF	зкаремак <i>т</i> есснес	HEL
GGGRTGIIE	TLFKDE	TETTDLFGEQAVI	dggavelvr	dgbavelvkagfetltedgkapemakf	зКАРЕМАК FECLHEL	HEL
GGGRTGIIE	TTFKDEF	ETTDLFGEQAVL	dGGTVELVF	CAGFETLVEAC	dggtvelvkagfetlveagghapemahfeclhel	НЕГ
GGGRSGVIE	RSGVIETTFREEVETD	ЕТРЕЕСАЧЕ	dggltalit	dggltalitagfetlteagkapema	зКАРЕМА <u></u> FFECMHEM	HEM
GGGRTGIIE	TFKA	EFETDLFGEQAVI	dggrsalic	dgglsaliqagfetlveagkepemak	зкеремак ғесінем	HEM
GGGRSGIIE	TTFREE	сепрісеричі	deerskrid	LFGEQVVIDGGLSKLIQYGFETLVEAGYAPEMAYF	зКАРЕМАК FECLHEV	НΕV
GATRAGVIP	TFKEE	тепрьясти	NGG IMELMF	FGEQUILNGSIMELMRAAFETLVEEGKOPEVAY	SY QPEVANFETINEL	NEL
GCTRAGWETT	TTFREETEND	ETIDLFGEQAVL		(TGYETLVDA	JGGVTSLVKTGYETLVDAGKSPEMAKFECLNEL	NEL
GATRAGVIETTFAEETETD	TLFAEEL	ETULIGEQIVL	NGGLMELIF	<u>М</u> GБLMELIKKGFEVLVEMGYQPEV <mark>A</mark> Y	5KQPEVAKFEVLNEA	NEA
GGARAGVIFTLF	TLFEAET	TVTDLFGEQAVL		WGFEVLTEAC	dgbteelvkvgfevlteagkepemakfevlhel	HEL
GGARAGVLETTFKEETETDLFG	ഥ	EQAV	dgglsalvr	UdgGLSALVKAGFETLTEAGYQPELAYF	зұ орецай ғесьнег	HEL
GATRAGVLETFEKE	TTEREET	TETDLFGEQAVL	dgpvtalv ^k	dgbutalukag fetludagk	зи орецай гестнет	НЕГ
GCTRAGVIE GSPFTF2	TTFOEE	ETDLFGEQTVI KSDTFGFPGTI	dggvtalvr i gavugive	AGFETLTEdo	GCTRAGVIETTFOEFETDLFGEDOTVLIGGVTALVKAGFETLTEGGKRPEIAYFECLHEL GSPFTFATTIFOFYKSDIFGEPGIIIGAVHCIVFGIEPDYTFSGMSEDIAVKNIVECI	HEL
			J ^ T 2 L ^ L 2 J - T		V I NN I ALU Z CINE	コンコ

1441004614404000004016441

Sequence ID

FIG. 9F

	370	380	390	400	410	420
		_		1		
17	K-LIVDLMYEGGIAN					
43	K-LIVDLMYEGGIAN					
44	K-LIVDLMYEGGIAN	MNY S ISNNAEY	GEYVTGPE	VINEESRKAMR	NALKRIQOGE	EYAKMF
16	K-LIVDLMYEGGHAN	MNY S‡ SNNAEY	GEYVTGPE	VINAESRAAMR	NALKRIQDGE	EYAKMF
35	K-LIVDLLYQQQIAN	MRYSISNTAEY	GDFTRGPR	VINEESREAMR	EILAEIQEGE	FAREF
39	K-LIVDLMYEGGIAN	MNY <mark>SI</mark> SNNAEY	GEYVTGPE	VINDQSRAAMR	NALKRIQDGE	EYAKMF
41	K-LIVDLMFEGGIAN	MNYS I SNNAEY	GEYVTGPE	VINEQSRQAMR	NALKRIQDG	EYAKMF
38	K-LIVDLMYEGGIAD	MNYSISNNAEY	GEYVTGPE	VINEQSREAMR	NALKRIQSG	EYAKMF
15	K-LIVDLIYEGGIGN	MNYSISNNAEY	GEYVTGPR	VVTAETKQAMK	QCLHDIQTGE	YAKS F
40	K-LIVDLMYEGGIAN	MNYSISNNAEY	GEYVTGVK	VINEQSRAAMK	ECLANIONG	AYAKRF
42	K-LIVDLMYEGGIAN	MNYSISNNAEY	GEYVTGPE	VINAESROAMR	NALKRIODG	Yakmf
37	K-LIVDLMYEGGIAN				- 1	
46	K-LIVDLMYEGGIAN	1 1				
34	K-LIVDLIYEAGIAN	1 1			-	
36	K-LIVDLIYOGGIAD				- 1	
33	K-LIVDLIYEGGIAN					
13	K-MLVDLVYEKGISG					
30	K-LIVDLMYEGGNSE					
14	K-LIMDLIWQRGIYG					
32	K-LIVDLMFEGGISN					
	11					
31	K-LIVDLMYEEGLAG					
47	K-LIVDLMYEGGLEN	11			11	
48	K-LIVDLMYEGGLTN					
18	TGVISKTISTKGMLA	LYNSLSEEGKI	K-DFQAAYS	SASYYPSMDILY	ECYEDVASG:	SEIRSV

FIG. 96

Sequence ID	
	430 440 450 460 470 480
	•••••••••••••••••••••••••••••••••••••••
17	ISEGATGYPSMTAKRRNNAAHGIE-IIGEQURSMMPWIGANKIVDKAKN
43	ITEGATGYPSMTAKRRNNAEHGIE-VÍG <mark>E</mark> KURSMMPWIAANKIVDKDKN
44	ISEGATNYPSMTAKRRNNAAHGIE-I[GEQURSMMPWISANKIVDKTKN
16	ITEGAANYPSMTAYRRNNAAHPIE-QIGEKIRAMMPWIAANKIVDKSKN
35	VLENQAGCPTLTARRRLAAEHEIE-VVGERURGMMPWINANKLVDKDKN
39	IAEGAHNYPSMTAYRRNNAAHPIE-QVGEKIRSMMPWIASNKIVDKSKN
41	ITEGAANYPSMTAYRRNNAAHQIE~VVGEKIRTMMPWIAANKIVDKTKN
38	ISEGATNYPSMTARRRNNAEHQIE-ITGAKURGMMPWIGGNKIIDKDKN
15	LLENKAGAPTLISRRRLTADHQIE-QVGAKURAMMPWIAKNKLVDQSKN
40	ILEGQANYPEMTAWRRNNAAHQIE-VVGAKURSMMPWIAANKLVDHSKN
42	ISEGATGYPSMTAKRRNNAAHGIE-ITGEKURSMMPWIAANKIVDKDKN
37	ISEGALNYPSMTARRRQNAAHEIE-TVGEKURSMMPWISANKIVDKDKN
46	ISEGATNYPSMTAKRRNNAAHGIE-IIGEQURSMMPWISANKIVDKTKN
34	VLDNRAGQPELKAARKRMAAHPIE-QVGARIRKMMPWIASNKLVDKARN
36	ILERRANFARMHAERKLMNDSLIE-KTGREURAMMPWISAKKLVDKDKN
33	MLECKAGQPSFKATRRIQXEHVIE-VVJGEKUJRGMMPWISKNKLVDKARN
13	VEEYGRGMPTVVNGLSNVQNSLEE-KIGNQURDLVQKGKPKS
30	ISENQAGRPSYKQLRAAEKNHDIE-AVGEDURALFAWGDDGDD
14	VEEYNRGAPTLRKLMEEARTHPIE-KVGEEMRKLLFGPGP
32	IANVENGNTELEGLRASYNNHPIE-ETGAKUR/DLMSWVKVDARAETA
31	IVENQVNRPRFNAINASENEHQIE-VVGRKUREMMPFVKQGKKKEAVVSVAQN~
47	IAEHKAGRPNFHATNEKENEHEIE-VVGRKIREMMPFV-QPRVKVGMK
48	ILENQAGRPTYNAMKKAEQNHQLE-KVGAELREMMSWIDAPKELVKK
18	VLAGRRFYEKEGLPAFPMGKIDQTRMWKVGEKVRSVRPAGDLGPLYPFTAGVYVAL

FIG. 9H

Sequence ID						
	490	500	510	520	530	540
17	· · · · · · · · · · · · · · · · · · ·		· · · · · · · · · · · · · · · · · · ·	••••••	••••	- ;
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44						ł
16						1
3 5						1
39						1
41						1
38			• • • • • • • • • • • • • • • • • • •			1
15						1
40					* # ! ! ! ! ! ! ! ! !	1
42						1
37						1
46						1
34						l l
						1
33						1
13						1
30						1
14						1
32						1
31						1
47			*****	1		1
48						1
18	MMAQIEILRKKGHSYSEIINESVIEAVDSLNPFMHARGVSFMVDNCSTTARLGSRKWAPR	EIINESVIEAVI	DSLNPEMHARG	VS FMV DNCS	TTARLGSRKWA	PR

FIG. 91

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	FDYTLSOOALWAVDNGAPTNODLTSNFTSDPVHEATGVCAOLBPSVDTSVTADADFVBF	TNODLTSNFL	SDPVHEAT	GVCAOLRPSV	DISVTADADI	FVRPF

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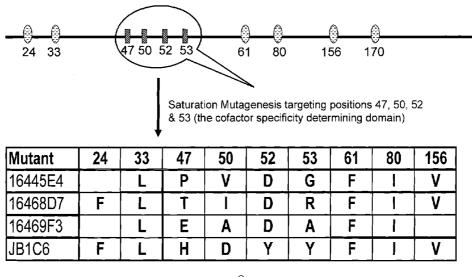
*->qMfafskVYYDkDadlsGhdeylikGKkVAvIGYGSQGHAHAqNLrD

				M kV+YDkD+dls +i+GKkVA+IGYGSQGHA+A+NL+D	
Sequence	١D	17	1	-MKVFYDKDCDLSIIQGKKVAIIGYGSQGHAQACNLKD 3	7
				SGVdVvVGIRkGsaSwakAeaaGfkVktvaEAvaqADvVmillPDefQae	
				SGVdV+VGIRkGsa++akAea+G+kV +va Ava+AD+VmiI+PDefQ++	
Sequence	ID	17	38	SGVDVTVGIRKGSATVAKAEAHGLKVTDVAAAVAGADLVMIITPDEFQSQ 8	7
				vYeeelepnLkpGatLaFAHGFNIHfqqIvPrafPkDiDViMVAPKgPGH	
				+Y++eIepn+k+GatLaF+HGF+IH++q+vPra D+DViM+APK+PGH	
Sequence	١D	17	88	LYKNEIEPNIKKGATLAFSHGFAIHYNQVVPRADLDVIMIAPKAPGH 1	34
				tVRreYvkGgGVPaLiAVyQDasGnAkdlALsYAkgiGggRAGvIETTFk	
				tVR+e+vkGgG+P+LiA+yQDasGnAk++ALsYA+g+GggR+G+IETTFk	
Sequence	D	17	135	TVRSEFVKGGGIPDLIAIYQDASGNAKNVALSYAAGVGGGRTGIIETTFK 1	84
				eETETDLFGEQaVLCGGvteLVkaGFETLVEaGYaPEmAYFECLHE1KLI	
				+ETETDLFGEQaVLCGG++eLVkaGFETLVEaGYaPEmAYFECLHELKLI	
Sequence	ID	17	105	-	24
00400100	10	11	192	DETETDLFGEQAVLCGGTVELVKAGFETLVEAGYAPEMAYFECLHELKLI 2	54

FIG. 10A

VDLmYEgGIanMrySiSdTAeYGdyvtGprVIdeeskeaMkevLkdIQsG VDLmYEgGIanM+ySiS++AeYG+yvtGp+VI++es++aM+++Lk+IQ+G Sequence ID 17 235 VDLMYEGGIANMNYSISNNAEYGEYVTGPEVINAESRQAMRNALKRIQDG 284 eFAkewilEnqaGyPketltalrrneaeHqIEWkVGekLRsmmpWIaanK e+Ak++i+E+++GyP ++ta rrn+a+H IE +Ge+LRsmmpWI anK Sequence ID 17 285 EYAKMFISEGATGYP--SMTAKRRNNAAHGIE-IIGEQLRSMMPWIGANK 331 lvdkdkn<-* +vdk+kn Sequence ID 17 332 IVDKAKN 338

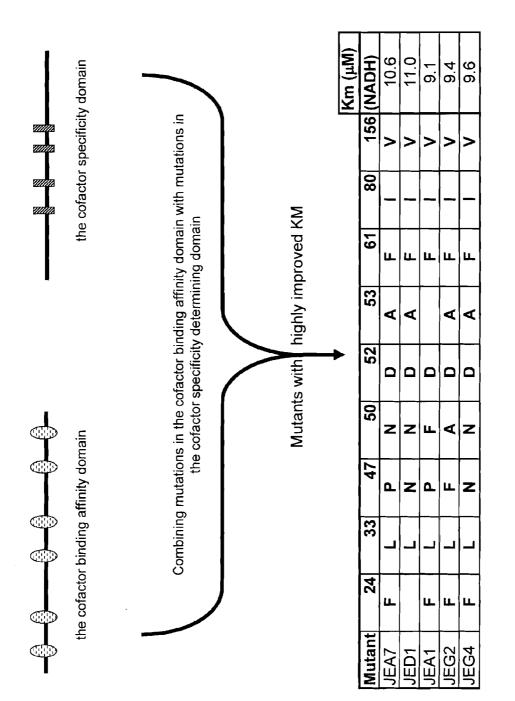
FIG. 10B

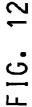


the cofactor specificity domain

the cofactor binding affinity domain

FIG. 11





KETOL-ACID REDUCTOISOMERASE USING NADH

[0001] This application is a continuation-in-part of U.S. Ser. No. 12/337,736, filed Dec. 18, 2008 and claims the benefit of the U.S. Provisional Applications, 61/015,346, filed Dec. 20, 2007, and 61/109,297, filed Oct. 29, 2008.

FIELD OF THE INVENTION

[0002] The invention relates to protein evolution. Specifically, ketol-acid reductoisomerase enzymes have been evolved to use the cofactor NADH instead of NADPH.

BACKGROUND OF THE INVENTION

[0003] Ketol-acid reductoisomerase enzymes are ubiquitous in nature and are involved in the production of valine and isoleucine, pathways that may affect the biological synthesis of isobutanol. Isobutanol is specifically produced from catabolism of L-valine as a by-product of yeast fermentation. It is a component of "fusel oil" that forms as a result of incomplete metabolism of amino acids by yeasts. After the amine group of L-valine is harvested as a nitrogen source, the resulting α -keto acid is decarboxylated and reduced to isobutanol by enzymes of the Ehrlich pathway (Dickinson, et al., J. Biol. Chem., 273: 25752-25756, 1998).

[0004] Addition of exogenous L-valine to the fermentation increases the yield of isobutanol, as described by Dickinson et al., supra, wherein it is reported that a yield of isobutanol of 3 g/L is obtained by providing L-valine at a concentration of 20 g/L in the fermentation. In addition, production of n-propanol, isobutanol and isoamylalcohol has been shown by calcium alginate immobilized cells of *Zymomonas mobilis* (Oaxaca, et al., Acta Biotechnol., 11: 523-532, 1991).

[0005] An increase in the yield of C3-C5 alcohols from carbohydrates was shown when amino acids leucine, isoleucine, and/or valine were added to the growth medium as the nitrogen source (WO 2005040392).

[0006] While methods described above indicate the potential of isobutanol production via biological means these methods are cost prohibitive for industrial scale isobutanol production. The biosynthesis of isobutanol directly from sugars would be economically viable and would represent an advance in the art. However, to date the only ketol-acid reductoisomerase (KARI) enzymes known are those that bind NADPH in its native form, reducing the energy efficiency of the pathway. A KARI that would bind NADH would be beneficial and enhance the productivity of the isobutanol biosynthetic pathway by capitalizing on the NADH produced by the existing glycolytic and other metabolic pathways in most commonly used microbial cells. The discovery of a KARI enzyme that can use NADH as a cofactor as opposed to NADPH would be an advance in the art.

[0007] The evolution of enzymes having specificity for the NADH cofactor as opposed to NADPH is known for some enzymes and is commonly referred to as "cofactor switching". See for example Eppink, et al. (J. Mol. Biol., 292: 87-96, 1999), describing the switching of the cofactor specificity of strictly NADPH-dependent p-Hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescens* by site-directed mutagenesis; and Nakanishi, et al., (J. Biol. Chem., 272: 2218-2222, 1997), describing the use of site-directed mutagenesis on a mouse lung carbonyl reductase in which

Thr-38 was replaced by Asp (T38D) resulting in an enzyme having a 200-fold increase in the K_M values for NADP(H) and a corresponding decrease of more than 7-fold in those for NAD(H). Co-factor switching has been applied to a variety of enzymes including monooxygenases, (Kamerbeek, et al., Eur. J, Biochem., 271: 2107-2116, 2004); dehydrogenases; Nishiyama, et al., J. Biol. Chem., 268: 4656-4660, 1993; Ferredoxin-NADP reductase, Martinez-Julvez, et al., Biophys. Chem., 115: 219-224, 2005); and oxidoreductases (US2004/0248250).

[0008] Rane et al., (Arch. Biochem. Biophys., 338: 83-89, 1997) discuss cofactor switching of a ketol acid reductoisomerase isolated from *E. coli* by targeting four residues in the enzyme for mutagenesis, (R68, K69, K75, and R76,); however the effectiveness of this method is in doubt.

[0009] Although the above cited methods suggest that it is generally possible to switch the cofactor specificity between NADH and NADPH, the methods are enzyme specific and the outcomes unpredictable. The development of a ketol-acid reductoisomerase having a high specificity for NADH with decreased specificity for NADPH would greatly enhance this enzyme's effectiveness in the isobutanol biosynthetic pathway and hence increase isobutanol production. However, no such KARI enzyme has been reported.

SUMMARY OF THE INVENTION

[0010] Applicants have solved the stated problem by identifying a number of mutant ketol-acid reductoisomerase enzymes that either have a preference for specificity for NADH as opposed to NADPH or use NADH exclusively in their reaction. The method involves mutagenesis of certain specific residues in the KARI enzyme to produce the cofactor switching.

[0011] Accordingly the invention provides A mutant ketolacid reductoisomerase enzyme comprising the amino acid sequence as set forth in SEQ ID NO: 29; a nucleic acid molecule encoding a mutant ketol-acid reductoisomerase enzyme having the amino acid sequence as set forth in SEQ ID NO:19; a mutant ketol-acid reductoisomerase enzyme as set for in SEQ ID NO:19; a mutant ketol-acid reductoisomerase enzyme having the amino acid sequence selected from the group consisting of SEQ ID NO: 24, 25, 26, 27, 28, 67, 68, 70, 75, 79, 80, 81 and 82; and a mutant ketol-acid reductoisomerase enzyme as set forth in SEQ ID NO:17 comprising at least one mutation at a residue selected from the group consisting of 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, 165, and 170.

[0012] In another embodiment the invention provides a method for the evolution of an NADPH binding ketol-acid reductoisomerase enzyme to an NADH using form comprising:

- [0013] a) providing a ketol-acid reductoisomerase enzyme which uses NADPH having a specific native amino acid sequence;
- [0014] b) identifying the cofactor switching residues in the enzyme of (a) based on the amino acid sequence of the *Pseudomonas fluorescens* ketol-acid reductoisomerase enzyme as set for the in SEQ ID NO:17 wherein the cofactor switching residues are at positions selected from the group consisting of: 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, 165, and 170; and
- [0015] c) creating mutations in at least one of the cofactor switching residues of (b) to create a mutant enzyme wherein said mutant enzyme binds NADH.

- **[0017]** a) providing a recombinant microbial host cell comprising the following genetic constructs:
 - **[0018]** i) at least one genetic construct encoding an acetolactate synthase enzyme for the conversion of pyruvate to acetolactate;
 - [0019] ii) at least one genetic construct encoding a ketol-acid reductoisomerase enzyme of either of Claims 1 or 6;
 - **[0020]** iii) at least one genetic construct encoding an acetohydroxy acid dehydratase for the conversion of 2,3-dihydroxyisovalerate to α -ketoisovalerate, (pathway step c);
 - **[0021]** iv) at least one genetic construct encoding a branched-chain keto acid decarboxylase, of the conversion of α -ketoisovalerate to isobutyraldehyde, (pathway step d);
 - **[0022]** v) at least one genetic construct encoding a branched-chain alcohol dehydrogenase for the conversion of isobutyraldehyde to isobutanol (pathway step e); and
- **[0023]** b) growing the host cell of (a) under conditions where iso-butanol is produced.

[0024] In another embodiment the invention provides a method for the evolution and identification of an NADPH binding ketol-acid reductoisomerase enzyme to an NADH using form comprising:

- **[0025]** a) providing a ketol-acid reductoisomerase enzyme which uses NADPH having a specific native amino acid sequence;
- [0026] b) identifying the amino acid residues in the native amino acid sequence whose side chains are in close proximity to the adenosyl 2'-phosphate of NADPH as mutagenesis targets;
- [0027] c) creating a library of mutant ketol-acid reductoisomerase enzymes from the class I ketol-acid reductoisomerase enzyme of step (a), having at least one mutation in at least one of the mutagenesis target sites of step (b); and
- **[0028]** d) screening the library of mutant ketol-acid reductoisomerase enzymes of step (c) to identify NADH binding mutant of ketol-acid reductoisomerase enzyme.

[0029] Alternatively the invention provides a method for evolution of an NADPH specific ketol-acid reductoisomerase enzyme to an NADH using form comprising:

- **[0030]** a) providing a mutant enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 28, 67, 68, 69, 70, and 84;
- **[0031]** b) constructing a site-saturation library targeting amino acid positions 47, 50, 52 and 53 of the mutant enzyme of (a); and
- **[0032]** c) screening the site-saturation library of (b) to identify mutants which accept NADH instead of NADPH as cofactor.

[0033] Similarly the invention provides a method for evolution of an NADPH specific ketol-acid reductoisomerase enzyme to an NADH using form comprising:

[0034] a) providing a DNA fragment encoding a mutant enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, and 98 containing mutations in cofactor specificity domain;

- **[0035]** b) producing a DNA fragment cofactor specificity domain of (a);
- **[0036]** c) providing a DNA fragment encoding a mutant enzyme having mutations in cofactor binding affinity domain selected from the group consisting of SEQ ID NOs: 28, 67, 68, 69, 70, 84 and 86;
- [0037] d) incorporating mutations of step (b) into mutants of step (c); and
- **[0038]** e) screening mutants of step (d) for mutant enzymes having a ratio of NADH/NADPH utilization is greater than one.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE DESCRIPTIONS

[0039] The invention can be more fully understood from the following detailed description, the Figures, and the accompanying sequence descriptions, which form part of this application.

[0040] FIGS. 1A and 1B—Show four different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f", "g", "h", "i", "j" and "k" represent the substrate to product conversions described below.

[0041] FIGS. 2A and 2B—Multiple sequence alignment (MSA) of KARI enzymes from different recourses; FIG. 2A—MSA among three NADPH-requiring KARI enzymes; FIG. 2B—MSA among PF5-KARI and other KARI enzymes, with promiscuous nucleotide specificity, where, MMC5—is from *Methanococcus maripaludis* C5; MMS2—is from *Methanococcus maripaludis* S2; MNSB is from *Methanococcus vanniellii* SB; ilv5—is from *Saccharomyces cerevisiae* ilv5; KARI-D1—is from *Sulfolobus solfataricus* P2 ilvC; KARI-D2—is from *Pyrobaculum aerophilum*

P2ilvC; and KARI S1—is from *Ralstonia solanacearum* GMI1000 ivIC.

[0042] FIG. **3**—Interaction of phosphate binding loop with NADPH based on homology modeling.

[0043] FIG. **4**—KARI activities of top performers from library C using cofactor NADH versus NADPH. Activity and standard deviation were derived from triple experiments. The mutation information is as follows: C3A7=R47Y/S50A/ T52D/V53W; C3A10=R47Y/S50A/T52G/V53W; C3B11=R47F/S50A/T52D/V53W; C3C8=R47G/S50M/ T52D/V53W; and C4D12=R47C/S50MT52D/V53W

[0044] FIGS. 5A and 5B—FIG. 5A—Comparison of KARI activities of top performers from libraries E, F and G using cofactors NADH and NADPH. FIG. 5B—KARI activities of positive control versus wild type Pf5-ilvC using cofactors NADH. Activity and standard deviation were derived from at least three parallel experiments. "Wt" represents the wild type of Pf5-ilvC and "Neg" means negative control. Experiments for NADH and NADPH reactions in FIG. 5A were 30 min; in FIG. 5B were 10 min.

[0045] FIG. **6**—Activities of top performers from library H using cofactors NADH versus NADPH. Activity and standard deviation were derived from triple experiments. Mutation information is as follows: 24F9=R47P/S50G/T52D; 68F10=R47P/T52S; 83G10=R47P/S50D/T52S; 39G4=R47P/S50C/T52D; 91A9=R47P/S50CT52D; and C3B11=R47F/S50A/T52D/V53W and Wt is wild type.

[0046] FIG. **7**—Thermostability of wild type PF5-ilvC. The remaining activity of the enzyme after heating at certain temperatures for 10 min was the average number of triple experiments and normalized to the activity measured at room temperature.

[0047] FIG. **8**—Multiple DNA sequence alignment among 5 naturally existing KARI molecules. The positions both bolded and boxed were identified by error prone PCR and the positions only boxed were targeted for mutagenesis.

[0048] FIGS. 9A through 9*k*-Alignment of the twenty-four functionally verified KARI sequences. The GxGXX(G/A) motif involved in the binding of NAD(P)H is indicated below the alignment.

[0049] FIGS. **10**A and **10**B—An example of the alignment of *Pseudomonas fluorescens* Pf-5 KARI to the profile HMM of KARI. The eleven positions that are responsible for co-factor switching are boxed.

[0050] FIG. **11**—(A) is a linear depiction of the KARI amino acid sequence with specific amino acids numbered. The cofactor specificity domain residues are shown in shaded rectangles. The cofactor binding domain is shown in dotted ovals. (Table A) shows changed amino acids, using single letter code, at numbered positions in four KARI mutants.

[0051] FIG. **12** (A) is a linear depiction of the KARI amino acid sequence with specific amino acids numbered. The cofactor specificity domain residues are shown in shaded rectangles. (B) Depicts the first PCR step amplifying the mutated cofactor specificity domain residues. (C) is a linear depiction of the KARI amino acid sequence with specific amino acids of the cofactor binding domain shown in dotted

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ovals. (D) Depicts incorporation of the domain swapping library into the mutants containing K_M improving mutations. Table (E) summaries the K_M values for NADH for mutations resulting from combining mutations in the cofactor binding affinity domain with mutations in the cofactor specificity determining domain.

[0052] Table 9—is a table of the Profile HMM of the KARI enzymes described in Example 3. The eleven positions in the profile HMM representing the columns in the alignment which correspond to the eleven cofactor switching positions in *Pseudomonas fluorescens* Pf-5 KARI are identified as positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170. The lines corresponding to these positions in the model file are highlighted in yellow. Table 9 is submitted herewith electronically and is incorporated herein by reference.

[0053] The following sequences conform with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures—the Sequence Rules") and are consistent with the World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

TABLE 1

-	Oligonucleotide Primers Used In This	Invention
EQUENCE No .	ID SEQUENCE	Description
1	TGATGAACATCTTCGCGTATTCGCCGTCCT	Reverse Primer for pBAD vector
2	GCGTAGACGTGACTGTTGGCCTGNNTAAAGGCNN GGCTNNCTGGGCCAAGGCT GAAGCCCACGGCTTG	Forward primer library C
3	GCGTAGACGTGACTGTTGGCCTGNNTAAAGGCTCG GCTACCGTTGCCAAGGCTGAAGCCCACGGCTTG	Forward primer for library E
4	GCGTAGACGTGACTGTTGGCCTGCGTAAAGGCNNT GCTACCGTTGCCAAGGCTGAAGCCCACGGCTTG	Forward primer for library F
5	GCGTAGACGTGACTGTTGGCCTGCGTAAAGGCTCG GCTNNTGTTGCCAAGGCTGAAGCCCACGGCTTG	Forward primer for library G
6	GCGTAGACGTGACTGTTGGCCTGNNTAAAGGCNNT GCTNNTGTTGCCAAGGCTGAAGCCCACGGCTTG	Forward primer for library H
7	AAGATTAGCGGATCCTACCT	Sequencing primer (forward)
8	AACAGCCAAGCTTTTAGTTC	Sequencing primer (reverse)
20	CTCTCTACTGTTTCTCCATACCCG	pBAD_266-021308f
21	CAAGCCGTGGGCTTCAGCCTTGGCKNN	PF5_53Mt022908r
22	CGGTTTCAGTCTCGTCCTTGAAG	pBAD_866-021308
49	GCTCAAGCANNKAACCTGAAGG	pBAD-405- C33_090808f
50	CCTTCAGGTTKNNTGCTTGAGC	pBAD-427- C33_090808r
51	GTAGACGTGNNKGTTGGCCTG	pBAD-435- T43_090808f

	Oligonucleotide Primers Used In This	Invention
SEQUENCE No .	ID SEQUENCE	Description
52	CAGGCCAACKNNCACGTCTAC	pBAD-456- T43_090808r
53	CTGAAGCCNNKGGCNNKAAAGTGAC	pBAD-484- H59L61_090808f
54	GTCACTTTKNNGCCKNNGGCTTCAG	pBAD-509- H59L61_090808r
55	GCAGCCGTTNNKGGTGCCGACT	pBAD-519- A71_090808f
56	AGTCGGCACCKNNAACGGCTGC	pBAD-541- A71_090808r
57	CATGATCCTGNNKCCGGACGAG	pBAD-545- T80_090808f
58	CTCGTCCGGKNNCAGGATCATG	pBAD-567- T80_090808r
59	CAAGAAGGGCNNKACTCTGGCCT	pBAD-608- A101_090808f
60	AGGCCAGAGTKNNGCCCTTCTTG	pBAD-631- A101_090808r
61	GTTGTGCCTNNKGCCGACCTCG	pBAD-663- R119_090808f
62	CGAGGTCGGCKNNAGGCACAAC	pBAD-685- R119_090808r
71	GTAGACGTGACTGTTGGCCTGNNKAAAGGCNNKGC TNNKNNKGCCAAGGCTGAAGCCCACGG	PF5_4Mt111008.f
72	CCGTGGGCTTCAGCCTTGGCKNNKNNAGCKNNGC CTTTKNNCAGGCCAACAGTCACGTCTAC	PF5_4Mt111008.r
73	AAGATTAGCGGATCCTACCT	pBAD_230.f
74	GAGTGGCGCCCTTCTTGATGTTCG	pBAD_601_021308r

TABLE 1-continued

[0054] Additional sequences used in the application are listed below. The abbreviated gene names in bracket are used in this disclosure.

SEQ ID NO: 9—*Methanococcus maripaludis* C5-ilvC (MMC5)—GenBank Accession Number NC_009135.1 Region: 901034...902026

SEQ ID NO: 10 is the *Methanococcus maripaludis* S2-ilvC (MMS2)—GenBank Accession Number NC_005791.1 Region: 645729 ... 646721

SEQ ID NO: 11 is the *Methanococcus vannielii* SB-ilv5 (MVSB)—GenBank Accession Number NZ_AAWX01000002.1 Region: 302214...303206

SEQ ID NO: 12 is the Saccharomyces cerevisiae ilv5 (ilv5)—

GenBank Accession Number NC_001144.4 Region: 838065 ... 839252

SEQ ID NO: 13 is the *Sulfolobus solfataricus* P2 ilvC (KARI-D1)—GenBank Accession Number NC_002754.1 Region: 506253... 507260

SEQ ID NO: 14 is the *Pyrobaculum aerophilum* str. IM2 ilvC (KARI-D2)—GenBank Accession Number NC_003364.1 Region: 1976281 . . . 1977267

SEQ ID NO: 15 is the *Ralstonia solanacearum* GMI1000 ilvC (KARI-S1)—GenBank Accession Number NC_003295.1 Region: 2248264 . . . 2249280

SEQ ID NO: 16 is the *Pseudomonas aeruginosa* PAO1 ilvC—GenBank Accession Number NC_002516 Region: 5272455...5273471

SEQ ID NO: 17 is the *Pseudomonas fluorescens* PF5 ilvC— GenBank Accession Number NC_004129 Region: 6017379 ... 6018395

SEQ ID NO: 18 is the *Spinacia oleracea* ilvC (Spinach-KARI)—GenBank Accession Number NC_002516 Region: 1 . . . 2050.

SEQ ID NO: 19 is the amino acid sequence of the mutant (Y24F/R47Y/S50A/T52D/V53A/L61F/G170A) of the ilvC native protein of *Pseudomonas fluorescens*.

SEQ ID NO: 23 is the DNA SEQ of the mutant (Y24F/R47Y/ S50A/T52D/V53A/L61F/G170A) of the ilvC native protein of *Pseudomonas fluorescens*.

SEQ ID NO: 24 is the amino acid SEQ of the mutant ZB1 (Y24F/R47Y/S50A/T52D/V53A/L61F/A156V)

SEQ ID NO: 25 is the amino acid SEQ of the mutant ZF3 (Y24F/C33L/R47Y/S50A/T52D/V53A/L61F)

SEQ ID NO: 26 is the amino acid SEQ of the mutant ZF2 (Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/A156V)

SEQ ID NO: 27 is the Amino Acid SEQ of the Mutant Zb3 (Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/G170A)

[0055] SEQ ID NO: 28 is the amino acid SEQ of the mutant Z4B8 (C33L/R47Y/S50A/T52D/V53A/L61F/T80I/A156V/G170A)

SEQ ID NO: 29 is a consensus amino acid sequence comprising all experimentally verified KARI point mutations as based on SEQ ID NO:17.

SEQ ID NO: 30 is the amino acid sequence for KARI from *Natronomonas pharaonis* DSM 2160

SEQ ID NO: 31 is the amino acid sequence for KARI from *Bacillus subtilis* subsp. *subtilis* str. 168

SEQ ID NO: 32 is the amino acid sequence for KARI from *Corynebacterium glutamicum* ATCC13032

SEQ ID NO: 33 is the amino acid sequence for KARI from *Phaeospirilum molischianum*

SEQ ID NO: 34 is the amino acid sequence for KARI from *Zymomonas mobilis* subsp. *mobilis* ZM4

SEQ ID NO: 35 is the amino acid sequence for KARI Alkalilimnicola ehrlichei MLHE-1

SEQ ID NO: 36 is the amino acid sequence for KARI from *Campylobacter lari* RM2100

SEQ ID NO: 37 is the amino acid sequence for KARI from *Marinobacter aquaeolei* VT8

SEQ ID NO: 38 is the amino acid sequence for KARI *Psychrobacter arcticus* 273-4

SEQ ID NO: 39 is the amino acid sequence for KARI from *Hahella chejuensis* KCTC2396

SEQ ID NO: 40 is the amino acid sequence for KARI from *Thiobacillus denitrificans* ATCC25259

SEQ ID NO: 41 is the amino acid sequence for KARI from *Azotobacter vinelandii* AvOP

SEQ ID NO: 42 is the amino acid sequence for KARI from *Pseudomonas syringae* pv. *syringae* B728a

SEQ ID NO: 43 is the amino acid sequence for KARI from *Pseudomonas syringae* pv. *tomato* str. DC3000

SEQ ID NO: 44 is the amino acid sequence for KARI from *Pseudomonas putida* KT2440

SEQ ID NO: 45 is the amino acid sequence for KARI from *Pseudomonas entomophila* L48

SEQ ID NO: 46 is the amino acid sequence for KARI from *Pseudomonas mendocina* ymp

SEQ ID NO: 47 is the amino acid sequence for KARI from *Bacillus cereus* ATCC10987 NP_977840.1

SEQ ID NO: 48 is the amino acid sequence for KARI from *Bacillus cereus* ATCC10987 NP_978252.1

SEQ ID NO: 63 is the amino acid sequence for KARI from *Escherichia coli*—GenBank Accession Number P05793

SEQ ID NO: 64 is the amino acid sequence for KARI from Marine Gamma *Proteobacterium* HTCC2207—GenBank Accession Number ZP_01224863.1

SEQ ID NO: 65 is the amino acid sequence for KARI from *Desulfuromonas acetoxidans*—GenBank Accession Number ZP_01313517.1

SEQ ID NO: 66 is the amino acid sequence for KARI from *Pisum sativum* (Pea)—GenBank Accession Number O82043 SEQ ID NO: 67 is the amino acid sequence for mutant 3361G8 (C33L/R47Y/S50A/T52D/V53A/L61F/T80I)

SEQ ID NO: 68 is the amino acid sequence for mutant 2H10 (Y24F/C33L/R47Y/S50A/T52D/V53I/L61F/T80I/A156V) SEQ ID NO: 69 is the amino acid sequence for mutant 1D2 (Y24F/R47Y/S50A/T52D/V53A/L61F/T80I/A156V.

SEQ ID NO: 70 is the amino acid sequence for mutant 3F12 (Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/T80I/A156V).

SEQ ID NO: 75 is the amino acid sequence for mutant JB1C6 (Y24F/C33L/R47H/S50D/T52Y/V53Y/L61F/T80I/A156V) SEQ ID NO: 76 is the amino acid sequence for mutant 16445E4 (C33L/R47P/S50V/T52D/V53G/L61F/T80I/ A156V)

SEQ ID NO: 77 is the amino acid sequence for mutant 16468D7 (Y24F/C33L/R47T/S50I/T52D/V53R/L61F/T80I/A156V)

SEQ ID NO: 78 is the amino acid sequence for mutant 16469F3 (C33L/R47E/S50A/T52D/V53A/L61F/T80I)

SEQ ID NO: 79 is the Amino Acid Sequence for Mutant JEA1 (Y24F/C33L/R47P/S50F/T52D/L61F/T80I/A156V)

[0056] SEQ ID NO: 80 is the amino acid sequence for mutant JEG2 (Y24 F/C33L/R47F/S50A/T52D/V53A/L61F/ T80I/A156V)

SEQ ID NO: 81 is the amino acid sequence for mutant JEG4 (Y24F/C33L/R47N/S50N/T52D/V53A/L61F/T80I/A156V) SEQ ID NO: 82 is the amino acid sequence for mutant JEA7 (Y24F/C33L/R47P/S50N/T52D/V53A/L61F/T80I/A156V) SEQ ID NO: 83 is the amino acid sequence for mutant JED1 (C33L/R47N/S50N/T52D/V53A/L61F/T80I/A156V)

SEQ ID NO: 84 is the Amino Acid Sequence for Mutant 3361E1

[0057] SEQ ID NO: 85 is the amino acid sequence for mutant C2F6

SEQ ID NO: 86 is the amino acid sequence for mutant C3B11 SEQ ID NO: 87 is the amino acid sequence for mutant C4D12 SEQ ID NO: 88 is the amino acid sequence for mutant SE1

SEQ ID NO: 89 is the amino acid sequence for mutant SE1 SEQ ID NO: 89 is the amino acid sequence for mutant SE2

SEQ ID NO: 90 is the amino acid sequence for mutant SB3

SEQ ID NO: 91 is the amino acid sequence for mutant SD3 SEQ ID NO: 92 is the amino acid sequence for mutant 9650E5

SEQ ID NO: 93 is the amino acid sequence for mutant 9667A11

SEQ ID NO: 94 is the amino acid sequence for mutant 9862B9

SEQ ID NO: 95 is the amino acid sequence for mutant 9875B9

SEQ ID NO: 96 is the amino acid sequence for mutant 11461D8

SEQ ID NO: 97 is the amino acid sequence for mutant 11463 SEQ ID NO: 98 is the amino acid sequence for mutant 11518B4

DETAILED DESCRIPTION OF THE INVENTION

[0058] The present invention relates to the generation of mutated KARI enzymes to use NADH as opposed to NADPH. Such co-factor switched enzymes function more effectively in microbial systems designed to produce isobutanol. Isobutanol is an important industrial commodity chemical with a variety of applications, where its potential as a fuel or fuel additive is particularly significant. Although only a four-carbon alcohol, butanol has the energy content

similar to that of gasoline and can be blended with any fossil fuel. Isobutanol is favored as a fuel or fuel additive as it yields only CO_2 and little or no SO_x or NO_x when burned in the standard internal combustion engine. Additionally butanol is less corrosive than ethanol, the most preferred fuel additive to date.

[0059] The following definitions and abbreviations are to be use for the interpretation of the claims and the specification.

[0060] The term "invention" or "present invention" as used herein is meant to apply generally to all embodiments of the invention as described in the claims as presented or as later amended and supplemented, or in the specification.

[0061] The term "isobutanol biosynthetic pathway" refers to the enzymatic pathway to produce isobutanol. Preferred isobutanol biosynthetic pathways are illustrated in FIG. **1** and described herein.

[0062] The term "NADPH consumption assay" refers to an enzyme assay for the determination of the specific activity of the KARI enzyme, involving measuring the disappearance of the KARI cofactor, NADPH, from the enzyme reaction.

[0063] "KARI" is the abbreviation for the enzyme ketolacid reducto-isomerase.

[0064] The term "close proximity" when referring to the position of various amino acid residues of a KARI enzyme with respect to the adenosyl 2'-phosphate of NADPH means amino acids in the three-dimensional model for the structure of the enzyme that are within about 4.5 Å of the phosphorus atom of the adenosyl 2'-phosphate of NADPH bound to the enzyme.

[0065] The term "ketol-acid reductoisomerase" (abbreviated "KARI"), and "acetohydroxy acid isomeroreductase" will be used interchangeably and refer to the enzyme having the EC number, EC 1.1.1.86 (Enzyme Nomenclature 1992, Academic Press, San Diego). Ketol-acid reductoisomerase catalyzes the reaction of (S)-acetolactate to 2,3-dihydroxyisovalerate, as more fully described below. These enzymes are available from a number of sources, including, but not limited to E. coli GenBank Accession Number NC-000913 REGION: 3955993 ... 3957468, Vibrio cholerae GenBank Accession Number NC-002505 REGION: 157441 . . . 158925, Pseudomonas aeruginosa, GenBank Accession Number NC-002516, (SEQ ID NO: 16) REGION: 5272455. ... 5273471, and Pseudomonas fluorescens GenBank Accession Number NC-004129 (SEQ ID NO: 17) REGION: 6017379 . . . 6018395. As used herein the term "Class I ketol-acid reductoisomerase enzyme" means the short form that typically has between 330 and 340 amino acid residues, and is distinct from the long form, called class II, that typically has approximately 490 residues.

[0066] The term "acetolactate synthase" refers to an enzyme that catalyzes the conversion of pyruvate to acetolactate and CO_2 . Acetolactate has two stereoisomers ((R) and (S)); the enzyme prefers the (S)-isomer, which is made by biological systems. Preferred acetolactate synthases are known by the EC number 2.2.1.6 9 (*Enzyme Nomenclature* 1992, Academic Press, San Diego). These enzymes are available from a number of sources, including, but not limited to, *Bacillus subtilis* (GenBank Nos: CAB15618, Z99122, NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence, respectively), *Klebsiella pneumoniae* (GenBank Nos: AAA25079, M73842 and *Lactococcus lactis* (GenBank Nos: AAA25161, L16975).

[0067] The term "acetohydroxy acid dehydratase" refers to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to α -ketoiso-valerate. Preferred acetohydroxy acid dehydratases are known by the EC number 4.2.1.9. These enzymes are available from a vast array of microorganisms, including, but not limited to, *E. coli* (GenBank Nos: YP_026248, NC_000913, *S. cerevisiae* (GenBank Nos: NP_012550, NC_001142), *M. maripaludis* (GenBank Nos: CAF29874, BX957219), and *B. subtilis* (GenBank Nos: CAB14105, Z99115).

[0068] The term "branched-chain α -keto acid decarboxylase" refers to an enzyme that catalyzes the conversion of α -ketoisovalerate to isobutyraldehyde and CO₂. Preferred branched-chain α -keto acid decarboxylases are known by the EC number 4.1.1.72 and are available from a number of sources, including, but not limited to, *Lactococcus lactis* (GenBank Nos: AAS49166, AY548760; CAG34226, AJ746364, *Salmonella typhimurium* (GenBank Nos: NP-461346, NC-003197), and *Clostridium acetobutylicum* (GenBank Nos: NP-149189, NC-001988).

[0069] The term "branched-chain alcohol dehydrogenase" refers to an enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol. Preferred branched-chain alcohol dehydrogenases are known by the EC number 1.1.1.265, but may also be classified under other alcohol dehydrogenases (specifically, EC 1.1.1.1 or 1.1.1.2). These enzymes utilize NADH (reduced nicotinamide adenine dinucleotide) and/or NADPH as electron donor and are available from a number of sources, including, but not limited to, *S. cerevisiae* (GenBank Nos: NP-010656, NC-001136; NP-014051, NC-001145), *E. coli* (GenBank Nos: NP-349892, NC_003030).

[0070] The term "branched-chain keto acid dehydrogenase" refers to an enzyme that catalyzes the conversion of α -ketoisovalerate to isobutyryl-CoA (isobutyryl-cofactor A), using NAD⁺ (nicotinamide adenine dinucleotide) as electron acceptor. Preferred branched-chain keto acid dehydrogenases are known by the EC number 1.2.4.4. These branched-chain keto acid dehydrogenases comprise four subunits, and sequences from all subunits are available from a vast array of microorganisms, including, but not limited to, *B. subtilis* (GenBank Nos: CAB14336, Z99116; CAB14335, Z99116; CAB14334, Z99116; and CAB14337, Z99116) and *Pseudomonas putida* (GenBank Nos: AAA65614, M57613; AAA65615, M57613; AAA65617, M57613; and AAA65618, M57613).

[0071] The terms " k_{cat} " and " K_M " are known to those skilled in the art and are described in Enzyme Structure and Mechanism, 2nd ed. (Ferst; W.H. Freeman Press, NY, 1985; pp 98-120). The term " k_{cat} ", often called the "turnover number", is defined as the maximum number of substrate molecules converted to products per active site per unit time, or the number of times the enzyme turns over per unit time. K_{cat}=Vmax/[E], where [E] is the enzyme concentration (Ferst, supra). The terms "total turnover" and "total turnover number" are used herein to refer to the amount of product formed by the reaction of a KARI enzyme with substrate.

[0072] The term "catalytic efficiency" is defined as the K_{cat}/K_M of an enzyme. Catalytic efficiency is used to quantify the specificity of an enzyme for a substrate.

[0073] The term "isolated nucleic acid molecule", "isolated nucleic acid fragment" and "genetic construct" will be used interchangeably and will mean a polymer of RNA or DNA that is single- or double-stranded, optionally containing syn-

thetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

[0074] The term "amino acid" refers to the basic chemical structural unit of a protein or polypeptide. The following abbreviations are used herein to identify specific amino acids:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	А
Arginine	Arg	R
Asparagine	Asn	Ν
Aspartic acid	Asp	D
Cysteine	Cys	С
Glutamine	Gln	Q
Glutamic acid	Glu	Ĕ
Glycine	Gly	G
Histidine	His	Н
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	М
Phenylalanine	Phe	F
Proline	Pro	Р
Serine	Ser	S
Threonine	Thr	Т
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0075] The term "gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' noncoding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of a microorganism. A "foreign" gene refers to a gene not normally found in the host microorganism, but that is introduced into the host microorganism by gene transfer. Foreign genes can comprise native genes inserted into a nonnative microorganism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

[0076] As used herein the term "coding sequence" refers to a DNA sequence that encodes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

[0077] The term "promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to

a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0078] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0079] The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

[0080] As used herein the term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host microorganism, resulting in genetically stable inheritance. Host microorganisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" microorganisms.

[0081] The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[0082] The term "site-saturation library" refers to a library which contains random substitutions at a specific amino acid position with all 20 possible amino acids at once.

[0083] The term "error-prone PCR" refers to adding random copying errors by imposing imperfect or 'sloppy' PCR reaction conditions which generate randomized libraries of mutations in a specific nucleotide sequence.

[0084] As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

[0085] The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host microorganism without altering the polypeptide encoded by the DNA.

Molecular Techniques

[0086] Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook et al. (Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) (hereinafter "Maniatis"); and by Silhavy et al. (*Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press Cold Spring Harbor, N.Y., 1984); and by Ausubel, F. M. et al., (*Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience, 1987).

[0087] The present invention addresses a need that arises in the microbial production of isobutanol where the ketol-acid reductoisomerase enzyme performs a vital role. Wild type ketol-acid reductoisomerase enzymes typically use NADPH as their cofactor. However, in the formation of isobutanol an excess of NADH is produced by ancillary metabolic pathways. The invention provides mutant Class I KARI enzymes that have been evolved to utilize NADH as a cofactor, overcoming the cofactor problem and increasing the efficiency of the isobutanol biosynthetic pathway.

[0088] Production of isobutanol utilizes the glycolysis pathway present in the host microorganism. During the production of two molecules of pyruvate from glucose during glycolysis, there is net production of two molecules of NADH from NAD⁺ by the glyceraldehyde-3-phosphate dehydrogenase reaction. During the further production of one molecule of isobutanol from two molecules of pyruvate, there is net consumption of one molecule of NADPH, by the KARI reaction, and one molecule of NADH by the isobutanol dehydrogenase reaction. The overall reaction of glucose to isobutanol thus leads to net production of one molecule of NADH and net consumption of one molecule of NADPH. The interconversion of NADH with NADPH is generally slow and inefficient; thus, the NADPH consumed is generated by metabolism (for example, by the pentose phosphate pathway) consuming substrate in the process. Meanwhile, the cell strives to maintain homeostasis in the NAD+/NADH ratio, leading to the excess NADH produced in isobutanol production being consumed in wasteful reduction of other metabolic intermediates; e.g., by the production of lactate from pyruvate. Thus, the imbalance between NADH produced and NADPH consumed by the isobutanol pathway leads to a reduction in the molar yield of isobutanol produced from glucose in two ways: 1) unnecessary operation of metabolism to produce NADPH, and 2) wasteful reaction of metabolic intermediates to maintain NAD+/NADH homeostasis. The solution to this problem is to invent a KARI that is specific for NADH as its cofactor, so that both molecules of NADH produced in glycolysis are consumed in the synthesis of isobutanol from pyruvate.

Keto Acid Reductoisomerase (KARI) Enzymes

[0089] Acetohydroxy acid isomeroreductase or ketol-acid reducto-isomerase (KARI; EC 1.1.1.86) catalyzes two steps in the biosynthesis of branched-chain amino acids and is a key enzyme in their biosynthesis. KARI is found in a variety of microorganisms and amino acid sequence comparisons across species have revealed that there are 2 types of this enzyme: a short form (class I) found in fungi and most bacteria, and a long form (class II) typical of plants.

[0090] Class I KARIs typically have between 330-340 amino acid residues. The long form KARI enzymes have about 490 amino acid residues. However, some bacteria such as *Escherichia coli* possess a long form, where the amino acid sequence differs appreciably from that found in plants. KARI is encoded by the ilvC gene and is an essential enzyme for growth of *E. coli* and other bacteria in a minimal medium. Typically KARI uses NADPH as cofactor and requires a divalent cation such as Mg⁺⁺ for its activity. In addition to utilizing acetolactate in the valine pathway, KARI also converts acetohydroxybutanoate to dihydroxymethylpentanoate in the isoleucine production pathway.

[0091] Class II KARIs generally consist of a 225-residue N-terminal domain and a 287-residue C-terminal domain. The N-terminal domain, which contains the NADPH-binding site, has an α/β structure and resembles domains found in other pyridine nucleotide-dependent oxidoreductases. The C-terminal domain consists almost entirely of α -helices and is of a previously unknown topology.

[0092] The crystal structure of the E. coli KARI enzyme at 2.6 Å resolution has been solved (Tyagi, et al., Protein Sci., 14: 3089-3100, 2005). This enzyme consists of two domains, one with mixed α/β structure which is similar to that found in other pyridine nucleotide-dependent dehydrogenases. The second domain is mainly α -helical and shows strong evidence of internal duplication. Comparison of the active sites of KARI of E. coli, Pseudomonas aeruginosa, and spinach showed that most residues in the active site of the enzyme occupy conserved positions. While the E. coli KARI was crystallized as a tetramer, which is probably the likely biologically active unit, the P. aeruginosa KARI (Ahn, et al., J. Mol. Biol., 328: 505-515, 2003) formed a dodecamer, and the enzyme from spinach formed a dimer. Known KARIs are slow enzymes with a reported turnover number (k_{cat}) of 2 s⁻¹ (Aulabaugh et al.; Biochemistry, 29: 2824-2830, 1990) or 0.12 s^{-1} (Rane et al., Arch. Biochem. Biophys. 338: 83-89, 1997) for acetolactate. Studies have shown that genetic control of isoleucine-valine biosynthesis in E. coli is different than that in Ps. aeruginosa (Marinus, et al., Genetics, 63: 547-56, 1969).

Identification of Amino Acid Target Sites for Cofactor Switching

[0093] It was reported that phosphate p2' oxygen atoms of NADPH form hydrogen bonds with side chains of Arg162, Ser165 and Ser167 of spinach KARI (Biou V., et al. The EMBO Journal, 16: 3405-3415, 1997). Multiple sequence alignments were performed, using vector NTI (Invitrogen Corp. Carlsbad, Calif.), with KARI enzymes from spinach, *Pseudomonas aeruginosa* (PAO-KARI) and *Pseudomonas fluorescens* (PF5-KARI). The NADPH binding sites are

shown in FIG. **2**A. The amino acids, argenine, threonine and serine appear to play similar roles in forming hydrogen bonds with phosphate p2' oxygen atoms of NADPH in KARI enzymes. Studies by Ahn et al., (J. Mol. Biol., 328: 505-515, 2003) had identified three NADPH phosphate binding sites (Arg47, Ser50 and Thr52) for *Pseudomonas aeruginosa* (PAO-KARI) following comparing its structure with that of the spinach KARI. Hypothesizing that these three NADPH phosphate binding sites of the three KARI enzymes used in the disclosure were conserved, Arg47, Ser50 and Thr52 of

homology modeling. [0094] Multiple sequence alignment among PF5-ilvC and several other KARI enzymes with promiscuous nucleotide specificity was also performed. As shown in FIG. 2B, the amino acids of glycine (G50) and tryptophan (W53), in other KARI enzymes in FIG. 2B, always appear together as a pair in the sequences of those enzymes. It was therefore assumed that the tryptophan 53 bulky residue was important in determining nucleotide specificity and by reducing the size of nucleotide binding pocket one could favor binding of the smaller nucleotide, NADH. Position 53 of PF5-ilvC was therefore chosen as a target for mutagenesis.

PF5-KARI were targeted as the phosphate binding sites for

this enzyme. This hypothesis was further confirmed through

[0095] Several site-saturation gene libraries were prepared containing genes encoding KARI enzymes by commercially available kits for the generation of mutants. Clones from each library were screened for improved KARI activity using the NADH consumption assay described herein. Screening resulted in the identification of a number of genes having mutations that can be correlated to KARI activity. The location of the mutations were identified using the amino acid sequence of the *Pseudomonas fluorescens* PF5 ilvC protein (SEQ ID NO:17). Mutants with improved KARI activity had mutations at one or more positions at amino acids: 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, 165, and 170. More specifically desirable mutations included the following substitutions:

- **[0096]** a) the residue at position 47 has an amino acid substitution selected from the group consisting of A, C, D, F, G, I, L, N, P, and Y;
- [0097] b) the residue at position 50 has an amino acid substitution selected from the group consisting of A, C, D, E, F, G, M, N, V, W;
- [0098] c) the residue at position 52 has an amino acid substitution selected from the group consisting of A, C, D, G, H, N, S;
- **[0099]** d) the residue at position 53 has an amino acid substitution selected from the group consisting of A, H, I, W;

[0100] In another embodiment, additional mutagenesis, using error prone

[0101] PCR, performed on the mutants listed above identified suitable mutation positions as: 156, 165, 61, 170, 115 and 24. More specifically the desirable mutants with lower K_M for NADH contained the following substitutions:

- **[0102]** e) the residue at position 156 has an amino acid substitution of V;
- **[0103]** f) the residue at position 165 has an amino acid substitution of M;
- **[0104]** g) the residue at position 61 has an amino acid substitution of F;
- **[0105]** h) the residue at position 170 has an amino acid substitution of A;

- $[0106]\quad i)$ the residue at position 24 has an amino acid substitution of F; and
- [0107] j) the residue at position 115 has an amino acid substitution of L.

[0108] In another embodiment, multiple sequence alignment of *Pseudomonas fluorescens* PF5-ilvC and *Bacillus cereus* ilvC1 and livC2 and spinach KARI was performed which allowed identification of positions 24, 33, 47, 50, 52, 53, 61, 80, 156 and 170 for further mutagenesis. More specifically mutants with much lower K_M for NADH were obtained. These mutations are also based on the *Pseudomonas fluorescens*, KARI enzyme (SEQ ID NO:17) as a reference sequence wherein the reference sequence comprises at least one amino acid substitution selected from the group consisting of:

- **[0109]** k) the residue at position 24 has an amino acid substitution of phenylalanine;
- **[0110]** 1) the residue at position 50 has an amino acid substitution of alanine;
- **[0111]** m) the residue at position 52 has an amino acid substitution of aspartic acid;
- **[0112]** n) the residue at position 53 has an amino acid substitution of alanine;
- **[0113]** o) the residue at position 61 has an amino acid substitution of phenylalanine;
- **[0114]** p) the residue at position 156 has an amino acid substitution of valine;
- **[0115]** q) the residue at position 33 has an amino acid substitution of leucine;
- **[0116]** r) the residue at position 47 has an amino acid substitution of tyrosine;
- **[0117]** s) the residue at position 80 has an amino acid substitution of isoleucine;
- [0118] and
- **[0119]** t) the residue at position 170 has an amino acid substitution of alanine.

[0120] The present invention includes a mutant polypeptide having KARI activity, said polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 24, 25, 26, 27 and 28.

[0121] A consensus sequence for the mutant ilvC was generated from the multiple sequence alignment and is provided as SEQ ID NO: 29 which represents all experimentally verified mutations of the KARI enzyme based on the amino acid sequence of the KARI enzyme isolated from *Pseudomonas fluorescens*, (SEQ ID NO:17)

[0122] Additionally the present invention describes mutation positions identified using a profile Hidden Markov Model (HMM) built based on sequences of 25 functionally verified Class I and Class II KARI enzymes. Profile HMM identified mutation positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170 (the numbering is based on the sequences of *Pseudomonas fluorescens* PF5 KARI). Thus, it will be appreciated by the skilled person that mutations at these positions, as well as those discussed above that have been experimentally verified will also give rise to KARI enzymes having the ability to bind NADH.

[0123] Furthermore, applicants have discovered that the ketol-acid reductoisomerase enzyme has two functionally related domains: one domain affecting nucleotide specificity and the other domain impacting the K_M for the cofactor (FIGS. **11** and **12**). To examine whether this characteristic could be exploited to engineer the desired KARI mutants (i.e.,

mutants with high NADH activity ($K_M < 20 \ \mu M$) and substantially decreased NADPH activity ($K_M > 100 \ \mu M$)), two libraries were created.

[0124] One library was a four-site saturation library targeting the NADH or NADPH binding positions, i.e., amino acids at positions 47, 50, 52 and 53 (FIG. 11). To build this library, mutants which possessed both NADH and NADPH activities and K_{M} ~10-20 μ M for NADH, were selected from a group consisting of SEQ ID NOs: 28, 67, 68, 69, 70 and 84, as templates. Further saturation mutagenesis generated new mutants (i.e., mutants with SEQ ID NOs: 75-78) that possessed mainly NADH activity with very low NADPH activity. **[0125]** The desirable mutants with higher NADH activity, following site saturation mutagenesis, comprised the following substitutions:

- **[0126]** u) the residue at position 24 has an amino acid substitution of phenylalanine;
- **[0127]** v) the residue at position 50 has an amino acid substitution of aspartic acid or valine or isoleucine or phenylalanine;
- **[0128]** w) the residue at position 52 has an amino acid substitution of tyrosine or aspartic acid;
- **[0129]** x) the residue at position 53 has an amino acid substitution of tyrosine or glycine, or argenine, or alanine;
- **[0130]** y) the residue at position 61 has an amino acid substitution of phenylalanine;
- **[0131]** z) the residue at position 156 has an amino acid substitution of valine;
- **[0132]** aa) the residue at position 33 has an amino acid substitution of leucine;
- **[0133]** bb) the residue at position 47 has an amino acid substitution of histidine, or proline, or threonine, or glutamic acid; and
- **[0134]** cc) the residue at position 80 has an amino acid substitution of isoleucine.

[0135] The K_M for NADH in the above mutants was still slightly high (e.g., JB1C6, SEQ ID NO: 74, has K_M of 22 μ M for NADH). To further improve the NADH K_M of the mutant KARIs, a "domain swapping library", which combined the nucleotide switching mutations and mutations with improved K_M for NADH, was created (FIG. **12**). More specifically, the beneficial mutations at positions 47, 50, 52 and 53 obtained in the site saturation experiment (see Tables 3 and 4), were transferred into mutants that possessed K_M-4-40 μ M for NADH (SEQ ID NOs:24-28 and 67-70 and 84, see Tables 6 and 7). The resultant new mutants accepted NADH as cofactor with very low K_M-10 μ M and greatly reduced NADPH activity. Examples of these mutants include: JEA1 (SEQ ID NO: 79), JEG2 (SEQ ID NO: 80), JEG4 (SEQ ID NO: 81), JEA7 (SEQ ID NO: 82) and JED1 (SEQ ID NO: 83).

[0136] Following domain swapping experiments, the mutants that possessed very low K_M for NADH had the following substitutions:

- **[0137]** dd) the residue at position 24 has an amino acid substitution of phenylalanine;
- **[0138]** ee) the residue at position 50 has an amino acid substitution of alanine, asparagine, or phenylalanine;
- **[0139]** ff) the residue at position 52 has an amino acid substitution of aspartic acid;
- **[0140]** gg) the residue at position 53 has an amino acid substitution of alanine;
- **[0141]** hh) the residue at position 61 has an amino acid substitution of phenylalanine;

- **[0142]** ii) the residue at position 156 has an amino acid substitution of valine;
- **[0143]** jj) the residue at position 33 has an amino acid substitution of leucine;

[0144] kk) the residue at position 47 has an amino acid substitution of asparagine, proline; and phenylalanine;

[0145] 1l) the residue at position 80 has an amino acid substitution of isoleucine.

[0146] In one embodiment the present method includes a mutant polypeptide having KARI activity, said polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 24,-28, 67-70, and 75-98,

[0147] In another embodiment the method provides an NADH utilizing KARI mutant with a K_M for NADH <15 μ M. **[0148]** In a preferred embodiment, the mutant KARI JEA1 (SEQ ID NO: 79) has the following substitutions:

Y24F/C33L/R47P/S50F/T52D/L61F/T80I/A156V

[0149] In another preferred embodiment, the mutant KARI JEG2 (SEQ ID NO: 80) has the following substitutions:

(Y24F/C33L/R47F/S50A/T52D/V53A/L61F/T80I/A156V)

[0150] In another preferred embodiment, the mutant KARI JEG4 (SEQ ID NO: 81), has the following substitutions:

(Y24F/C33L/R47N/S50N/T52D/V53A/L61F/T80I/A156V)

[0151] In another preferred embodiment, the mutant KARI JEA7 (SEQ ID NO: 82), has the following substitutions:

(Y24F/C33L/R47P/S50N/T52D/V53A/L61F/T80I/A156V)

[0152] In another preferred embodiment, the mutant KARI JED1 (SEQ ID NO: 83) has the following substitutions:

(C33L/R47N/S50N/T52D/V53A/L61F/T80I/A156V)

[0153] In another embodiment the method provides an NADH accepting KARI mutant wherein the ratio of NADH/ NADPH activity is greater than one. A consensus sequence for the mutant ilvC was generated from the multiple sequence alignment and is provided as SEQ ID NO: 29 which represents all experimentally verified mutations of the KARI enzyme based on the amino acid sequence of the KARI enzyme isolated from *Pseudomonas fluorescens* (SEQ ID NO:17).

The Host Strains for KARI Engineering

[0154] Two host strains, E. coli TOP10 from Invitrogen and E. coli Bw25113 (ΔilvC, an ilvC gene-knockout), were used for making constructs over-expressing the KARI enzyme in this disclosure. In the Bw25113 strain, the entire ilvC gene of the E. coli chromosome was replaced by a Kanamycin cassette using the Lambda red homology recombination technology described by Kirill et al., (Kirill A. Datsenko and Barry L. Wanner, Proc. Natl. Acad. Sci. USA, 97: 6640-6645, 2000). Homology Modeling of PF5 KARI with Bound Substrates [0155] The structure of PF5-KARI with bound NADPH, acetolactate and magnesium ions was built based on the crystal structure of P. aeruginosa PAO1-KARI (PDB ID 1NP3, Ahn H. J. et al., J. Mol. Biol., 328: 505-515, 2003) which has 92% amino acid sequence homology to PF5 KARI. PAO1-KARI structure is a homo-dodecamer and each dodecamer consists of six homo-dimers with extensive dimer interface. The active site of KARI is located in this dimer interface. The biological assembly is formed by six homo-dimers positioned on the edges of a tetrahedron resulting in a highly symmetrical dodecamer of 23 point group symmetry. For simplicity, only the dimeric unit (monomer A and monomer B) was built for the homology model of PF5-KARI in this study because the active site is in the homo-dimer interface.

[0156] The model of PF5-KARI dimer was built based on the coordinates of monomer A and monomer B of PAO1-KARI and sequence of PF5-KARI using DeepView/Swiss PDB viewer (Guex, N. and Peitsch, M. C., Electrophoresis, 18: 2714-2723, 1997). This model was then imported to program O (Jones, T. A. et al, Acta Crystallogr. A 47: 110-119, 1991) on a Silicon Graphics system for further modification. [0157] The structure of PAO1-KARI has no NADPH, substrate or inhibitor or magnesium in the active site. Therefore, the spinach KARI structure (PDB ID 1yve, Biou V. et al., The EMBO Journal, 16: 3405-3415, 1997.), which has magnesium ions, NADPH and inhibitor (N-Hydroxy-N-isopropyloxamate) in the acetolacate binding site, was used to model these molecules in the active site. The plant KARI has very little sequence homology to either PF5- or PAO1 KARI (<20% amino acid identity), however the structures in the active site region of these two KARI enzymes are very similar. To overlay the active site of these two KARI structures, commands LSQ_ext, LSQ_improve, LSQ_mol in the program O were used to line up the active site of monomer A of spinach KARI to the monomer A of PF5 KARI model. The coordinates of NADPH, two magnesium ions and the inhibitor bound in the active site of spinach KARI were extracted and incorporated to molecule A of PF5 KARI. A set of the coordinates of these molecules were generated for monomer B of PF5 KARI by applying the transformation operator from monomer A to monomer B calculated by the program.

[0158] Because there is no NADPH in the active site of PAO1 KARI crystal structure, the structures of the phosphate binding loop region in the NADPH binding site (residues 44-45 in PAO1 KARI, 157-170 in spinach KARI) are very different between the two. To model the NADPH bound form, the model of the PF5-KARI phosphate binding loop (44-55) was replaced by that of 1yve (157-170). Any discrepancy of side chains between these two was converted to those in the PF5-KARI sequence using the mutate_replace command in program O, and the conformations of the replaced side-chains were manually adjusted. The entire NADPH/Mg/inhibitor bound dimeric PF5-KARI model went through one round of energy minimization using program CNX (ACCELRYS San Diego Calif., Burnger, A. T. and Warren, G. L., Acta Crystallogr., D 54: 905-921, 1998) after which the inhibitor was replaced by the substrate, acetolactate (AL), in the model. The conformation of AL was manually adjusted to favor hydride transfer of C4 of the nicotinamine of NADPH and the substrate. No further energy minimization was performed on this model (coordinates of the model created for this study are attached in a separate word file). The residues in the phosphate binding loop and their interactions with NADPH are illustrated in FIG. 3.

Application of a "Profile Hidden Markov Model" for Identification of Residue Positions Involved in Cofactor Switching in KARI Enzymes

[0159] Applicants have developed a method for identifying KARI enzymes and the residue positions that are involved in cofactor switching from NADPH to NADH. To structurally characterize KARI enzymes, a Profile Hidden Markov Model

(HMM) was prepared as described in Example 5 using amino acid sequences of 25 KARI proteins with experimentally verified function as outlined in Table 6. These KARIs were from [Pseudomonas fluorescens Pf-5 (SEQ ID NO: 17), Sulfolobus solfataricus P2 (SEQ ID NO: 13), Pyrobaculum aerophilum str. IM2 (SEQ ID NO: 14), Natronomonas pharaonis DSM 2160 (SEQ ID NO: 30), Bacillus subtilis subsp. subtilis str. 168 (SEQ ID NO: 31), Corynebacterium glutamicum ATCC 13032 (SEQ ID NO: 32), Phaeospririlum molischianum (SEQ ID NO: 33), Ralstonia solanacearum GMI1000 (SEQ ID NO: 15), Zymomonas mobilis subsp. mobilis ZM4 (SEQ ID NO: 34), Alkalilimnicola ehrlichei MLHE-1 (SEQ ID NO: 35), Campylobacter lari RM2100 (SEQ ID NO: 36), Marinobacter aquaeolei VT8 (SEQ ID NO: 37), Psychrobacter arcticus 273-4 (SEQ ID NO: 38), Hahella chejuensis KCTC 2396 (SEQ ID NO: 39), Thiobacillus denitrificans ATCC 25259 (SEQ ID NO: 40), Azotobacter vinelandii AvOP (SEQ ID NO: 41), Pseudomonas syringae pv. syringae B728a (SEQ ID NO: 42), Pseudomonas syringae pv. tomato str. DC3000 (SEQ ID NO: 43), Pseudomonas putida KT2440 (Protein SEQ ID NO: 44), Pseudomonas entomophila L48 (SEQ ID NO: 45), Pseudomonas mendocina ymp (SEQ ID NO: 46), Pseudomonas aeruginosa PAO1 (SEQ ID NO: 16), Bacillus cereus ATCC 10987 (SEQ ID NO: 47), Bacillus cereus ATCC 10987 (SEQ ID NO: 48), and Spinacia oleracea (SEQ ID NO: 18). [0160] In addition using methods disclosed in this application, sequences of Class II KARI enzymes such as E. coli (SEQ ID NO: 63—GenBank Accession Number P05793), marine gamma Proteobacterium HTCC2207 (SEQ ID NO: 64-GenBank Accession Number ZP_01224863.1), Desulfuromonas acetoxidans (SEQ ID NO: 65-GenBank Accession Number ZP_01313517.1) and Pisum sativum (pea) (SEQ ID NO: 66-GenBank Accession Number O82043) could be mentioned.

[0161] This Profile HMM for KARIs may be used to identify any KARI related proteins. Any protein that matches the Profile HMM with an E value of $<10^{-3}$ using hmmsearch program in the HMMER package is expected to be a functional KARI, which can be either a Class I and Class II KARI. Sequences matching the Profile HMM given herein are then analyzed for the location of the 12 positions in Pseudomonas fluorescens Pf-5 that switches the cofactor from NADPH to NADH. The eleven nodes, as defined in the section of Profile HMM building, in the profile HMM representing the columns in the alignment which correspond to the eleven co-factor switching positions in Pseudomonas fluorescens Pf-5 KARI are identified as node 24, 33, 47, 50, 52, 53, 61, 80, 115, 156 and 170. The lines corresponding to these nodes in the model file are identified in Table 9. One skilled in the art will readily be able to identify these 12 positions in the amino acid sequence of a KARI protein from the alignment of the sequence to the profile HMM using hmm search program in HMMER package.

[0162] The KARI enzymes identified by this method, include both Class I and Class II KARI enzymes from either microbial or plant natural sources. Any KARI identified by this method may be used for heterologous expression in microbial cells.

[0163] For example each of the KARI encoding nucleic acid fragments described herein may be used to isolate genes encoding homologous proteins. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include,

but are not limited to: 1) methods of nucleic acid hybridization; 2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR) (Mullis et al., U.S. Pat. No. 4,683,202); ligase chain reaction (LCR) (Tabor, S. et al., Proc. Acad. Sci. USA 82:1074, 1985); or strand displacement amplification (SDA) (Walker, et al., Proc. Natl. Acad. Sci. U.S.A., 89: 392, 1992); and 3) methods of library construction and screening by complementation.

[0164] Although the sequence homology between Class I and Class II KARI enzymes is low, the three dimensional structure of both Classes of the enzymes, particularly around the active site and nucleotide binding domains is highly conserved (Tygai, R., et al., Protein Science, 34: 399-408, 2001). The key amino acid residues that make up the substrate binding pocket are highly conserved between these two Classes even though they may not align well in a simple sequence comparison. It can therefore be concluded that the residues affecting cofactor specificity identified in Class I KARI (e.g., positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170 of PF5 KARI) can be extended to Class II KARI enzymes.

Isobutanol Biosynthetic Pathways

[0165] Carbohydrate utilizing microorganisms employ the Embden-Meyerhof-Parnas (EMP) pathway, the Entner and Doudoroff pathway (EDP) and the pentose phosphate pathway (PPP) as the central, metabolic routes to provide energy and cellular precursors for growth and maintenance. These pathways have in common the intermediate glyceraldehyde-3-phosphate and, ultimately, pyruvate is formed directly or in combination with the EMP pathway. Subsequently, pyruvate is transformed to acetyl-cofactor A (acetyl-CoA) via a variety of means. Acetyl-CoA serves as a key intermediate, for example, in generating fatty acids, amino acids and secondary metabolites. The combined reactions of sugar conversion to pyruvate produce energy (e.g., adenosine-5'-triphosphate, ATP) and reducing equivalents (e.g., reduced nicotinamide adenine dinucleotide, NADH, and reduced nicotinamide adenine dinucleotide phosphate, NADPH). NADH and NADPH must be recycled to their oxidized forms (NAD+ and NADP⁺, respectively). In the presence of inorganic electron acceptors (e.g. O₂, NO₃⁻ and SO₄²⁻), the reducing equivalents may be used to augment the energy pool; alternatively, a reduced carbon byproduct may be formed.

[0166] There are four potential pathways for production of isobutanol from carbohydrate sources with recombinant microorganisms as shown in FIG. **1**. All potential pathways for conversion of carbohydrates to isobutanol have been described in the commonly owned U.S. patent application Ser. No. 11/586,315, which is incorporated herein by reference.

[0167] The preferred pathway for conversion of pyruvate to isobutanol consists of enzymatic steps "a", "b", "c", "d", and "e" (FIGS. 1A and 1B) and includes the following substrate to product conversions:

- **[0168]** a) pyruvate to acetolactate, as catalyzed for example by acetolactate synthase,
- **[0169]** b) (S)-acetolactate to 2,3-dihydroxyisovalerate, as catalyzed for example by acetohydroxy acid isomeroreductase,
- **[0170]** c) 2,3-dihydroxyisovalerate to α-ketoisovalerate, as catalyzed for example by acetohydroxy acid dehydratase,

- [0171] d) α-ketoisovalerate to isobutyraldehyde, as catalyzed for example by a branched-chain keto acid decarboxylase, and
- **[0172]** e) isobutyraldehyde to isobutanol, as catalyzed for example by, a branched-chain alcohol dehydrogenase.

[0173] This pathway combines enzymes involved in wellcharacterized pathways for valine biosynthesis (pyruvate to α -ketoisovalerate) and valine catabolism (α -ketoisovalerate to isobutanol). Since many valine biosynthetic enzymes also catalyze analogous reactions in the isoleucine biosynthetic pathway, substrate specificity is a major consideration in selecting the gene sources. For this reason, the primary genes of interest for the acetolactate synthase enzyme are those from Bacillus (alsS) and Klebsiella (budB). These particular acetolactate synthases are known to participate in butanediol fermentation in these microorganisms and show increased affinity for pyruvate over ketobutyrate (Gollop et al., J. Bacteriol., 172: 3444-3449, 1990); and (Holtzclaw et al., J. Bacteriol., 121: 917-922, 1975). The second and third pathway steps are catalyzed by acetohydroxy acid reductoisomerase and dehydratase, respectively. These enzymes have been characterized from a number of sources, such as for example, E. coli (Chunduru et al., Biochemistry, 28: 486-493,1989); and (Flint et al., J. Biol. Chem., 268: 14732-14742, 1993). The final two steps of the preferred isobutanol pathway are known to occur in yeast, which can use valine as a nitrogen source and, in the process, secrete isobutanol. a-Ketoisovalerate can be converted to isobutyraldehyde by a number of keto acid decarboxylase enzymes, such as for example pyruvate decarboxylase. To prevent misdirection of pyruvate away from isobutanol production, a decarboxylase with decreased affinity for pyruvate is desired. So far, there are two such enzymes known in the art (Smit et al., Appl. Environ. Microbiol., 71: 303-311, 2005); and (de la Plaza et al., FEMS Microbiol. Lett., 238: 367-374, 2004). Both enzymes are from strains of Lactococcus lactis and have a 50-200-fold preference for ketoisovalerate over pyruvate. Finally, a number of aldehyde reductases have been identified in yeast, many with overlapping substrate specificity. Those known to prefer branched-chain substrates over acetaldehyde include, but are not limited to, alcohol dehydrogenase VI (ADH6) and Ypr1p (Larroy et al., Biochem. J., 361: 163-172, 2002); and (Ford et al., Yeast, 19: 1087-1096, 2002), both of which use NADPH as electron donor. An NADPH-dependent reductase, YqhD, active with branched-chain substrates has also been recently identified in E. coli (Sulzenbacher et al., J. Mol. Biol., 342: 489-502, 2004).

[0174] Two of the other potential pathways for isobutanol production also contain the initial three steps of "a", "b" and "c" (FIG. 1A). One pathway consists of enzymatic steps "a", "b", "c", "f", "g", "e" (FIGS. 1A and 1B). Step "f" containing a "branched-chain keto acid dehydrogenase (EC1.2.4.4). Step "g" containing an "acylating aldehyde dehydrogenase" (EC1.2.1.10) and 1.2.1.57 in addition to step "e" containing the "branched chain alcohol dehydrogenase". The other potential pathway consists of steps "a", "b", "c", "h", "i", "j", "e" (FIGS. 1A and 1B). The term "transaminase" (step "h") EC numbers 2.6.1.42 and 2.6.1.66. Step "h" consists of either a "valine dehydrogenase" (EC1.4.1.8 and EC1.4.1.9) or step "i", a "valine decarboxylase" with an EC number 4.1.1.14. Finally step "j" will use an "omega transaminase" (EC2.6.1. 18) to generate isobutyraldehyde which will be reduced by

step "e" to produce isobutanol. All potential pathways for conversion of pyruvate to isobutanol are depicted in FIGS. 1A and 1B.

[0175] Additionally, a number of microorganisms are known to produce butyrate and/or butanol via a butyryl-CoA intermediate (Dürre, et al., FEMS Microbiol. Rev., 17: 251-262, 1995); and (Abbad-Andaloussi et al., Microbiology, 142: 1149-1158, 1996). Therefore isobutanol production in these microorganisms will take place using steps "k", "g" and "e" shown in FIG. 1B. Step "k" will use an "isobutyryl-CoA mutase" (EC5.4.99.13). The nest step will involve using the "acylating aldehyde dehydrogenase" (EC 1.2.1.10 and EC1. 2.1.57) to produce isobutyraldehyde followed by enzymatic step "e" to produce isobutanol. All these pathways are fully described in the commonly owned patent application Ser. No. 11/586,315, herein incorporated by reference.

[0176] Thus, in providing multiple recombinant pathways from pyruvate to isobutanol, there exist a number of choices to fulfill the individual conversion steps, and the person of skill in the art will be able to use publicly available sequences to construct the relevant pathways.

Microbial Hosts for Isobutanol Production

[0177] Microbial hosts for isobutanol production may be selected from bacteria, cyanobacteria, filamentous fungi and yeasts. The microbial host used for isobutanol production should be tolerant to isobutanol so that the yield is not limited by butanol toxicity. Microbes that are metabolically active at high titer levels of isobutanol are not well known in the art. Although butanol-tolerant mutants have been isolated from solventogenic Clostridia, little information is available concerning the butanol tolerance of other potentially useful bacterial strains. Most of the studies on the comparison of alcohol tolerance in bacteria suggest that butanol is more toxic than ethanol (de Cavalho, et al., Microsc. Res. Tech., 64: 215-22, 2004) and (Kabelitz, et al., FEMS Microbiol. Lett., 220: 223-227, 2003, Tomas, et al., J. Bacteriol., 186: 2006-2018, 2004) report that the yield of 1-butanol during fermentation in Clostridium acetobutylicum may be limited by 1-butanol toxicity. The primary effect of 1-butanol on Clostridium acetobutylicum is disruption of membrane functions (Hermann et al., Appl. Environ. Microbiol., 50: 1238-1243, 1985).

[0178] The microbial hosts selected for the production of isobutanol should be tolerant to isobutanol and should be able to convert carbohydrates to isobutanol. The criteria for selection of suitable microbial hosts include the following: intrinsic tolerance to isobutanol, high rate of glucose utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

[0179] Suitable host strains with a tolerance for isobutanol may be identified by screening based on the intrinsic tolerance of the strain. The intrinsic tolerance of microbes to isobutanol may be measured by determining the concentration of isobutanol that is responsible for 50% inhibition of the growth rate (IC_{50}) when grown in a minimal medium. The IC_{50} values may be determined using methods known in the art. For example, the microbes of interest may be grown in the presence of various amounts of isobutanol and the growth rate monitored by measuring the optical density at 600 nanometers. The doubling time may be calculated from the logarithmic part of the growth curve and used as a measure of the growth rate. The concentration of isobutanol that produces 50% inhibition of growth may be determined from a graph of the percent inhibition of growth versus the isobutanol con-

centration. Preferably, the host strain should have an IC_{50} for isobutanol of greater than about 0.5%.

[0180] The microbial host for isobutanol production should also utilize glucose at a high rate. Most microbes are capable of metabolizing carbohydrates. However, certain environmental microbes cannot metabolize carbohydrates to high efficiency, and therefore would not be suitable hosts.

[0181] The ability to genetically modify the host is essential for the production of any recombinant microorganism. The mode of gene transfer technology may be by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative plasmids and drug resistance markers are available. The cloning vectors are tailored to the host microorganisms based on the nature of antibiotic resistance markers that can function in that host.

[0182] The microbial host also has to be manipulated in order to inactivate competing pathways for carbon flow by deleting various genes. This requires the availability of either transposons to direct inactivation or chromosomal integration vectors. Additionally, the production host should be amenable to chemical mutagenesis so that mutations to improve intrinsic isobutanol tolerance may be obtained.

[0183] Based on the criteria described above, suitable microbial hosts for the production of isobutanol include, but are not limited to, members of the genera Clostridium, Zymomonas, Escherichia, Salmonella, Rhodococcus, Pseudomonas, Bacillus, Vibrio, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, Brevibacterium, Pichia, Candida, Hansenula and Saccharomyces. Preferred hosts include: Escherichia coli, Alcaligenes eutrophus, Bacillus licheniformis, Paenibacillus macerans, Rhodococcus erythropolis, Pseudomonas putida, Lactobacillus plantarum, Enterococcus faecium, Enterococcus faecalis, Bacillus subtilis and Saccharomyces cerevisiae.

Construction of Production Host

[0184] Recombinant microorganisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a fermentable carbon substrate to isobutanol may be constructed using techniques well known in the art. In the present invention, genes encoding the enzymes of one of the isobutanol biosynthetic pathways of the invention, for example, acetolactate synthase, acetohydroxy acid isomeroreductase, acetohydroxy acid dehydratase, branched-chain α -keto acid decarboxylase, and branched-chain alcohol dehydrogenase, may be isolated from various sources, as described above.

[0185] Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard primerdirected amplification methods such as polymerase chain reaction (U.S. Pat. No. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors. Tools for codon optimization for expression in a heterologous host are readily available. Some tools for codon optimization are available based on the GC content of the host microorganism. [0186] Once the relevant pathway genes are identified and isolated they may be transformed into suitable expression hosts by means well known in the art. Vectors or cassettes useful for the transformation of a variety of host cells are common and commercially available from companies such as EPICENTRE® (Madison, Wis.), Invitrogen Corp. (Carlsbad, Calif.), Stratagene (La Jolla, Calif.), and New England Biolabs, Inc. (Beverly, Mass.). Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the specific species chosen as a production host.

[0187] Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements is suitable for the present invention including, but not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, ara, tet, trp, IP_L , IP_R , T7, tac, and trc (useful for expression in *Escherichia coli, Alcaligenes*, and *Pseudomonas*) as well as the amy, apr, npr promoters and various phage promoters useful for expression in *Bacillus subtilis, Bacillus licheniformis*, and *Paenibacillus macerans*.

[0188] Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary, however, it is most preferred if included.

[0189] Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three related vectors-pRK437, pRK442, and pRK442(H) are available. These derivatives have proven to be valuable tools for genetic manipulation in Gram-negative bacteria (Scott et al., Plasmid, 50: 74-79, 2003). Several plasmid derivatives of broad-host-range Inc P4 plasmid RSF1010 are also available with promoters that can function in a range of Gram-negative bacteria. Plasmid pAYC36 and pAYC37, have active promoters along with multiple cloning sites to allow for the heterologous gene expression in Gram-negative bacteria.

[0190] Chromosomal gene replacement tools are also widely available.

[0191] For example, a thermosensitive variant of the broadhost-range replicon pWV101 has been modified to construct a plasmid pVE6002 which can be used to effect gene replacement in a range of Gram-positive bacteria (Maguin et al., J. Bacteriol., 174: 5633-5638, 1992). Additionally, in vitro transposomes are available to create random mutations in a variety of genomes from commercial sources such as EPI-CENTRE®.

[0192] The expression of an isobutanol biosynthetic pathway in various preferred microbial hosts is described in more detail below.

Expression of an Isobutanol Biosynthetic Pathway in E. coli

[0193] Vectors or cassettes useful for the transformation of *E. coli* are common and commercially available from the companies listed above. For example, the genes of an isobu-

tanol biosynthetic pathway may be isolated from various sources, cloned into a modified pUC19 vector and transformed into *E. coli* NM522.

Expression of an Isobutanol Biosynthetic Pathway in *Rhodo-coccus ervthropolis*

[0194] A series of *E. coli-Rhodococcus* shuttle vectors are available for expression in *R. erythropolis*, including, but not limited to, pRhBR17 and pDA71 (Kostichka et al., Appl. Microbiol. Biotechnol., 62: 61-68, 2003). Additionally, a series of promoters are available for heterologous gene expression in *R. erythropolis* (Nakashima et al., Appl. Environ. Microbiol., 70: 5557-5568, 2004 and Tao et al., Appl. Microbiol. Biotechnol., 68: 346-354, 2005). Targeted gene disruption of chromosomal genes in *R. erythropolis* may be created using the method described by Tao et al., supra, and Brans et al. (Appl. Environ. Microbiol., 66: 2029-2036, 2000).

[0195] The heterologous genes required for the production of isobutanol, as described above, may be cloned initially in pDA71 or pRhBR71 and transformed into *E. coli*. The vectors may then be transformed into *R. erythropolis* by electroporation, as described by Kostichka et al., supra. The recombinants may be grown in synthetic medium containing glucose and the production of isobutanol can be followed using methods known in the art.

Expression of an Isobutanol Biosynthetic Pathway in *B. subtilis*

[0196] Methods for gene expression and creation of mutations in *B. subtilis* are also well known in the art. For example, the genes of an isobutanol biosynthetic pathway may be isolated from various sources, cloned into a modified pUC19 vector and transformed into *Bacillus subtilis* BE1010. Additionally, the five genes of an isobutanol biosynthetic pathway can be split into two operons for expression. The three genes of the pathway (bubB, ilvD, and kivD) can be integrated into the chromosome of *Bacillus subtilis* BE1010 (Payne, et al., J. Bacteriol., 173, 2278-2282, 1991). The remaining two genes (ilvC and bdhB) can be cloned into an expression vector and transformed into the *Bacillus* strain carrying the integrated isobutanol genes

Expression of an Isobutanol Biosynthetic Pathway in *B. licheniformis*

[0197] Most of the plasmids and shuttle vectors that replicate in *B. subtilis* may be used to transform *B. licheniformis* by either protoplast transformation or electroporation. The genes required for the production of isobutanol may be cloned in plasmids pBE20 or pBE60 derivatives (Nagarajan et al., Gene, 114: 121-126, 1992). Methods to transform *B. licheniformis* are known in the art (Fleming et al. Appl. Environ. Microbiol., 61: 3775-3780, 1995). The plasmids constructed for expression in *B. subtilis* may be transformed into *B. licheniformis* to produce a recombinant microbial host that produces isobutanol.

Expression of an Isobutanol Biosynthetic Pathway in Paenibacillus macerans

[0198] Plasmids may be constructed as described above for expression in *B. subtilis* and used to transform *Paenibacillus macerans* by protoplast transformation to produce a recombinant microbial host that produces isobutanol.

Expression of the Isobutanol Biosynthetic Pathway in Alcaligenes (Ralstonia) eutrophus

[0199] Methods for gene expression and creation of mutations in *Alcaligenes eutrophus* are known in the art (Taghavi et al., Appl. Environ. Microbiol., 60: 3585-3591, 1994). The genes for an isobutanol biosynthetic pathway may be cloned in any of the broad host range vectors described above, and electroporated to generate recombinants that produce isobutanol. The poly(hydroxybutyrate) pathway in *Alcaligenes* has been described in detail, a variety of genetic techniques to modify the *Alcaligenes eutrophus* genome is known, and those tools can be applied for engineering an isobutanol biosynthetic pathway.

Expression of an Isobutanol Biosynthetic Pathway in *Pseudomonas putida*

[0200] Methods for gene expression in *Pseudomonas putida* are known in the art (see for example Ben-Bassat et al., U.S. Pat. No. 6,586,229, which is incorporated herein by reference). The butanol pathway genes may be inserted into pPCU18 and this ligated DNA may be electroporated into electrocompetent *Pseudomonas putida* DOT-T1 C5aAR1 cells to generate recombinants that produce isobutanol.

Expression of an Isobutanol Biosynthetic Pathway in Saccharomyces cerevisiae

[0201] Methods for gene expression in Saccharomyces cerevisiae are known in the art (e.g., Methods in Enzymology, Volume 194, Guide to Yeast Genetics and Molecular and Cell Biology, Part A, 2004, Christine Guthrie and Gerald R. Fink, eds., Elsevier Academic Press, San Diego, Calif.). Expression of genes in yeast typically requires a promoter, followed by the gene of interest, and a transcriptional terminator. A number of yeast promoters can be used in constructing expression cassettes for genes encoding an isobutanol biosynthetic pathway, including, but not limited to constitutive promoters FBA, GPD, ADH1, and GPM, and the inducible promoters GAL1, GAL10, and CUP1. Suitable transcriptional terminators include, but are not limited to FBAt, GPDt, GPMt, ERG10t, GAL1t, CYC1, and ADH1. For example, suitable promoters, transcriptional terminators, and the genes of an isobutanol biosynthetic pathway may be cloned into E. coliyeast shuttle vectors.

Expression of an Isobutanol Biosynthetic Pathway in Lactobacillus plantarum

[0202] The Lactobacillus genus belongs to the Lactobacillales family and many plasmids and vectors used in the transformation of Bacillus subtilis and Streptococcus may be used for lactobacillus. Non-limiting examples of suitable vectors include pAM_{β1} and derivatives thereof (Renault et al., Gene 183:175-182, 1996); and (O'Sullivan et al., Gene, 137: 227-231, 1993); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al., Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267, 2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Several plasmids from Lactobacillus plantarum have also been reported (van Kranenburg R, et al. Appl. Environ. Microbiol., 71: 1223-1230, 2005).

Expression of an Isobutanol Biosynthetic Pathway in Various *Enterococcus* Species (*E. faecium*, *E. gallinarium*, and *E. faecalis*)

[0203] The *Enterococcus* genus belongs to the Lactobacillales family and many plasmids and vectors used in the transformation of Lactobacilli, Bacilli and Streptococci species may be used for *Enterococcus* species. Non-limiting examples of suitable vectors include pAM β 1 and derivatives thereof (Renault et al., Gene, 183: 175-182, 1996); and (O'Sullivan et al., Gene, 137: 227-231, 1993); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267, 2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Expression vectors for *E. faecalis* using the nisA gene from *Lactococcus* may also be used (Eichenbaum et al., Appl. Environ. Microbiol., 64: 2763-2769, 1998). Additionally, vectors for gene replacement in the *E. faecuum* chromosome may be used (Nallaapareddy et al., Appl. Environ. Microbiol., 72: 334-345, 2006).

Fermentation Media

[0204] Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally the carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. In addition to one and two carbon substrates methylotrophic microorganisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., Microb. Growth C1 Compd., [Int. Symp.], 7th (1993), 415-32. (eds): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of Candida will metabolize alanine or oleic acid (Sulter et al., Arch. Microbiol., 153: 485-489, 1990). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of microorganism.

[0205] Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are glucose, fructose, and sucrose.

[0206] In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for growth of the cultures and promotion of the enzymatic pathway necessary for isobutanol production.

Culture Conditions

[0207] Typically cells are grown at a temperature in the range of about 25° C. to about 40° C. in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Medium (YM) broth. Other defined or synthetic growth media may also be used, and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or

indirectly, e.g., cyclic adenosine 2',3'-monophosphate (cAMP), may also be incorporated into the fermentation medium.

[0208] Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred for the initial condition.

[0209] Fermentations may be performed under aerobic or anaerobic conditions, where anaerobic or microaerobic conditions are preferred.

Industrial Batch and Continuous Fermentations

[0210] The present process employs a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired microorganism or microorganisms, and fermentation is permitted to occur without adding anything to the system. Typically, however, a "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

[0211] A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass., or Deshpande, Mukund (Appl. Biochem. Biotechnol., 36: 227, 1992), herein incorporated by reference.

[0212] Although the present invention is performed in batch mode it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

[0213] Continuous fermentation allows for modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth may be altered continuously while the cell concentration, measured by medium turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to the medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, supra.

[0214] It is contemplated that the present invention may be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isobutanol production. Methods for Isobutanol Isolation from the Fermentation Medium

[0215] The biologically produced isobutanol may be isolated from the fermentation medium using methods known in the art for Acetone-butanol-ethanol (ABE) fermentations (see for example, Durre, Appl. Microbiol. Biotechnol. 49: 639-648, 1998), and (Groot et al., Process. Biochem. 27: 61-75, 1992 and references therein). For example, solids may be removed from the fermentation medium by centrifugation, filtration, decantation and isobutanol may be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

EXAMPLES

[0216] The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

General Methods:

[0217] Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987. Materials and Methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for use in the following Examples may be found in Manual of Methods for General Bacteriology, Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, D.C., 1994, or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, Mass., 1989. All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee,

Wis.), BD Diagnostic Systems (Sparks, Md.), Life Technologies (Rockville, Md.), or Sigma Chemical Company (St. Louis, Mo.), unless otherwise specified.

[0218] The meaning of abbreviations used is as follows: "A" means Angstrom, "min" means minute(s), "h" means hour(s), "µl" means microliter(s), "ng/µl" means nano gram per microliter, "µmol/µl" means pico mole per microliter, "ml" means milliliter(s), "L" means liter(s), "g/L" mean gram per liter, "ng" means nano gram, "sec" means second(s), "ml/min" means milliliter per minute(s), "w/v" means weight per volume, "v/v" means volume per volume, "nm" means nanometer(s), "mm" means millimeter(s), "cm" means centimeter(s), "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" means micromole(s), g" means gram(s), "µg" means microgram(s), "mg" means milligram(s), "g" means the gravitation constant, "rpm" means revolutions per minute, "HPLC" means high performance liquid chromatography, "MS" means mass spectrometry, "HPLC/MS" means high performance liquid chromatography/mass spectrometry, "EDTA" means ethylendiamine-tetraacetic acid, "dNTP" means deoxynucleotide triphosphate, "º C." means degrees Celsius, and "V" means voltage.

[0219] The oligonucleotide primers used in the following Examples have been described herein (see Table 1).

High Throughput Screening Assay of Gene Libraries

[0220] High throughput screening of the gene libraries of mutant KARI enzymes was performed as described herein: $10\times$ freezing medium containing 554.4 g/L glycerol, 68 mM of $(NH_4)_2SO_4$, 4 mM MgSO_4, 17 mM sodium citrate, 132 mM KH_2PO_4, 36 mM K_2HPO_4 was prepared with molecular pure water and filter-sterilized. Freezing medium was prepared by diluting the $10\times$ freezing medium with the LB medium. An aliquot ($200\,\mu$ I) of the freezing medium was used for each well of the 96-well archive plates (cat #3370, Corning Inc. Corning, N.Y.).

[0221] Clones from the LB agar plates were selected and inoculated into the 96-well archive plates containing the freezing medium and grown overnight at 37° C. without shaking. The archive plates were then stored at -80° C. *E. coli* strain Bw25113 transformed with pBAD-HisB (Invitrogen) was always used as the negative control. For libraries C, E, F and G, mutant T52D of (PF5-ilvC) was used as the positive control. The mutant T52D was a mutant of PF5-ilvC in which the threonine at position 52 was changed to aspartic acid. For library H, mutant C3B11 (R47F/S50A/T52D/v53W of PF5-ilvC) was used as the positive control.

[0222] Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0 μ l of cells from thawed archive plates, 300 μ l of the LB medium containing 100 μ g/ml ampicillin and 0.02% (w/v) arabinose as the inducer. Cells were the grown overnight at 37° C. with 80% humidity while shaking (900 rpm), harvested by centrifugation (4000 rpm, 5 min at 25° C.). (Eppendorf centrifuge, Brinkmann Instruments, Inc. Westbury, N.Y.) and the cell pellet was stored at -20° C. for later analysis.

[0223] The assay substrate, (R,S)-acetolactate, was synthesized as described by Aulabaugh and Schloss (Aulabaugh and Schloss, Biochemistry, 29: 2824-2830, 1990): 1.0 g of 2-acetoxy-2-methyl-3-oxobutyric acid ethyl ester (Aldrich, Milwaukee, Wis.) was mixed with 10 ml NaOH (1.0 M) and stirred at room temperature. When the solution's pH became neutral, additional NaOH was slowly added until pH ~8.0 was maintained. All other chemicals used in the assay were purchased from Sigma.

[0224] The enzymatic conversion of acetolactate to α , β dihydroxy-isovalerate by KARI was followed by measuring the disappearance of the cofactor, NADPH or NADH, from the reaction at 340 nm using a plate reader (Molecular Device, Sunnyvale, Calif.). The activity was calculated using the molar extinction coefficient of 6220 M⁻¹ cm⁻¹ for either NADPH or NADH. The stock solutions used were: K₂HPO₄ (0.2 M); KH₂PO₄ (0.2 M); EDTA (0.5 M); MgCl₂ (1.0 M); NADPH (2.0 mM); NADH (2.0 mM) and acetolactate (45 mM). The 100 ml reaction buffer mix stock containing: 4.8 ml K₂HPO₄, 0.2 ml KH₂PO₄, 4.0 ml MgCl₂, 0.1 ml EDTA and 90.9 ml water was prepared.

[0225] Frozen cell pellet in deep-well plates and BugBuster were warmed up at room temperature for 30 min at the same time. Each well of 96-well assay plates was filled with 120 µl of the reaction buffer and 20 µl of NADH (2.0 mM), 150 µl of BugBuster was added to each well after 30 min warm-up and cells were suspended using Genmate (Tecan Systems Inc. San Jose, Calif.) by pipetting the cell suspension up and down (×5). The plates were incubated at room temperature for 20 min and then heated at 60° C. for 10 min. The cell debris and protein precipitates were removed by centrifugation at 4,000 rpm for 5 min at 25° C. An aliquot (50 µl) of the supernatant was transferred into each well of 96-well assay plates, the solution was mixed and the bubbles were removed by centrifugation at 4,000 rpm at 25° C. for 1 min. Absorbance at 340 nm was recorded as background, 20 µl of acetolactate (4.5 mM, diluted with the reaction buffer) was added to each well and mixed with shaking by the plate reader. Absorbance at 340 nm was recoded at 0, and 60 minutes after substrate addition. The difference in absorbance (before and after substrate addition) was used to determine the activity of the mutants. Mutants with higher KARI activity compared to the wild type were selected for re-screening.

[0226] About 5,000 clones were screened for library C and 360 top performers were selected for re-screen. About 92 clones were screened for library E and 16 top performers were selected for re-screening. About 92 clones were screened for library F and 8 top performers were selected for re-screening. About 92 clones were screened for library G and 20 top performers were selected for re-screening. About 92 clones were screened for library H and 62 top performers were selected for re-screening was described below as secondary assay.

Secondary Assay of Active Mutants

[0227] Cells containing pBad-ilvC and its mutants identified by high throughput screening were grown overnight, at 37° C., in 3.0 ml of the LB medium containing 100 µg/ml ampicillin and 0.02% (w/v) arabinose as the inducer while shaking at 250 rpm. The cells were then harvested by centrifugation at 18,000×g for 1 min at room temperature (Sigma micro-centrifuge model 1-15, Laurel, Md.). The cell pellets were re-suspended in 300 µl of BugBuster Master Mix (EMD Chemicals). The reaction mixture was first incubated at room temperature for 20 min and then heated at 60° C. for 10 min. The cell debris and protein precipitate were removed by centrifugation at 18,000×g for 5 min at room temperature.

[0228] The reaction buffer (120 μ l) prepared as described above was mixed with either NADH or NADPH (20 μ l) stock and cell extract (20 μ l) in each well of a 96-well assay plate.

The absorbance at 340 nm at 25° C. was recorded as background. Then 20 µl of acetolactate (4.5 mM, diluted with reaction buffer) was added each well and mixed with shaking by the plate reader. The absorbance at 340 nm at 0 min, 2 min and 5 min after adding acetolactate was recorded. The absorbance difference before and after adding substrate was used to determine the activity of the mutants. The mutants with high activity were selected for sequencing.

[0229] Five top performers from "Library C" were identified and sequenced (FIG. **4**). The best performer was mutant R47F/S50A/T52D/V53W, which completely reversed the nucleotide specificity. The best performers from "Libraries E, F and G" were R47P, S50D and T52D respectively (FIG. **5**). For "Library H", 5 top performers were identified and sequenced (FIG. **6**) and the best performer was R47P/S50G/T52D, which also completely reversed the nucleotide specificity. Enzymes containing activities higher than the background were considered positive.

KARI Enzyme Assay

[0230] KARI enzyme activity can be routinely measured by NADH or NADPH oxidation as described above, however to measure formation of the 2,3-dihydroxyisovalerate product directly, analysis of the reaction was performed using HPLC/MS.

[0231] Protein concentration of crude cell extract from Bugbuster lysed cells (as described above) was measured using the BioRad protein assay reagent (BioRad Laboratories, Inc., Hercules, Calif. 94547). A total of 0.5 micrograms of crude extract protein was added to a reaction buffer consisting of 100 mM HEPES-KOH, pH 7.5, 10 mM MgCl₂, 1 mM glucose-6-phosphate (Sigma-Aldrich), 0.2 Units of Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase (Sigma-Aldrich), and various concentrations of NADH or NADPH, to a volume of 96 µL. The reaction was initiated by the addition of 4 µL of acetolactate to a final concentration of 4 mM and a final volume of 100 μ L. After timed incubations at 30° C., typically between 2 and 15 min, the reaction was quenched by the addition of 10 µL of 0.5 M EDTA, pH 8.0 (Life Technologies, Grand Island, N.Y. 14072). To measure the $K_{\mathcal{M}}$ of NADH, the concentrations used were 0.03, 0.1, 0.3, 1, 3, and 10 mM.

[0232] To analyze for 2,3-dihydroxyisovalerate, the sample was diluted 10x with water, and 8.0 μ l was injected into a Waters Acquity HPLC equipped with Waters SQD mass spectrometer (Waters Corporation, Milford, Mass.). The chromatography conditions were: flow rate (0.5 ml/min), on a Waters Acquity HSS T3 column (2.1 mm diameter, 100 mm length). Buffer A consisted of 0.1% (v/v) in water, Buffer B was 0.1% formic acid in acetonitrile. The sample was analyzed using 1% buffer B (in buffer A) for 1 min, followed by a linear gradient from 1% buffer B at 1 min to 75% buffer B at 1.5 min. The reaction product, 2,3-dihydroxyiso-valerate, was detected by ionization at m/z=133, using the electrospray ionization devise at -30 V cone voltage. The amount of product 2,3-dihydroxyisovalerate was calculated by comparison to an authentic standard.

[0233] To calculate the K_M for NADH, the rate data for DHIV formation was plotted in Kaleidagraph (Synergy Software, Reading, Pa.) and fitted to the single substrate Michaelis-Menton equation, assuming saturating acetolactate concentration.

Example 1

Construction of Site-Saturation Gene Libraries to Identify Mutants Accepting NADH as Cofactor

[0234] Seven gene libraries were constructed (Table 2) using two steps: 1) synthesis of Megaprimers using commer-

cially synthesized oligonucleotides described in Table 1; and 2) construction of mutated genes using the Megaprimers obtained in step 1. These primers were prepared using high fidelity pfu-ultra polymerase (Stratagene, La Jolla, Calif.) for one pair of primer containing one forward and one reverse primer. The templates for libraries C, E, F, G and H were the wild type of PF5_ilvc. The DNA templates for library N were those mutants having detectable NADH activity from library C while those for library O were those mutants having detectable NADH activity from library H. A 50 µl reaction mixture contained: 5.0 µl of 10× reaction buffer supplied with the pfu-ultra polymerase (Stratagene), 1.0 µl of 50 ng/µl template, 1.0 µl each of 10 pmol/µl forward and reverse primers, 1.0 µl of 40 mM dNTP mix (Promega, Madison, Wis.), 1.0 µl pfu-ultra DNA polymerase (Stratagene) and 39 µl water. The mixture was placed in a thin well 200 µl tube for the PCR reaction in a Mastercycler gradient equipment (Brinkmann Instruments, Inc. Westbury, N.Y.). The following conditions were used for the PCR reaction: The starting temperature was 95° C. for 30 sec followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 54° C. for 1 min, and 70° C. for 2 min. At the completion of the temperature cycling, the samples were kept at 70° C. for 4 min more, and then held awaiting sample recovery at 4° C. The PCR product was cleaned up using a DNA cleaning kit (Cat#D4003, Zymo Research, Orange, Calif.) as recommended by the manufacturer.

TABLE 2

		Gene Libraries	
Library name	Templates	Targeted position(s) of Pf5_ilvC	Primers used
с	PF5 ilve	47, 50, 52 and 53	SEQ ID No: 1 and 2
Е	PF5_ilvc	47	SEQ ID No: 1 and 3
F	PF5_ilvc	50	SEQ ID No: 1 and 4
G	PF5_ilvc	52	SEQ ID No: 1 and 5
Η	PF5_ilvc	47, 50, and 52	SEQ ID No: 1 and 6
Ν	Good mutants from library C	53	SEQ ID NO: 20 and 21
0	Good mutants from library H	53	SEQ ID NO: 20 and 21

[0235] The Megaprimers were then used to generate gene libraries using the QuickChange II XL site directed mutagenesis kit (Catalog #200524, Stratagene, La Jolla Calif.). A 50 µl reaction mixture contained: 5.0 µl of 10× reaction buffer, 1.0 µl of 50 ng/µl template, 42 µl Megaprimer, 1.0 µl of 40 mM dNTP mix, 1.0 µl pfu-ultra DNA polymerase. Except for the Megaprimer and the templates, all reagents used here were supplied with the kit indicated above. This reaction mixture was placed in a thin well 200 µl-capacity PCR tube and the following reactions were used for the PCR: The starting temperature was 95° C. for 30 sec followed by 25 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 1 min, and 68° C. for 6 min. At the completion of the temperature cycling, the samples were kept at 68° C. for 8 min more, and then held at 4° C. for later processing. Dpn I restriction enzyme $(1.0 \ \mu l)$ (supplied with the kit above) was directly added to the finished reaction mixture, enzyme digestion was performed at 37° C. for 1 h and the PCR product was cleaned up using a DNA cleaning kit (Zymo Research). The cleaned PCR product (10 µl) contained mutated genes for a gene library.

[0236] The cleaned PCR product was transformed into an electro-competent strain of *E. coli* Bw25113 (Δ ilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on agar plates containing the LB medium and 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Dozens of clones were randomly chosen for DNA sequencing to confirm the quality of the library.

TABLE 3

	List of some mutants having NADH activity identified from saturation libraries			tified from
Mutant	Position 47	Position 50	Position 52	Position 53
SD2	R47Y	S50A	Т52Н	V53W
SB1	R47Y	S50A	T52G	V53W
SE1	R47A	S50W	T52G	V53W
SH2	R47N	S50W	T52N	V53W
SB2	R47I		T52G	V53W
SG1	R47Y		T52G	V53W
SB3	R47G	S50W	T52G	V53W
SE2	R47P	S50E	T52A	V53W
SD3	R47L	S50W	T52G	V53W
C2A6	R47I	S50G	T52D	V53W
C3E11	R47A	S50M	T52D	V53W
C3A7	R47Y	S50A	T52D	V53W
C3B11	R47F	S50A	T52D	V53W
C4A5	R47Y	S50A	T52S	V53W
C3B12	R47I		T52D	V53W
C4H7	R47I		T52S	V53W
C1D3	R47G	S50M	T52D	V53W
C4D12	R47C	S50W	T52G	V53W
C1G7	R47P	S50G	T52D	V53W
C2F6	R47P	S50V	T52D	V53W
C1C4	R47P	S50E	T52S	V53W
6924F9	R47P	S50G	T52D	
6881E1	1 R47P	S50N	T52C	
6868F1	0 R47P		T52S	
6883G1	0 R47P	S50D	T52S	
6939G4	R47P	S50C	T52D	
11463D	98 R47P	S50F	T52D	
9667A1	1 R47N	S50N	T52D	V53A
9675C8	R47Y	S50A	T52D	V53A
9650E5	R47N	S50W	T52G	V53H
9875B9	R47N	S50N	T52D	V53W
9862B9	R47D	S50W	T52G	V53W
9728G1	1 R47N	S50W	T52G	V53W
11461D	8 R47F	S50A	T52D	V53A
11461A	.2 R47P	S50F	T52D	V53I

Example 2

Construction of Error Prone PCR Library

[0237] Mutants obtained in Example 1, with mutations in their cofactor binding sites which exhibited relatively good NADH activities, were used as the DNA template to prepare the error prone (ePCR) libraries using the GeneMorph II kit (Stratagene) as recommended by the manufacturer. All the epPCR libraries target the N-terminal (which contains the NADPH binding site) of PF5_KARI. The forward primer (SEQ ID No: 20) and the reverse primer (SEQ ID No: 22) were used for all ePCR libraries.

[0238] The DNA templates for the n^{th} epPCR library were mutants having good NADH activity from the $(n-1)^{th}$ epPCR library. The templates of the first epPCR library were mutants having relatively good NADH activity from libraries N and O. The mutations rate of library made by this kit was controlled by the amount of template added in the reaction mixture and the number of amplification cycles. Typically, 1.0 ng of each

DNA template was used in 100 µl of reaction mixture. The number of amplification cycles was 70. The following conditions were used for the PCR reaction: The starting temperature was 95° C. for 30 sec followed by 70 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 30 min, and 70° C. for 2 min. After the first 35 heating/cooling cycles finished, more dNTP and Mutazyme II DNA polymerase were added. The PCR product was cleaned up using a DNA cleaning kit (Cat#D4003, Zymo Research, Orange, Calif.) as recommended by the manufacturer. The cleaned PCR product was treated as Megaprimer and introduced into the vector using the Quickchange kit as described in Example 1. Table 4 below lists the KARI mutants obtained and the significant improvement observed in their NADH binding ability. The K_M was reduced from 1100 μ M for mutant C3B11 to 50 µM for mutant 12957G9.

TABLE 4

List of some mutants with their measured K_M values		
Mutant	Mutation Locations	$\begin{array}{c} \text{NADH} \\ \text{K}_{\mathcal{M}}(\mu\text{M}) \end{array}$
C3B11	R47F/S50A/T52D/V53W	1100
SB3	R47G/S50W/T52G/V53W	500
11518B4	R47N/S50N/T52D/V53A/A156V	141
11281G2	R47N/S50N/T52D/V53A/A156V/L165M	130
12985F6	R47Y/S50A/T52D/V53A/L61F/A156V	100
13002D8	R47Y/S50A/T52D/V53A/L61F/A156V/G170A	68
12957G9	Y24F/R47Y/S50A/T52D/V53A/L61F/G170A	50
12978D9	R47Y/S50A/T52D/V53A/L61F/Q115L/A156V	114

Example 3

Identification of Amino Acids for Cofactor Specificity Switching Using Bioinformatic Tools

[0239] To discover if naturally existing KARI sequences could provide clues for amino acid positions that should be targeted for mutagenesis, multiple sequence alignment (MSA) using PF5_KARI, its close homolog PAO1_KARI and three KARI sequences with measureable NADH activity, i.e., B. Cereus ilvC1 and ilvC2 and spinach KARI were performed (FIG. 8). Based on the multiple sequence alignment, positions 33, 43, 59, 61, 71, 80, 101, and 119 were chosen for saturation mutagenesis. Saturation mutagenesis on all of these positions was performed simultaneously using the QuickChange II XL site directed mutagenesis kit (Catalog #200524, Stratagene, La Jolla Calif.) with the manufacturer's suggested protocol. Starting material for this mutagenesis was a mixed template consisting of the mutants already identified in Example 2, Table 4. The primers used are listed in Table 5. The library of mutants thus obtained were named "library Z". Mutants with good NADH activity from this library were identified using high throughput screening and their KARI activity and the K_M for NADH were measured as described above. These mutants (Table 6) possess much lower K_M s for NADH compared to the parent templates (Table 4). A Megaprimer, using primers (SEQ ID Nos. 20 and 58), was created and mutations at positions 156 and 170 were eliminated. Further screening of this set of mutants identified mutant 3361G8 (SEQ ID NO: 67) (Table 7). The hits from library Z were further subjected to saturation mutagenesis at position 53 using primers (SEQ ID Nos. 20 and 21), and subsequent screening identified the remaining mutants in Table 7. As shown in Table 7 the new mutants possessed much lower K_M for NADH (e.g., 4.0 to 5.5 μ M) compared to mutants listed in Table 6 (e.g., 14-40 µM).

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	Primers for Example 5
Targeted position(s) of Pf5_ilvC	Primers
33	pBAD-405-C33_090808f: GCTCAAGCANNKAACCTGAAGG (SEQ ID NO: 49) pBAD-427-C33_090808r: CCTTCAGGTTKNNTGCTTGAGC (SEQ ID NO: 50)
43	pBAD-435-T43_090808f: GTAGACGTGNNKGTTGGCCTG (SEQ ID NO: 51) pBAD-456-T43_090808r: CAGGCCAACKNNCACGTCTAC (SEQ ID NO: 52)
59 and 61	<pre>pBAD-484-H59L61_090808f: CTGAAGCCNNKGGCNNKAAAGTGAC (SEQ ID NO: 53) pBAD-509-H59L61_090808r: GTCACTTTKNNGCCKNNGGCTTCAG (SEQ ID NO: 54)</pre>
71	pBAD-519-A71_090808f: GCAGCCGTTNNKGGTGCCGACT (SEQ ID NO: 55) pBAD-541-A71_090808r: AGTCGGCACCKNNAACGGCTGC (SEQ ID NO: 56)
80	pBAD-545-T80_090808f: CATGATCCTGNNKCCGGACGAG (SEQ ID NO: 57) pBAD-567-T80_090808r: CTCGTCCGGKNNCAGGATCATG (SEQ ID NO: 58)
101	pBAD-608-A101_090808f: CAAGAAGGGCNNKACTCTGGCCT (SEQ ID NO: 59) pBAD-631-A101_090808r: AGGCCAGAGTKNNGCCCTTCTTG (SEQ ID NO: 60)
119	<pre>pBAD-663-R119_090808f: GTTGTGCCTNNKGCCGACCTCG (SEQ ID NO: 61) pBAD-685-R119_090808r: CGAGGTCGGCKNNAGGCACAAC (SEQ ID NO: 62)</pre>

TABLE 6

List of some mutants with their measured K_M values (positions to be mutated in this library were indentified by bioinformatic tools)

Mutant	Mutation Locations	$\begin{array}{l} {\rm NADH} \\ {\rm K}_{\mathcal{M}}(\mu {\rm M}) \end{array}$
ZB1	Y24F/R47Y/S50A/T52D/V53A/L61F/A156V (SEQ ID NO: 24)	40
ZF3	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F (SEQ ID NO: 25)	21
ZF2	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/A156V (SEQ ID NO: 26)	17
ZB3	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/G170A (SEQ ID NO: 27)	17
Z4B8	C33L/R47Y/S50A/T52D/V53A/L61F/T80I/A156V (SEQ ID NO: 28)	14

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Mutant	Mutants further optimized for improved K_M (for NA Mutation Locations	NADH) NADH K _m (µM)
3361G8	C33L/R47Y/S50A/T52D/V53A/L61F/T80I	5.5
2H10	(SEQ ID NO: 67) Y24F/C33L/R47Y/S50A/T52D/V53I/L61F/T80I/ A156V (SEQ ID NO: 68)	5.3

TABLE 7-continued

	Mutants further optimized for improved K_M (for NADH)		
Mutant	Mutation Locations	NADH K _m (µM)	
1D2	Y24F/R47Y/S50A/T52D/V53A/L61F/T80I/ A156V (SEQ ID NO: 69)	4.1	
3F12	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/T80I/ A156V (SEQ ID NO: 70)	4.0	
3361E1	Y24F/R47Y/S50A/T52D/V53I/L61F (SEQ ID NO: 84)	4.5	

[0240] Further analyses using bioinformatic tools were therefore performed to expand the mutational sites to other KARI sequences as described below.

Sequence Analysis

[0241] Members of the protein family of ketol-acid reducoisomorase (KARI) were identified through BlastP searches of publicly available databases using amino acid sequence of *Pseudomonas fluorescens* PF5 KARI (SEQ ID NO:17) with the following search parameters: E value=10, word size=3, Matrix=Blosum62, and Gap opening=11 and gap extension=1, E value cutoff of 10^{-3} . Identical sequences and sequences that were shorter than 260 amino acids were removed. In addition, sequences that lack the typical GxGXX

(G/A) motif involved in the binding of NAD(P)H in the N-terminal domain were also removed. These analyses resulted in a set of 692 KARI sequences.

[0242] A profile HMM was generated from the set of the experimentally verified Class I and Class II KARI enzymes from various sources as described in Table 8. Details on building, calibrating, and searching with this profile HMM are provided below. Any sequence that can be retrieved by HMM search using the profile HMM for KARI at E-value above $1E^{-3}$ is considered a member of the KARI family. Positions in a KARI sequence aligned to the following in the profile HMM nodes (defined below in the section of profile HMM building) are claimed to be responsible for NADH utilization: 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170 (the numbering is based on the sequences of *Pseudomonas fluorescens* PF5 KARI).

Preparation of Profile HMM

[0243] A group of KARI sequences were expressed in *E. coli* and have been verified to have KARI activity These KARIs are listed in Table 6. The amino acid sequences of these experimentally verified functional KARIs were analyzed using the HMMER software package (The theory behind profile HMMs is described in R. Durbin, S. Eddy, A.

Krogh, and G. Mitchison, Biological sequence analysis: probabilistic models of proteins and nucleic acids, Cambridge University Press, 1998; Krogh et al., J. Mol. Biol. 235:1501-1531, 1994), following the user guide which is available from HMMER (Janelia Farm Research Campus, Ashburn, Va.). The output of the HMMER software program is a profile Hidden Markov Model (profile HMM) that characterizes the input sequences. As stated in the user guide, profile HMMs are statistical descriptions of the consensus of a multiple sequence alignment. They use position-specific scores for amino acids (or nucleotides) and position specific scores for opening and extending an insertion or deletion. Compared to other profile based methods, HMMs have a formal probabilistic basis. Profile HMMs for a large number of protein families are publicly available in the PFAM database (Janelia Farm Research Campus, Ashburn, Va.).

[0244] The profile HMM was built as follows:

Step 1. Build a Sequence Alignment

[0245] The 25 sequences for the functionally verified KARIs listed above were aligned using Clustal W (Thompson, J. D., Higgins, D. G., and Gibson T. J., Nuc. Acid Res. 22: 4673 4680, 1994) with default parameters. The alignment is shown in FIG. **9**.

TABLE	8
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	25 Exp	erimentally ver	rified KARI enzymes
GI Number	Accession	SEQ ID NO:	Microorganism
70732562	YP_262325.1	17	Pseudomonas fluorescens Pf-5
15897495	NP_342100.1	13	Sulfolobus solfataricus P2
18313972	NP_560639.1	14	Pyrobaculum aerophilum str. IM2
76801743	YP_326751.1	30	Natronomonas pharaonis DSM 2160
16079881	NP_390707.1	31	Bacillus subtilis subsp. subtilis str. 168
19552493	NP_600495.1	32	Corynebacterium glutamicum ATCC 13032
6225553	O32414	33	Phaeospririlum molischianum
17546794	NP_520196.1	15	Ralstonia solanacearum GMI1000
56552037	YP_162876.1	34	Zymomonas mobilis subsp. mobilis ZM4
114319705	YP_741388.1	35	Alkalilimnicola ehrlichei MLHE-1
57240359	ZP_00368308.1	36	Campylobacter lari RM2100
120553816	YP_958167.1	37	Marinobacter aquaeolei VT8
71065099	YP_263826.1	38	Psychrobacter arcticus 273-4
83648555	YP_436990.1	39	Hahella chejuensis KCTC 2396
74318007	YP_315747.1	40	Thiobacillus denitrificans ATCC 25259
67159493	ZP_00420011.1	41	Azotobacter vinelandii AvOP
66044103	YP_233944.1	42	Pseudomonas syringae pv. syringae B728a
28868203	NP_790822.1	43	Pseudomonas syringae pv. tomato str.
			DC3000
26991362	NP_746787.1	44	Pseudomonas putida KT2440
104783656	 YP_610154.1	45	Pseudomonas entomophila L48
146306044	 YP_001186509.1	46	Pseudomonas mendocina ymp
15599888		16	Pseudomonas aeruginosa PAO1
42780593	NP_977840.1	47	Bacillus cereus ATCC 10987
42781005	NP 978252.1	48	Bacillus cereus ATCC 10987
266346	Q01292	18	Spinacia oleracea

Step 2. Build a Profile HMM

[0246] The hmmbuild program was run on the set of aligned sequences using default parameters. hmmbuild reads the multiple sequence alignment file, builds a new profile HMM, and saves the profile HMM to file. Using this program an un-calibrated profile was generated from the multiple sequence alignment for twenty-four experimentally verified KARIs as described above.

[0247] The following information based on the HMMER software user guide gives some description of the way that the hmmbuild program prepares a profile HMM. A profile HMM is a linear state machine consisting of a series of nodes, each of which corresponds roughly to a position (column) in the multiple sequence alignment from which it is built. If gaps are ignored, the correspondence is exact, i.e., the profile HMM has a node for each column in the alignment, and each node can exist in one state, a match state. The word "match" here implies that there is a position in the model for every position in the sequence to be aligned to the model. Gaps are modeled using insertion (I) states and deletion (D) states. All columns that contain more than a certain fraction x of gap characters will be assigned as an insert column. By default, x is set to 0.5. Each match state has an I and a D state associated with it. HMMER calls a group of three states (M/D/I) at the same consensus position in the alignment a "node"

[0248] A profile HMM has several types of probabilities associated with it. One type is the transition probability—the probability of transitioning from one state to another. There are also emissions probabilities associated with each match state, based on the probability of a given residue existing at that position in the alignment. For example, for a fairly well-conserved column in an alignment, the emissions probability for the most common amino acid may be 0.81, while for each of the other 19 amino acids it may be 0.01.

[0249] A profile HMM is completely described in a HMMER2 profile save file, which contains all the probabilities that are used to parameterize the HMM. The emission probabilities of a match state or an insert state are stored as log-odds ratio relative to a null model: $\log_2 (p_x)/(null_x)$. Where p_x is the probability of an amino acid residue, at a particular position in the alignment, according to the profile HMM and null_x is the probability according to the Null model. The Null model is a simple one state probabilistic model with pre-calculated set of emission probabilities for each of the 20 amino acids derived from the distribution of amino acids in the SWISSPROT release 24. State transition scores are also stored as log odds parameters and are proportional to $\log_2(t_x)$. Where t_x is the transition probability of transiting from one state to another state.

Step 3. Calibrate the Profile HMM

[0250] The profile HMM was read using hmmcalibrate which scores a large number of synthesized random sequences with the profile (the default number of synthetic sequences used is 5,000), fits an extreme value distribution (EVD) to the histogram of those scores, and re-saves the HMM file now including the EVD parameters. These EVD parameters (μ and λ) are used to calculate the E-values of bit scores when the profile is searched against a protein sequence database. Hmmcalibrate writes two parameters into the HMM file on a line labeled "EVD": these parameters are the μ (location) and λ (scale) parameters of an extreme value distribution (EVD) that best fits a histogram of scores calcu-

lated on randomly generated sequences of about the same length and residue composition as SWISS-PROT. This calibration was done once for the profile HMM.

[0251] The calibrated profile HMM for the set of KARI sequences is provided appended hereto as a profile HMM Excel chart (Table 9). In the main model section starting from the HMM flag line, the model has three lines per node, for M nodes (where M is the number of match states, as given by the LENG line). The first line reports the match emission logodds scores: the log-odds ratio of emitting each amino acid from that state and from the Null model. The first number if the node number (1 . . . M). The next K numbers for match emission scores, one per amino acid. The highest scoring amino acid is indicated in the parenthesis after the node number. These log-odds scores can be converted back to HMM probabilities using the null model probability. The last number on the line represents the alignment column index for this match state. The second line reports the insert emission scores, and the third line reports on state transition scores: $M \rightarrow M, M \rightarrow I, M \rightarrow D; I \rightarrow M, I \rightarrow I; D \rightarrow M, D \rightarrow D; B \rightarrow M;$ M→E.

Step 4. Test the Specificity and Sensitivity of the Built Profile HMMs

[0252] The Profile HMM was evaluated using hmmsearch, which reads a Profile HMM from hmmfile and searches a sequence file for significantly similar sequence matches. The sequence file searched contained 692 sequences (see above). During the search, the size of the database (Z parameter) was set to 1 billion. This size setting ensures that significant E-values against the current database will remain significant in the foreseeable future. The E-value cutoff was set at 10.

[0253] An hmmersearch, using hmmsearch, with the profile HMM generated from the alignment of the twenty-five KARIs with experimentally verified function, matched all 692 sequences with an E value $<10^{-3}$. This result indicates that members of the KARI family share significant sequence similarity. A hmmersearch with a cutoff of E value 10^{-3} was used to separate KARIs from other proteins.

Step 5. Identify Positions that are Relevant for NAD(P)H Utilization.

[0254] Eleven positions have been identified in KARI of *Pseudomonas fluorescens* Pf-5 that switches the cofactor from NADPH to NADH. Since the KARI sequences share significant sequence similarity (as described above), it can be reasoned that the homologous positions in the alignment of KARI sequences should contribute to the same functional specificity. The profile HMM for KARI enzymes has been generated from the multiple sequence alignment which contains the sequence of *Pseudomonas fluorescens* Pf-5 KARI. The eleven positions in the profile HMM representing the columns in the alignment which correspond to the eleven cofactor switching positions in *Pseudomonas fluorescens* Pf-5 KARI are identified as positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170. The lines corresponding to these positions in the model file are highlighted in yellow in Table 0

[0255] For any query sequence, hmm search is used to search the profile HMM for KARI against the query sequence and the alignment of the query to the HMM is recorded in the output file. In the alignment section of the output, the top line is the HMM consensus. The amino acid shown for the consensus is the highest probability amino acid at that position according to the HMM (not necessarily the highest scoring

amino acid). The center line shows letters for "exact" matches to the highest probability residue in the HMM, or a "+" when the match has a positive score. The third line shows the sequence itself. The positions in the query sequence that are deemed as relevant for cofactor switching are identified as those that are aligned to these eleven nodes in the profile HMM as described above. An example of the alignment of *Pseudomonas fluorescens* Pf-5 KARI to the profile HMM of KARI is shown in FIG. **10** and the eleven positions that are responsible for cofactor switching are shaded in grey.

Example 4

Construction of a Site-Saturation Gene Library for Complete Cofactor Switching to NADH

[0256] To construct the site-saturation gene library for KARI mutants, mutants 3361E1, 3361G8, 1D2, 2H10, 3F12, & Z4B8 (see Example 3, Tables 6 and 7) were used as templates. The library was constructed using QuickChange kit (Cat#200524, Stratagene, La Jolla, Calif.). The concentration of each mutant in the template mixture was 5.0 ng/ μ l. The two primers (2.5 nM) introducing saturation mutagenesis at positions 47, 50, 52 and 53, were PF5_4Mt111008.f (SEQ ID NO: 71) and PF5_4Mt111008.r (SEQ ID NO: 72).

The PCR Reaction Mixture Contained:

[0257]

$10 \times \text{reaction buffer}$	5.0 µl
PF5_4Mt111008.f	2.0 µl
PF5_4Mt111008.r	2.0 µl
50 x dNTP	1.0 µl
DNA Template	1.0 µl
PfuUltra	1.0 µl
Water	38 µl

The	PCR	Reaction	Program	was:
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[0258]

1)	95° C.	30 sec	
2)	95° C.	30 sec	
3)	55° C.	1.0 min	
4)	68° C.	6.0 min	
5)	Go to step (2)	Repeat 35 times	
6)	68° C.	8.0 min	
7)	4° C.	press Enter	
,			

[0259] The mixture was placed in a thin well 200 µl tube for the PCR reaction in a Mastercycler gradient equipment (Brinkmann Instruments, Inc. Westbury, N.Y.). After the PCR reaction, $1.0 \,\mu$ l Dpn I restriction enzyme (supplied with the kit above) was directly added into the PCR reaction mixture, which was then incubated at 37° C. for 1 h to remove the DNA templates. The Dpn I digested PCR product was cleaned up by the Zymo DNA clearance kit (Cat#D4003, Zymo Research, Orange, Calif.) as recommended by the manufacturer.

[0260] The cleaned PCR product was transformed into an electro-competent strain of *E. coli* Bw25113 (Δ ilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on agar plates

containing the LB medium and 100 μ g/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Dozens of clones were randomly chosen for DNA sequencing to confirm the quality of the library. Several mutants identified in this library (Table 10 and FIG. 11) had very low NADPH activity while they had good NADH activity. Their cofactor consumption is listed in Table 11 (The data was based on three parallel measurements). "Negative" in the following Tables refers to an empty pBAD vector without the KARI gene.

TABLE 10

	List of some of the mutants identified in Example 1
Mutant	Mutation Locations
JB1C6 16445E4 16468D7 16469F3	Y24F/C33L/R47H/S50D/T52Y/V53Y/L61F/T80I/A156V C33L/R47P/S50V/T52D/V53G/L61F/T80I/A156V Y24F/C33L/R47T/S50I/T52D/V53R/L61F/T80I/A156V C33L/R47E/S50A/T52D/V53A/L61F/T80I

TABLE 11

The cofactor consumption of some mutants following	a 5 min
reaction (decrease in OD ₃₄₀ nm)	

	0.2 mM	NADH	0.2 mM NADPH		
Mutants	average	stdev	average	stdev	
JB1C6	-0.232	0.127	-0.019	0.009	
16445E4	-0.152	0.057	-0.013	0.001	
16468D7	-0.153	0.012	-0.039	0.020	
16469F3	-0.054	0.069	-0.025	0.016	
Z4B8	-0.178	0.042	-0.170	0.013	
PF5_WT	-0.078	0.014	-0.320	0.024	
Negative	-0.061	0.029	-0.015	0.014	

Example 5

Construction of a Domain Swapping Library

[0261] In this Example the beneficial mutations outside the cofactor binding sites and the beneficial mutations within the cofactor binding sites were combined to create a domain swapping library.

[0262] Mutants, which had mutations in the cofactor binding site and exhibited only NADH activity (SE1, SB3, SE2, SD3, C2F6, C3B11, C4D12, 9650E5, 9667A11, 9862B9, 9875B9, 11461D8, 11463D8, 11518B4, SEQ ID NOs: 85-98), were used to obtain additional beneficial mutations in the cofactor binding site. Two primers, pBAD_230f (SEQ ID NO: 73) and pBAD_601_021308r (SEQ ID NO: 74), were used to amplify the mutants listed in Table 12. PCR reagents used were from Invitrogen (Cat#10572-014, Invitrogen, Carlsbad, Calif.).

The PCR Reaction Mixture Contained:

[0263]

PCR SuperMix pBAD_230.f (18 nM)

-continued

	pBAD_601_021308r (10 nM) Template mix (5.0 ng/µl)	9.0 µl 6.0 µl
--	--	------------------

The PCR Reaction Program was:

[0264]

(1)	95° C.	30 sec	
(2)	95° C.	20 sec	
(3)	55° C.	20 sec	
(4)	72° C.	60 sec	
(5)	Go to step (2)	repeat 35 times	
(6)	72° C.	4 min	
(7)	4° C.	press enter	

[0265] After the PCR reaction, 1.0 μ l Dpn I restriction enzyme (supplied with the kit above) was directly added into the PCR reaction mixture, which was then incubated at 37° C. for 1 h to remove the DNA templates. The Dpn I digested PCR product was cleaned up by the Zymo DNA clearance kit (Cat#D4003, Zymo Research, Orange, Calif.) as recommended by the manufacturer and 42 μ l cleaned DNA product containing beneficial mutations in the cofactor binding sites obtained was designated as Megaprimer.

[0266] The Megaprimers thus obtained were then used to generate the domain swapping library using the Quick-Change II XL site directed mutagenesis kit (Catalog #200524, Stratagene, La Jolla Calif.). The templates used in Example 4 were also used in this experiment. A 50 µl reaction mixture containing: $5.0 \,\mu$ l of $10 \times$ reaction buffer, $1.0 \,\mu$ l of 5.0ng/µl template, 42 µl Megaprimer, 1.0 µl of 40 mM dNTP mix, 1.0 µl pfu-ultra DNA polymerase was prepared. Except for the Megaprimer and the templates, all reagents used here were supplied with the purchased kit. This reaction mixture was placed in a thin well 200 µl-capacity PCR tube and the following reactions were used for the PCR. The starting temperature was 95° C. for 30 sec followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 1 min, and 68° C. for 6 min. At the completion of the temperature cycling, the samples were kept at 68° C. for 8 min, and then stored at 4° C. for later processing. Dpn I restriction enzyme $(1.0 \ \mu l)$ (supplied with the kit above) was directly added to the finished reaction mixture, enzyme digestion was performed at 37° C. for 1 h and the PCR product was cleaned up using a DNA cleaning kit (Zymo Research). The cleaned PCR product (10 µl) contained mutated genes for a gene library.

[0267] The mutated genes were transformed into an electro-competent strain of *E. coli* Bw25113 (Δ ilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on LB agar plates containing 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Dozens of clones were randomly chosen for DNA sequencing to confirm the quality of the library.

[0268] This library yielded many mutants with high NADH activity (low K_M for NADH), which also had very low NADPH activity. (Table 12 and FIG. **12**). Their cofactor consumption is also shown in Table 13 (The data was based on three parallel measurements).

TABLE 12

swapping library						
Mutant	Mutation Locations	NADH K _M (μM)				
JEA1	Y24F/C33L/R47P/S50F/T52D/L61F/T80I/A156V	9.1				
JEG2	Y24F/C33L/R47F/S50A/T52D/V53A/L61F/T80I/ A156V	9.4				
JEG4	Y24F/C33L/R47N/S50N/T52D/V53A/L61F/T80I/ A156V	9.6				
JEA7	Y24F/C33L/R47P/S50N/T52D/V53A/L61F/T80I/ A156V	10.6				
JED1	C33L/R47N/S50N/T52D/V53A/L61F/T80I/ A156V	11.0				

TABLE 13

	0.2 mM	NADH	0.2 n NAD	
Mutants	average	stdev	average	stdev
JEA1	-0.285	0.030	-0.110	0.025
JED1	-0.287	0.032	-0.074	0.014
JEG2	-0.261	0.009	-0.078	0.009
JEG4	-0.227	0.016	-0.050	0.016
JEA7	-0.205	0.079	-0.038	0.009
Z4B8	-0.178	0.042	-0.170	0.013
PF5 WT	-0.078	0.014	-0.320	0.024
Negative	-0.061	0.029	-0.015	0.014

Example 6

Thermostability of PF5-ILVC and its Mutants

[0269] The wildtype PF5-ILVC and various cells containing mutated pBad-ilvC were grown overnight at 37° C. in 25 ml of the LB medium containing 100 µg/ml ampicillin and 0.02% (w/v) arabinose inducer while shaking at 250 rpm. The cells were then harvested by centrifugation at $18,000 \times g$ for 1 min at room temperature and the cell pellets were re-suspended in 300 µl of BugBuster Master Mix (EMD Chemicals). The reaction mixture was first incubated at room temperature for 20 min and aliquots of this cell mixture (e.g. 50 µl) were incubated at different temperatures (from room temperature to 75° C.) for 10 min. The precipitate was removed by centrifugation at 18,000×g for 5 min at room temperature. The remaining activity of the supernatant was analyzed as described above. As shown in FIG. 7, pBad-ilvC was very stable with T_{50} at 68° C. (T_{50} is the temperature, at which 50% of protein lost its activity after 10 min incubation).

[0270] The thermostability of PF5-ilvC allowed destruction of most of the other non-KARI NADH oxidation activity within these cells, reducing the NADH background consumption and thus facilitating the KARI activity assays. This heat treatment protocol was used in all screening and re-screening assays. The mutants thus obtained were all thermostable which allowed easier selection of the desirable mutants.

Example 7

Stoichiometric Production of 2,3-Dihydroxyisovalerate by KARI During Consumption of NADH or NADPH as Cofactors

[0271] Screening and routine assays of KARI activity rely on the 340 nm absorption decrease associated with oxidation of the pyridine nucleotides NADPH or NADH. To insure that this metric was coupled to the formation of the reaction product (i.e., 2,3-dihydroxyisovalerate), oxidation of both pyridine nucleotide and formation of 2,3-dihydroxyisovalerate were measured in the same samples.

[0272] The oxidation of NADH or NADPH was measured at 340 nm in a 1 cm path length cuvette on a Agilent model 8453 spectrophotometer (Agilent Technologies, Wilmington Del.). Crude cell extract (0.1 ml) prepared as described above containing either wild type PF5 KARI or the C3B11 mutant, was added to 0.9 ml of K-phosphate buffer (10 mM, pH 7.6), containing 10 mM MgCl₂, and 0.2 mM of either NADPH or NADH. The reaction was initiated by the addition of aceto-lactate to a final concentration of 0.4 mM. After 10-20%

decrease in the absorption (about 5 min), 50 μ l of the reaction mixture was rapidly withdrawn and added to a 1.5 ml Eppendorf tube containing 10 μ l 0.5 mM EDTA to stop the reaction and the actual absorption decrease for each sample was accurately recorded. Production of 2,3-dihydroxyisovalerate was measured and quantitated by HPLC/MS as described above. **[0273]** The coupling ratio is defined by the ratio between the amount of 2,3-dihydroxyisovalerate (DHIV) produced and the amount of either NADH or NADPH consumed during the experiment. The coupling ratio for the wild type enzyme (PF5-ilvC), using NADPH, was 0.98 DHIV/NADPH, while that for the mutant (C3B11), using NADH, was on average around 1.10 DHIV/NADPH underlining the high activity of the mutant enzyme to consume NADH and produce DHIV.

	LABLE 9
HMMER2.0 [2.2g]	File format version: a unique identifier for this save file format.
NAME Functionally Verified KARIs	Name of the profile HMM
LENG 334 AT DIT A minim	Mode length: the number of match states in the model. Sumbal algorithms of determinant the analysis of algorithms and the class of the analysis
ALFH AIIIII0	symbol alphapet: this determines the symbol alphapet and the size of the symbol emission probability distributions. Amino, the alphabet size
	Is set to 20 and the symbol alphabet to "ACDEFGHIKLMNPQRSTVWY" (alphabetic order).
MAP yes	Map annotation flag: If set to yes, each line of data for the match state/consensus column
	in the main section of the file is followed by an extra
	number. This number gives the index of the alignment column that the match state was
	made from. This information provides a "map" of the
	match states (1M) onto the columns of the alignment (1. alen). It is used for
	quickly aligning the model back to the original alignment, e.g. when using hmmalign-mapali.
COM hmmbuild-n Functionally Verified KARIs exp-KARI.hmm exp-KARI_mod.aln	Command line for every HMMER command that modifies the save file:
	This one means that hmmbuild (default patrameters) was applied to
	generate the save file.
COM hmmcalibrate exp-KARI.hmm	Command line for every HMMER command that modifies the save file:
	This one means that hmmcalibrate (default parametrs) was applied to the save profile.
NSEQ 25	Sequence number: the number of sequences the HMM was trained on
DATE Mon Dec. 8 17:34:51 2008	Creation date: When was the save file was generated.
$\rm XT$ -8455-4-1000-1000-8455-4-8455-4	Eight "special" transitions for controlling parts of the algorithm-specific parts of the
	Plan7 model. The null probability used to convert these
	back to model probabilities is 1.0.
	The order of the eight field is N->B, N->N, E->C, E->I, C->T, C->C, J->B, J->I.
NULT-4-8455	The transition probability distribution for the null model (single G state).
NULE 595-1558 85336-294-453-1158 197 249 902-1085-142-21-313 45 531 201 384-1998-644 EVID 333 71 7708 0 110102	The extreme value distribution parameters μ and lambda respectively; both floating point values. These values are set when the model is collibrated with humoolibrate
	ллех чанов ад хак милен цилоса на саполахся мни пиписациях. Т Пак это посай то distermine E-volues of bit contracts

Position in alignment	7100%	7200%	%0096	8700%	8800%	%0006	9100%	9200%	9300%	9400%
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M	-1542 -294	-4103 -296	305 -294	-2038 -294	-774 -295	1320 -294	-2577 -294	-3962 -294	-581 -294	-2009 -294
>	-1239 -369	-3529 -368	-389 -369	-3	-50 -369	1117 -369	-2040 -369	3023 -369	-3643 -369	-2436 -369
H	-684 117	-4459 121	$-1350 \\ 117$	640 117	-338 118	476 117	224 117	-82 117	-4533 117	1039 117
s	-643 359	-4692 361	-1617 359	-488 369	-631 359	1715 359	829 359	-4080 359	-4313 359	-2258 359
м	122 96	-4823 95	-1798 96	-383 96	-883 96	-1358 96	458 95	-4628 96	-4458 96	-1078 96
ð	3263 46	-4977 44	-1503 45	154 45	-451 45	-1013 45	1146 45	-4417 45	-3835 45	-1513 45
Ч	-1495 394	-4790 396	-2278 394	-1658 394	-1705 394	-1964 394	-2010 394	-4600 394	-4920 394	-3206 394
z	-227 275	-5052 276	-1626 275	-252 275	-731 275	-1258 275	57 275	-4442 275	-3726 275	-2051 275
Μ	-911 -720	5320 -722	66 -720	-911 -720	125 -721	19 -720	-1502 -720	-1318 -720	-2838 -720	-2098 -720
	-1417 -466	-2613 -467	64 -466	-1765 -466	-482 -466	-584 -466	-2420 -466	-151 -466	-392 -466	-2674 -466
K m->e	-321 210 *	-5113 209 *	-1891 210 *	937 210 *	-624 210 *	-1204 210 *	2435 210 *	-4574 210 *	-5065 210 *	906 210 *
I b- >m	-1455 -626 -650	-3232 -625 *	-196 -626 *	-1686 -626 *	-167 -626 *	1279 -626 *	-2483 -626 *	2241 -626 *	-3424 -626 *	-2628 -626 *
р < -р Н	-219 105 -1378	-4528 104 -1378	-244 106 -1060	-262 106 -314	-384 106 -444	-954 106 -3378	-558 106 -1378	-4391 106 -1378	-1332 105 -1378	-1481 106 -1378
G d- >m	-1166 399 -701	-4370 397 -701	-2093 399 -943	-1540 399 -2352	-1540 398 -1916	-1740 399 -146	-1919 399 -701	-4789 399 -701	-5069 399 -701	-2988 399 -701
F i->i	-1453 -381 -1115	-3438 -382 -136	3518 -381 -1115	-2015 -381 -1115	2092 -381 -3527	-821 -381 -1115	-2743 -381 -1115	-2534 -381 -1115	2423 -381 -1115	-1555 -1115
E - >m	-44 43 -894	-5402 42 -3473	-2120 43 -894	33 43 -894	-712 43 -131	-1415 43 -894	501 43 -894	-4702 43 -894	-5505 43 -894	-2097 43 -894
D m->d	-1463 -136 -136 -6882	-5216 232 -325	-2227 233 -6882	1125 233 -1125	1084 235 -7567	-1937 233 -7995	-803 233 -9181	-5089 -533 -9181	-5210 233 -9181	-2489 -233 -0181
C m->i	* -1356 -500 -5840	-3929 -501 -3318	-1104 -500 -5840	-1744 -500 -7402	2578 -500 -1006	-586 -500 -6953	-2411 -500 -8139	-2010 -500 -8139	-3685 -500 -8139	-2625 -500 -8139
A m- >m	-650 -648 -149 -38	-4231 -147 -3303	-1308 -149 -38	1616 -149 -901	-346 -149 -1009	800 -149 -17	-956 -149 -8	-2472 -149 -8	-4673 -149 -8	-2170 -149 -8
MMH		2(M)	3(F) —	4(A)			7(K)	8(V) 	9(Y) 	10(Y)

	9500%	9600%	9/00/6	9800%	%0066	10000%	10100%	10200%	10300%	10400%
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	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
	-8	-8	-8	-8	-8	-8	-2336	-38	-38	-38
	11(D)	12(K)	13(D)	14(C)	15(D)	16(L)	17(S)	18(G)	19(H)	20(D)
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	10500%	10600%	0700%	%00%	%00601	11000%	12600%	.2700%	12800%	12900%
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	45	-1111 - 45	-3538 -	-4454 - 45 -	2257 45	-1- 44	- 1076 - 45	1301 45	-4890 - 46	-3694 - 45
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LABLE 9-continued	-1919	-769	1990	1593	-2619	-3937	-3617	-2865	-1532	-4428
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	-626	-626	-626	-626	-626	-625	-626	-626	-626	-626
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	-293 106 -179	$\begin{array}{c} 121\\ 106\\ -3775 \end{array}$	-3320 106 -1378	-4849 106 -1378	-719 108 -1378	-1580 104 -1378	-1490 106 -1378	-923 106 -1378	-5131 106 -1378	-3657 106 -1378
	-1029	1957	-4227	-5164	-2141	2903	-3647	-2535	-5101	656
	399	399	399	399	399	399	399	399	399	399
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	-38	-38	-8	-8	-8	-155	-8	-8	-8	-8
	21(E)	22(Y)	23(I)	24(I)	25(K)	26(G)	27(K)	28(K)	29(V)	30(A)
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-1886 -626 *	-3116 -626 *	-5082 -626 * -4365	┟┼┥┝┼┼┥	-36 -626 *	-3350 -626 *	1415 -626 *	2623 -626 *	-4486 -626 *	-543 -626 *	-3905
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	236 117	$141 \\ 117$	-1368 117	-2700 117	-2509	687 119	-767 117	$-172 \\ 117$	$\frac{1827}{117}$	945 117	-829 117	-655 117	931 117	-58 117
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	446 43 -894	432 43 -894	-4279 43 -894	396 43 -894	-4951 43 -894	-4970 43 -894	-2708 43 -894	-4470 43 -894	-5628 43 -894	-3262 -43 -894	-3710 43 -894	-3014 43 -894	2715 43 -894	-14 -34 -894
	-959 233 -9181	815 233 -9181	-3998 233 -9181	3813 233 -9181	-5338 233 -9181	-5306 233 -9181	-3317 233 -9181	-5017 233 -9181	-5638 233 -9181	-3846 -333 -9181	-3396 233 -9181	4174 233 -9181	-983 233 -9181	-2012 233 -9181
	-2501 -500 -8139	-2347 -500 -8139	-1860 -500 -8139	-4795 -500 -8139	-2220 -500 -8139	-2129 -500 -8139	-1208 -500 -8139	-2177 -500 -8139	-3800 -500 -8139	-1286 -500 -8139	-2214 -500 -8139	-4701 -500 -8139	-2199 -500 -8139	-1137 -500 -8139
	1714 -149 -8	265 -149 -8	3391 -149 -8	-2747 -149 -8	-2717 -149 -8	-2635 -149 -8	-1340 -149 -8	-2566 -149 -8	-4414 -149 -8	1212 -149 -8	-1614 -149 -8	-4580 -149 -8	-1123 -149 -8	399 -149 -8
	81(K) 	82(W) 	83(A) 	84(D) 	85(V) 	86(V) 	87(M) —	88(I) 	89(L)	(1)06	91(P) 	92(D) 	93(E) 	94(H) —

1000000	20600%		20700%			20800%			20900%			21000%	0.000 **		2110007	0/00117]	21200%			21300%			21400%		21500%			21600%	
1	-2/11		-1883	-249		-1852	-249		-2442	-249		4377	-249		1011	070-	2 4		-2207	-249		-1917	-249		-3814	647-	-1862	-249		-2693	-249
- I +	- 5292 -			-294		-2535	-294		-2796	-		3325	-294	ì	I⊢	0007-	- 22		-2948	-294		-	-294		-4202	-294		-294		-	-294
. I ⊦	692-			-369		-623			2330	_		-3883	-369		∣⊢	0/61-	62		\vdash	-369		-1023	-369		2240	<i>6</i> 00-		-369		-	-369
	-2844		-874	117		250	117		-2000	117		-4800			۱Ļ	117			-1185	117		-885	117		-2642	/11	-855	117		-1644	117
	-2894			359		-760			-3105	_		-4357	-			350			265			109			-4470					-469	
	/ -1866 5 96		1 445			7 382			1 -3565	-		4 -4501			۱L	0 200 5			-10	5 96		φ	5 96		-49	2 20	1 -618	5 96		2 -1635	45 96
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- I H	ŗ		57 1217			9 -1947	275 394		804 -3870	_		24064	-		I to	207 - 1922			-2	275 39		8 -1994	275 39		4	60 017	17	75 394		18 2974	75 394
	Ŷ.			-720 275		40 -489	-720 27		-826 8(37 -3773							-	-720 27		'	-720 27		4	17 071-	36 -529	-720 27		20 -848	-720 27
	-2456 -2353 -466 -720	4	350 -1445	-466 -7		-2367 -1440	-466 -7		460 -8			-3041 -3132	t		۱H	-756 -1445	1		-2773 -1886	-466 -7		-2437 -1515	-466 -7		297 -1251		-2347 -1436	-466 -7		-3274 -2420	-466 -7
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	-2460	-1378	-579	106	-1378	1062	106	-1378	-3049	106	-1378	-1300	106	-1378	000	000	-1378		-775	106	1077-	-567	1278	0/61-	-4900	-1378	876	106	-1378	-1216	106 -1378
	-3344 309	-701	-531	399	-701	-1854	399	-701	-3939	399	-701	-5109	665	10/-	1000	600E	-701		-1913	<u>668</u>	8 <u>6</u> 6-	-436	399	TN/-	-5119	-701	-1887	399	-701	-489	102- 399
	-2/90	-1115	-2645	-381	-1115	-2671	-381	-1115	-1846	-381	-1115	1808	-381	-1115	1020	-28/4	-1115		-3060		c111-	-2740	-381		-2520	-1115	628		-1115	-3538	-381 -1115
	-2681	-894	-268	43	-894	1381	43	-894	-3939	43	-894	-5581	43	-894	1 201	1001	-894		2042	43	- 894	2138	43 804	100-	-4965	-894	2003	43	-894	442	43 -894
	-2/66	-9181	-809	233	-9181	1394	233	-9181	-4456	233	-9181	-5230	233	-9181		-/- 233	-2649		862	233	- 6668-	863	233 0181	101/-	-5316	-9181	-760	233	-9181	1561	233 -9181
	-5142 -500	-8139	-2315	-500	-8139	-2351	-500	-8139	-1706	-500	-8139	-3766	-500	-8139	0000	-500	-8139			-500		-2422	-500 e130	CCT0-	-2156	-200	-2341	-500	-8139	-3144	-500 -8139
	-2/42 -149	+		-149	%	491		8-	-2039			-4840			1	-140	╈	1		-149	-		-149 s	-	-2660	-	1068	\vdash	%		-149 -8
	(<u>)</u> (()		96(A)			97(D)			98(V)			00/01			1001	100(E)			101(E)			102(E)			103(I)		104(E)			105(P)	

continued
5
TABLE

21700%	21800%	21900%	22000%	22100%	22200%	22300%	22400%	22500%	2260%
647	-3194	-2622	-1935	-4521	-1863	-1182	-3351	-1442	-292
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-2486	-3071	-3002	-2610	-4703	-2540	-1581	-3264	-1913	-1356
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-198	69	-2858	-2050	-3974	-303	354	506	1305	-2374
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-1120	-3292	-90	-913	-2674	-840	1843	-3236	-913	-4016
117	117		117	117	117	117	117	117	117
-957 -1093	-4776	-1817	-480	-2418	790	334	-4864	-1111	-4547
96 359	359	359	359	359	359	359	359	359	359
	-4613 96	1261 96	5 -655 96	96	s -630 96	-1501 96	<u>-4762</u> 96	1135 96	-4561 96
8 -415	8 -4005	5 1457	1 1446	7 -3243	5 788	8 -1111	7 -4172	8 660	1 -3987
4 45	4 45	4 45	4 45		45	4 45	4 45	4 45	4 45
1 -2218	81 -4778	9 -2766	2 1941	<u>3 -3507</u>	4 -1966	75 -2388	2 -4857	0 -2228	0 -4871
5 394	75 394	5 394	5 394	75 - 394	5 394	75 -394	5 394	5 394	5 394
9 3151 0 275	-51	8 489 0 275	<u>5 -552</u> 0 275	2.	14 414 10 275	-140	32 -5232 30 275	2 -1040 0 275	04 -4290 0 275
86 -1509 86 -720	21 2728 56 -720	3010 -2208 -466 -720	1525 1525 1525	5053 -4315 -466 -720		1024 1167 -466 -720	2935 -282 -466 -720	187 -712 -466 -720	53 -1124 56 -720
9 -2336 0 -466 *	2 2621 0 -466		9 -2440 0 -466 *					╵┝╼┼╋╌┥	4 563 9 -466
-479	-5022	3059	1139	-3678	927	-1275	-5103	-752	-5074
210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*
-2279	1361	-3210	-2491	-5005	-2405	758	1096	-1181	-1742
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
1767	-4282	-1025	-589	-3349	-535	-1111	-4502	-891	-2159
106	106	106	106	106	-1378	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	1278
-2071	-5488	-2740	-1913	3554	-1868	-2314	-5535	148	-5143
399	399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	701
-2376	-1352	-3650	-2747	-4832	-2661	-1155	-1506	-1603	4216
-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	1115
827	-5252	-1232	964	-2709	-198	-1461	-5293	-857	-5431
43	43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894	804
-814	-5826	-1997	-740	-2347	958	-14	-5806	158	-5436
233	233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	0101
-2375 -500 -8139	-2890 - -500 -	-3098 - -500 - -8139 -	-2426 -500 -8139	-2907 - -500 -	-2349 -500 -8139	-1149 -500 -8139	-2796 -500 -8139	-1525 -500 -8139	-3437 -500
-1173 - -149 - -8 -	-3415 - -149 -	-1941 - -149 -	1129 - -149 -	-2276 - -149 -8 -	1730 - -149 -	1350 - -149 - -8 -	-3333 - -149 - -8 -	1769 - -149 -	-4110 . -149 .
106(N)	(W) 	108(K) —	109(P) —	110(G) 	.11(A) 	.12(T) —	(13(L) 	.14(A) 	(15(F)

22700%	22800%	22900%	23000%	23100%	23200%	23300%	23400%	23500%	23600%
-4539	-3641	-5849	-523	-2737	-3791	-3759	3677	-2021	-2414
-249	-249	-249	-249	-249	-249	-249	-249	-249	
-4632	-4063	-4924	-1536	-3194	-4158	-4170	-547	-2708	2789
-294	-294	-294	-294	-294	-294	-294	-294	-294	
-2983	-6022	-5862	-2200	-2139	1969	-4793	-3870	-2133	-1719
-369	-369	-369	-369	-369	-369	-369	-369	-369	
-1679	-5395 117	-4815 117	-3934 117	-1437 117	-2653 117	-3702 117	-4669 117	-1011 117	1964
1514	-5391	-4727	-4839	413	-4472	-3508	-4344	-938	2137
359	359	359	359	359	359	359	359	359	
5 -3927	1 -4732	5 -5385	8 -4592	8 -2141	1 -4961) -3879	1 -4483	9 -802	5 - 1738
5 96	5 96	5 -96	5 96	5 96	5 96	5 96	5 96	96	
6 -3656	0 -5011	4 -5546	0 -3998	3 -1678	4 -4681	5 -3770	5 -3851	0 -229	3 3585
4 45	4 45	4 45	4 45	4 45	4 45	4 45	4 45	4 45	
798 -2896	954 -4960	141 -4804	<u>43</u> -4880	468 -2633	780 -4824	481 -4185	715 -4955	553 -2090	7 3153
275 394	275 394	275 394	75 394	275 394	275 394	275 394	275 -394	275 -394	
-2,	4	- C	-868 -4443 -720 275	ň	4	<u>е</u> р			21 1 - 2187
-4469 -3523 -466 -720		-6297 -5970 -466 -720	1089 -8. -466 -7.	3 -2052 -466 -720	358 -1211 -466 -72(-284 -1622 -466 -720	1011-19651
						Υ Υ			100
-41:	-4911 210 *	-5765 210 *	-51	-18	4	ή	-5		-1588
-4216	-6314	-6627	-1514	-2602	3293	-5496	-3719	-2566	0151
-626	-626	-626	-626	-626	-626	-626	-626	-626	
*	*	*	*	*	*	*	*	*	
-3637	5435	-5028	-2370	-1925	-4876	5216	2153	998	-2019
106	106	106	106	106	106	106	106	106	
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	
119	-4506	3834	-5246	-2135	-5123	638	-5097	1844	12842
399	399	399	399	399	399	399	399	399	
-701	-701	-701	-701	-701	-701	-701	-701	-701	
-4413	-4036	-5893	4093	-2956	-2477	-4166	3410	-2820	1 87062
-381	-381	-381	-381	-381	-381	-381	-381	-381	
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	
-4219	-5009	-5462	-5444	-1781	-4969	-3491	-5549	-272	-2186
43	43	43	43	43	43	43	43	43	
-894	-894	-894	-894	-894	-894	-894	-894	-894	
-3998 - 233 - -9181	-4720 - 233 - 9181	-5092 . 233 .	-5534 - 233 - 9181	-2020 - 233 - -9181	-5324 . 233 .	-3197 - 233 - 9181	-5210 - 233 - 9181	948 233 -9181	-2466
-1829 -3 -500 -3 -8139 -9	-4539 -4 -500 -4 -8139 -4	-4203 -5 -500 -5 -6139 -5	-3387 -5 -500 -8139 -9	-1899 -2 -500 -	-2169 -5 -500 -5 -8139 -5	-3805 -3 -500 -3 -8139 -5	-3757 -5 -500 -5 -8139 -5	-2519 -500 -8139 -9	-2285
3091 -18 -149 -2 -8 -8	-5197 -4; -149 -; -8 -8;	-4435 -47 -149 -5 -8 -8	-4044 -3. -149 -3.	885 -18 -149	-2673 -2 -149 -2 -8 -8		-4816 -3 -149 -3 -8 -8	-1065 -2: -149 -: -8 -8	412 2
116(A) 30 1 	17(H) -51 — -1	18(G) -44 	119(F) -40 	120(N) 8 1	121(I) -26 — -1	122(H) -3381 — -149 —	123(Y) -48 1	124(G) -10 — -1	125(0)

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LABLE 9-COMMING	3248 -4248 -1515 -1324 -4004 -4259 -4063 -4255 230 -2003 -3673 -3237 23700% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 23700%	-93 1370 -300 -1337 465 -1974 655 -646 -794 -827 1255 -2448 -1798 23800% -626 210 -466 -720 275 394 45 96 359 117 -369 -294 -249 23800% * * * * -566 359 117 -369 -294 -249 23800%	-4274 -4053 -3598 -3770 3775 -3593 -3011 -1550 -16770 -3667 -4647 -4548 23900% -626 210 -466 -720 275 394 45 96 359 117 -369 -294 -249 23900% * * * * -456 -720 275 394 45 96 359 117 -369 -294 -249 23900%	-2610 -289 -5766 -1713 -884 2338 1247 2195 51 -1147 -2184 -2757 -24000% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 24000% * * * * * 246 -720 275 394 45 96 359 117 -369 -249 2400%	-2736 1785 -2680 -1776 -623 -2161 -319 -936 297 -1120 -2285 -2346 24100% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 24100% * * * -466 -720 275 394 45 96 359 117 -369 -249 24100%	-196 -1891 64 66 -1626 -2278 -1503 -1798 -1617 -1350 -389 305 1335 24200% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 -249 *	-1737 -1074 -1874 -1416 -992 3539 -1065 -1192 789 -866 -1383 -1765 -1661 24300% -626 210 -466 -720 275 394 45 96 359 117 -369 -294 -249 * * * * * * 559 117 -369 -294 -249	-1605 2889 -1630 -1021 -349 -1569 232 698 -786 -759 -1637 -1317 24400% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 24400% *	-4307 -1947 -4207 -3514 2151 -2936 -1416 -2754 -2102 -3802 -4324 637 24500% -626 210 -720 275 394 45 96 359 117 -369 -249 24500% * * * * -456 354 45 96 359 117 -369 -249 24500%	2461 -3697 1581 -797 -3593 -3371 -342 -2009 1904 -2784 -2461 24600% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 -2490 * * * * 394 45 96 359 117 -369 -249 -249	-44 -1632 -3675 -2915 685 -2782 -1248 -2305 478 -3088 -3191 -3098 24700% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 24700% *	-905 -5060 -2626 -2570 -4662 -4579 -4940 -4923 -4013 -3397 3796 -4414 -4190 24800% -626 210 -466 -720 275 394 45 96 359 117 -369 -294 -249 * * * * * * * * *	2613 -2236 -723 -81 -2157 -2608 -1885 -2086 -1633 276 -271 1325 844 24900% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 -4900%	-697 -3877 816 4679 -3879 -3924 -3361 -3576 -3242 -2531 -1114 -2746 -2629 25000% -626 210 -466 -720 275 394 45 96 359 117 -369 -249 25000% * * * * * 45 96 359 117 -369 -249 25000%
5					-7									
onunue	-1324 -720		-3598 -720			66 -720			-3514 -720			-2570 -720	-81 -720	4679 -720
										1581 -466				816 -466
IAI	-4248 210 *	1370 210 *	-4053 210 *	-289 210 *	1785 210 *	-1891 210 *	-1074 210 *		-1947 210 *	-3697 210 *	-1632 210 *	-5060 210 *	-2236 210 *	-3877 210 *
	3248 -626 *		-4274 -626 *	-2610 -626 *	-2736 -626 *	-196 -626 *	-1737 -626 *	-1605 -626 *	-4307 -626 *	2461 -626 *	-44 -626 *	-905 -626 *	2613 -626 *	-697 -626 *
	-3932 106 -1378	-546 106 -1378	-3594 106 -1378	-848 106 -1378	-762 106 -1378	-244 106 -179	-1092 106 -179	-101 106 -3775	-1744 106 -1378	-3081 106 -1378	-1596 106 -1378	-4687 106 -1378	-1422 106 -1378	-3301 106 -1378
	-4221 399 -701	-1881 399 -701	653 399 -701	-2106 399 -701	-1992 399 -701	-2093 399 -3098	-1041 399 -3098	-1335 399 -109	-261 399 -701	-3943 399 -701	-2331 399 -701	-4180 399 -701	-393 399 -701	-4001 399 -701
	-2466 -381 -1115	2504 	7 -4464 5 -381 1 -1115	7 -2915 5 -381 1 -1115	0 -2976 5 -381 1 -1115) 3516 5 -381 1 -1115	s -1832 -381 -1115) -1920 5 -381 4 -1115	-4260 -381 -1115	5 -1821 5 -381 1 -1115) -3868 5 -381 1 -1115) -3522 5 -381 1 -1115	b 1867 5 -381 1115	b -1396
	-4439 43 -894	1172 43 -894	-3897 43 -894	-637 43 -894	529 43 -894	-2120 43 -894	-1058 43 -894	-230 43 -894	-651 43 -894		-680 43 -894	-5160 43 -894		-4279 43 -894
	-4813 233 -9181	334 233 -9181	-3618 233 -9181	-1173 233 -9181	1377 233 -325	-2227 233 -6882	-997 233 -6882	-564 233 -6882	3349 233 -9181	-4504 233 -9181	3495 233 -9181	-5092 233 -9181	-3230 233 -9181	-4754 233 -9181
	-1916 -500 -8139	-2234 -500 -8139	-1925 -500 -8139	-2398 -500 -8139	-2663 -500 -8139	-1104 -500 -5840	-937 -500 -5840	-1483 -500 -5840	-4159 -500 -8139	-1713 -500 -8139	-3444 -500 -8139	-2888 -500 -8139	-875 -500 -8139	-2345 -500 -8139
	-2254 -149 -8		715 -149 -8	479 -149 -8	1787 -149 -2336	-1308 -149 -38	-603 -149 -38	-804 -149 -38	-2405 -149 -8	-2047 -149 -8	-2024 -149 -8	-3122 -149 -8	-53 -149 -8	315 -149 -8
	126(I) 	127(K) 	128(P) 	129(P) —	130(A) 	131(F) 	132(P) —	133(K) 	134(D) —	135(I) —	136(D) —	137(V) 	138(I) 	139(M)

25100%	25200%	25300%	25400%	25500%	25600%	25700%	25800%	25900%	2600%
-3991	-4519	-5786	-4729	-4556	-3787	-5849	-2990	-2351	-4190
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-4506	-4572	-4900	-4403	-4647	-4027	-4924	-3434	-2682	-4414
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
3206	-2929	-6092	-5264	-2994	-3249	-5862	-3482	-1484	3796
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-2619	-1844	-5194	-4408	-1682	-2963	-4815	-2510	2687	-3297
117	117	117	117	117	117	117	117	117	117
-4483	-1643	-5166	-4529	910	-2883	-4727	-2470	567	-4013
359	359	359	359	359	359	359	359	359	359
-5102	-4112	-5396	-2169	-3939	-3822	-5385	-748	-2684	-4923
96	96	96	96	96	96	96	96	96	96
-4890	-3976	-5648	-3079	-3661	-3912	-5546	-1305	-2399	-4940
45	45	45	45	45	45	45	45	45	45
-4869	-3052	4310	-4535	-2898	4036	-4804	1551	-2747	-4579
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-4945	-4447	-5780	3994	-4146	-3912	-5765	634	-2652	-5060
210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*
2415	-3901	-6679	-5555	-4238	-3353	-6627	-3830	-1754	-905
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-5132	-3851	-5077	-3307	-3642	-3767	-5028	4731	-2291	-4687
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-4990	-4795	-5573	-3992	-4199	-4235	-5462	-1702	-2899	-5160
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(40(V)	[41(A)	[42(P)	143(K) 	144(G) —	145(P) —	.46(G) —	47(H) 	148(T) —	149(V)

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contin
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26100%	26200%	26300%	26400%	26500%	26600%	26700%	27300%	27400%	27500%
-4993	-1919	537	4052	-1626	-1856	-4826	-2033	-4766	-3678
-249	-249	-249	-249	-249	-249	-250	-249	-249	-249
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-294	-294	-294	-294	-294	-294	-295	-294	-294	-294
-5644	-2023	-287	-3867	1871	-1976	-4837	-2149	-3384	2986
-369	-369	-369	-369	-369	-369	-368	-369	-369	-369
-4832	-66	1303	-4679	-344	-822	-3532	-1024	-2095	825
117	117	117	117	117	117	117	117	117	117
-4989	1224	-858	-4356	443	-764	-3211	-962	-1874	-4115
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4219	2308	-736	-4500	-796	419	-4377	471	-4170	-4752
96	96	96	96	96	96	95	96	96	96
-3672 45	-128	816	-3868	695	1878	-3187	-224	-3901	-4554
	45	45	45	45	45	45	45	45	45
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-8139	-8139	-9181	-8139	-8139	-8139	-3318	-8139	-8139	-8139
-4845	-962	-902	-4820	129	576	-3239	753	-52	-2485
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
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150(R) 	151(R) —	[52(E)	153(Y) —	[54(V)	55(Q) 	[56(G) 	.57(G) 	.58(G) 	159(V)

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-4362	-2102	-2755	-3040	-4942	-3991	3315	-4751	-3946	635
-249	-249	-249	-249	-249	-249	-249	-249	-249	
-4583	-2701	-3145	-3342	-4724	-4506	-981	-4577	-4932	-1968
-294	-294	-294	-294	-294	-294	-294	-294	-294	
-4148	-1946	-1973	2176	-3852	3200	-2272	-5612	-4491	1275
-369	-369	-369	-369	-369	-369	-369	-369	-369	
-2911	-1043	-2414	-2488	-2762	-2619	-2233	-4772	-3009	-894
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44 -3757 45 96	5 -1093 5 96	3 -35 <i>6</i> 7 5 96	3 -4320 5 96	7 -4545	1 -5102 5 96	115 -2362 45 96	5 -3826 5 -96	2 -3575 5 96	-538 -1013 -1056
-32	199 -545 394 45	26 -3433 34 45	39 -4023 34 45	728 -4477 394 45	869 -4891 394 45	-18	693 4575 394 45	<u>235 -1872</u> 394 45	- 535
1199 4031 275 394	-891 -2199 275 394	-3360 -3626 275 -394	-4323 -4439 275 394	Ϋ́	4	ς-	-4230 -4693 276 394	428 -3235 275 394	-943 1212
-4393 11	-1576 -8	-1200 -33	-841 -43	-4365 -3727	-1474 -4791	-1863 -2047	-5304 -42	-4387 4	6~ 082-
-720 2	-720 2	-720 2	-720 2	-720 275	-720 275	-720 275	-720 2	-720 2	
-5058 -4 -466 -	-2434 -1 -466 -	3041 -1 -466 -		-5025 -4 -466 -	-1532 -1. -466 -	-2342 -1 -466 -	-5564 -5 -466 -	-4922 -4 -466 -	-1526
-3527	-617 -5 210 -5	-3689 3 210 *	-4413 210 *	-4818	-4949 -3 210 *	-2056 -2 210 *	-3840	-2604 210 *	150
-5055	-2359	-1687	-2668	-4781	2426	-2386	-5973	-5087	-1295
-626	-626	-626	-626	-626	-626	-626	-626	-626	
*	*	*	*	*	*	*	*	*	
-3342	891	-3283	-3911	-4271	-5132	3753	-4099	-2157	-816
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-8	-8	-8	-8	-8	-8	-8	-8	-8	
160(P) 	161(C)	162(L)	163(I) 	164(A) 	165(V) 	(H)991		168(D) —	169(A)

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	28600%	28700%	28800%	28900%	29000%	29100%	29200%	29300%	29400%	29500%	29600%	29700%	29800%
	-3560	-4749	-1855	-4942	-2165	-2087	627	-4508	-3690	-4523	4349	-4534	-1726
	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
	-4143	-4622	-2534	-4724	-2690	-2793	-1425	-4599	-3665	-4623	2928	-4636	-2330
	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
	-3045	-4492	-539	-3852	-2245	-2231	1669	-2949	-2629	-2982	-2621	-3001	-1628
	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
	2313	-3317	1230	-2762	1094	-1058	-311	409	-4399	-1675	-3536	-1718	14
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	9 -2758 5 96	2 -3140 5 96	6 799 5 96	7 -4545 5 96	59 1012 45 96	<u>5 -869</u>	50 -2151 45 96	<u>889 -3929</u> 45 96	750 -5002 45 96	3 -3895 5 96	2 -4004 5 96	29 -3959 45 96	<u>5 -736</u> 5 96
	814 -1909 394 -45	16 -3312 14 45	54 436 04 45	728 -4477 394 45	<u></u>	115 345 394 45	-19	-36	-47	12 -3613 14 45	494 -3522 394 -45	-37	022 -205 394 45
	-7	-3174 -3946 275 394	1880 -1954 275 394	Ϋ́	-958 -2360 275 394	1408 -2115 275 394	-2	-7	4	82 -2892 75 394	4	-7	-632 -2022 275 394
nanun	-3103 -1627	-4565 -31	-1440 18	-4365 -3727	-1706 -9	-1712 14	-124 -2232	-3484 -2820	-1236 -5514	-3514 -2782	-1998 -3545	-3565 -2837	-1194 -6
	-720 275	-720 2	-720 2	-720 275	-720 2	-720 2	-720 275	-720 275	-720 275	-720 275	-720 275	-720 275	-720 2
NABLE 9-continued	-3938 -3	-5188 -4. -466 -	-2363 -1	-5025 -4	851 -1	-2624 -1	- <u>1388</u>	-4412 -3 -466 -	<u>3316 -1</u> -466 -	-4462 -3. -466 -	-250 -1	-4490 -3.	-2040 -1 -466 -
TABLE	-2264 -3	15 -5	711 -2	-4818 -5	1975 -	544 -2	1006 1	-4159 -4	-5423 3	-4076 -4	-4437 -	-4197 -4	1888 -2
	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -	210 -
	$\left + + + \right $			4			1266 1 -626 -				233 -4 -626 -		324 1 -626 *
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	5 -2206 1 106) -3454) 106 -1378) -518) 106 -1378	2 -4271 9 106 1 -1378	≓ Ț	2 1973 5 106 1 -1378	-15 -13	-3651 106 -1378	4 1-	5 -3608 5 -3608 1 -1378	7 -1405 9 106 -1378	3 -3684 1 -1378 1 -1378	5 -615 9 106 1 -1378
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	-2420	-3461	-2349	-2768	-2597	-2613	-936	-1826	-3800	-1831	-3050	-1860	307
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	170(S)	171(G)	172(N)	173(A)	174(K)	175(D)	176(V)	177(A)	178(L)	179(S)	180(Y)	181(A)	182(K)
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29900%	3000%	30200%	30300%	30400%	30500%	30600%
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-3191	1742 -369 -5862 -369	-2914 -359	-2867 -369	-3813 -369	-2713 -369	-5613 -369
-1898	-2148 117 -4815 117	-1656 117	2334 117	-3671 117	1907 117	-4560 117
-1679 359	-3388 359 -4727 -4727	712	-1441 359	-3768	1088 359	-4461 359
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ξ		-37	986 -3302 394 45	-26	-33	-53
£.		-7		4	-7	-4896 -4606 275 394
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3934 233 9181	-4630 233 -9181 -5092 233 -5092	-4353 233 -9181	-3738 233 -9181	-4490 233 -3345	-3814 233 -9034	-4855 233 -9034
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2572 149 -8	-2173 -149 -8 -8 -4435 -149 -149 -8	1392 -149 -8	855 -149 -8	-3706 -149 -155	2844 -149 -8	-4176 -149 -8
[83(G)	184(I) 	186(G) —	187(G) —	188(R) —	189(A) —	90(G)

	30700%	30800%	30900%	31000%	31100%	31200%	31300%	31400%	31500%	31600%	31700%	31800%
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	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
	-4191	-3223	-4121	-4684	-4405	-1356	-2858	-3909	-4878	-1933	-3398	-4554
	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
	2919	757	-3305	-4113	-2969	-2374	-2544	-3331	-5786	195	-171	-2989
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-8139 -9181 -894 -1115 -701- -1378

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-5894	-1279	-1899	-5862	-5786	-390	932	3416	-2629	1347
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203(D)	204(L) —	205(F) —	206(G) 	207(E)	208(Q) 	209(A) —	210(V) 	211(L) 	212(C) —

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223(F)	224(E)	225(T)	226(L)	227(V)	228(E)	229(A)	230(G)	231(Y)	232(Q)
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	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-3318
	-3403	-2870	-3089	3631	-4797	-3828	-2775	-1407	-3370	-2519	-3177	-85	-2513
	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-155
	233(P)	234(E)	235(M)	236(A)	237(Y)	238(F)	239(E)	240(C)	241(L)	242(H)	243(E)	244(L)	245(K)
	—		—	—				—		—	—	—	—

36300%	-36400%	36500%	3660%	36700%	36800%	36900%	37000%	37100%	37200%
-3239	-3397	-2504	-3962	-3067	-3210	4375	-2960	-2378	-5849
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-3082	-3627	-2874	-4770	-3154	-3321	2413	-3709	-2933	-4924
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
742	-64	3332	-4467	-1545	766	-3662	-3162	-2521	-5862
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-3436	-2949	-1807	-3060	699	-2764	-4432	692	-1435	-4815
117	117	117	117	117	117	117	117	117	117
-4963	-4598	367	-2624	-3399	-4251	381	-1757	-1397	-4727
359	359	359	359	359	359	359	359	359	359
-4689	-4799	-3320	-3072	-3989	-4487	-4393	-1666	658	-5385
96	96	96	96	96	96	96	96	96	96
-4044	-4427	-3145	-1891	-3740	-4076	-3786	1879	-486	-5546
45	45	45	45	45	45	45	45	45	45
-4852	-4833	-3419	-3314	-4179	-4601	-4868	-2716	-2459	-4804
394	394	394	394	394	394	394	394	394	
-5358	-4933	-3123	-1424	-4150	-4649 275	275	-1000	-1002	-5141
1671	-781	1383	-4260	-562	4005	-2986	-2751	-1972	-5970
-720	-720	-720	-720	-720	-720	-720	-720	-720	
2962	287	-1488	-4822	3056	173	-2963	-3547	-2822	-6297
-466	-466	-466	-466	-466	-466	-466	-466	-466	-766
-5138 210 *	-4915 210	-3402 210 *	767 210 *	-4207 210 *	-4663 210 *	-4968 210 *	-1165 210 *	1474 210 *	-5765
-632 -626 *	3728 -626 *	-227 -626 *	-4998 -626 *	-1038 -626 *	2587 -626 *	-3544 -626 *	-3622 -626 *	-2921 -626	-6627
-4390	-4587	-2893	-2187	-3790	-4151	-1300	-1410	-921	-5028
106	106	106	106	106	106	106	106	106	
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	
-5646 399 -701	-5196 399 -701	-3082 399 -701	-2751 399 -701	-3998 -701	-4985 399 -701	-4992 399 -701	-2347 399 -701	2635 399 -701	3834 300
-1321	-1958	-2081	-4953	-1675	-1759	1516	-3841	-2353	-5893
-381	-381	-381	-381	-381	-381	-381	-381	-381	
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	
-5375 43 -894	-5109 43 -894	-3732 43 -894	-1039 43 -894	-4633 43 -894	-4861 43 -894	-5401 43 -894	3135 43 -894	201 43 -894	-5462 43
-5954	-5473	-4095	3864	-4842	-5342	-5142	-568	-1215	-5092
233	233	233	233	233	233	233	233	233	
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	
-3023	-2484	-1668	-4569	-2715	-2356	-3630	-3457	-2818	-4203
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-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	
-3571 -149 -8	-2980 -149 -8	-1685 -149 -8	-2963 -149 -8	-2768 -149 -8	-2822 -149 -8	-4562 -149 -8	-1959 -149 -8	-347 -149 -8	-435 -140
246(L)	247(I)	248(V)	249(D)	250(L)	251(M)	252(Y)	253(E)	254(G)	255(G)
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37300%	37400%	37500%	37600%	37700%	37800%	37900%	3800%	38100%	38200%
-2073	624	-3144	-3030	1546	3695	-4566	-3821	-4605	-3794
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-2325	-2002	-4027	-3044	1995	-2924	-4661	-4218	-4616	-4919
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-344	-1149	-3433	-1557	-1468	-2582	-309	2521	-3902	-4325
-369	-369	-369	-369	-369	-369		-369	-369	-369
-1980 117	$\frac{162}{117}$	-2131 117	-3558 117	-1535 117	-1509	-1692 117	-2636 117	-2664 117	-2821 117
-2884	1037	-1845	-4860	-1789	-1389	3391	-4466	-3681	-2334
359	359	359	359	359	359	359	359	359	359
8 -3221 96	<u>3 -993</u>	8 -2243 5 96	96 -4539	5 2408 96	0 -1264 5 96	3 -3936 5 96	8 -4978 5 96	8 -4355 5 96	2 -3311 5 96
3 -2958	5 -498	3 -1148	8 -4039	4 -1215	6 -659	3 -3648	7 -4713	8 -4203	9 -1612
4 45	4 45	4 45	4 45	4 45	4 45	4 45	4 45	4 45	7 45
194 -3653	<u>905 -2155</u>	219 -2763	248 -4838	239 -2814	645 -2446	782 -2903	770 -4827	492 -3638	15 -3069
275 394	275 394	275 394	275 394	275 - 394	275 394	275 -394	275 -394	275 -394	15 -3069
1999 19 -720 27	-817 -905 -720 275	~	4920 -5248 -720 275	5		-2	4	Ϋ́	4140 3045 -720 275
1017 19	-49 -8	-3838 -3055	-948 49	380 -1133	-2941 -2110		332 -1267	-5102 -4364	-4718 -4140
-466 -7	-466 -7	-466 -720	-466 -7	-466 -720	-466 -720		-466 -720	-466 -720	-466 -720
┃┝┼┼┼┥	$\left + + + \right $				441 -29 210 -4 *		$\left + + \right $	┝┝┼┼┥	
<u>6</u>		-	4			4	4	4	-7
313 -62	-135 -62	-393	-62	-159	-2986 -626 *	-426 -62	-62 -62	-498 -62	-488 -62
-2668	-793	-1518	-4248	-1089	$1172 \\ 106 \\ -1378$	-3634	-4905	-4045	-1922
106	106	106	106	106		106	106	106	106
-1378	-1378	-1378	-1378	-1378		-1378	-1378	-1378	-1378
-3753	-661	1196	-5421	-2765	-2207	136	-5114	-2899	-2486
399	399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	-701
-1316	-1748	-4083	-1349	-886	-2846	-4448	-2538	-4697	-4896
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-3740	128	557	-5350	-1663	-509	-4131	-4961	-4348	990
43	43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894	-894
-4321	-1237	1001	-5816	-2260	568	-3877	-5311	-4019	2906
233	233	233	233	233	233	233	233	233	233
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-1769 - -500 - -8139 -	-1640 - -500 - -8139 -	-3809 -500 -8139 -	-3159 - -500 - -8139 -	-1949 - -500 -	-2973 -500 -8139 -	-1844 - -500 -8139 -	-2149 - -500 - -8139 -	-2711 - -500 - -8139 -	-4778 -500 -8139
-2042 -1 -149 -8	1914 -] -149 -8 -8 -8	365	-3656 -3 -149 -8 -8 -8	-1614 -1 -149 -	-1548 -2 -149 -8	279 -1 -149 -8 -8 -8	-2853 -2 -149 -	-2212 -2 -149 -8	-2725 -149
256(I) -	257(A) 	258(N) 	259(M) -	260(R)	261(Y)	262(S) —	263(I) -	264(S)	265(N)

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9-cont	
LE	
B	
Σ	

38300%	38400% 38500%	38600%	38700%	38800%	38900%	3900%	39100%
-3271 -249	-4687 -249 -249 -249	4211 -249	-5635 -249	-3791 -249	3508 -249	-1553 -249	-2006 -249
-4055 -294	-4697 -294 -3455 -294	2997 -294	-4754 -294	-4894 -294	-1615 -294	-1907 -294	-2454 -294
-3351 -369	-3194 -369 -369 -369	-3637 -369	-5613 -369	-4301 -369	-1282 -369	2578 -369	822 -369
3157 117	-1908 117 -2032 117	-4391 117	-4560 117	-2809 117	-2410 117	1701 117	2345 117
-1897 359	-1690 359 -1923 359	-4199 359	-4461 359	-2330 359	-3181 359	-2129 359	1341 359
-2277 96	-4099 96 -773 96	-4299 96	-5178 96	-3297 96	-3468 96	-2514 96	887 96
-1369 45	-3858 -3758 -3758	-3741	-5312	-1621	-3109	942 45	-1149
-2850 394		-4847 394	-4606 394	3073 - 394	-3886 394	-3060 394	-2421 394
3 2125 0 275	1 -2978 275 5 -1209 75	5 -3649) 275	-4896) 275	5 -1084) 275	3 -3537 3 275) -2568	5 -1442) 275
-3103		-2965	-5741	-4115	1558 -720	-429	-1265 -720
-3859 -466	-4660 -466 -3417 -466	-2951 -466	-6087 -466	-4703 -466	892 -466	-1 -466	-2016 -466
-1685 210 *	-4332 210 * 1878 210 210 *	634 210 *	-5533 210 *	-2320 210 *	-3766 210 *	-2570 210 *	-1234 210 *
-3874 -626 *	-4400 -626 * -3596 -626 *	-3522 -626 *	-6386 -626 *	-4863 -626 *	255 -626 *	691 -626 *	-1769 -626 *
-1713 106 -1378	-3798 -1378 -1378 -1284 -1284 -1378	-1299 106 -1378	-4823 106 -1980	-1932 106 -1378	-2215 106 -1378	-1955 106 -1378	-1387 106 -1378
-2367 399 -701	659 399 -701 -2588 399 -701	-4972 399 -701	3828 399 -422	1758 399 -701	-4046 399 -701	-3035 399 -701	-2112 399 -701
-4071 -381 -1115	-4573 -381 -1115 -335 -1115 -381 -1115	1910 -381 -1115	-5686 -381 -1115	-4880 -381 -1115	2447 -381 -1115	-1283 -381 -1115	-2170 -381 -1115
903 43 -894	-4290 -43 -894 -894 2964 -894	-5310 43 -894	-5222 43 -894	1828 43 -894	-4137 43 -894	-2894 43 -894	-1401 43 -894
-596 233 -9181	-3979 -333 -9181 -9181 -1036 -9181	-5100 233 -3345	-4855 233 -9034	3025 233 -9181	-4651 233 -9181	-3480 233 -9181	-1918 233 -9181
-3396 -500 -8139	-2035 -500 -8139 -8139 -3488 -3488 -3488 -300 -8139	-3618 -500 -8139	-3995 -500 -7992	-4705 -500 -8139	-2175 -500 -8139	-1250 -500 -8139	-1643 -500 -6139
-2061 -149 -8	3410 -149 -8 -8 -2118 -149 -8	-4524 -149 -155	-4176 -149 -8	-2710 -149 -8	-2497 -149 -8	-1425 -149 -8	516 -149 -8
266(T) 	267(A) 	269(Y) 	270(G) 	271(D) —	272(Y) —	273(V) —	274(T)

continued
9
ILE
TAB

39200%	39300%	39400%	39500%	39600%	39700%	39800%	39900%	4000%	40100%
-4735	1093	-2084	-3717	-3250	1327	1152	-1871	-3270	-3189
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-4725	-2467	-2671	-4202	-3688	-3366	-2885	-2555	-3549	-3267
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-3297	-873	-2191	3125	2182	-2804	-2310	-1994	1397	-3556
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-2005	-939 117	-1133 117	-2487 117	-2288 117	$\frac{1851}{117}$	-1162 117	-837 117	2366 117	-2650 117
-1784	465	417	-4102	226	-1561	-1065	116	2251	-2874
359	359	359	359	359	359	359	359	359	359
-4137	-802	2862	-4784	-4274	-1742	-992	-620	-3214	2705
96	96	96	96	96	96	96	96	96	96
-3871	-260	-273	-4585	-4082	-899	-358	1356	-2916	-937
45	45	45	45	45	45	45	45	45	45
-3149	2813	-2228	-4649	-4279	-2573	-2190	478	-2821	-3430
394	394	394	394	394	394	394	394	394	394
-3009	-651	-795	-4511	-4066	2295	-640	590	-2557	-2188
275	275	275	275	275	275	275	275	275	275
-3857	-1351	-1653	-1443	-1326	-2403	-1808	-1462	-2328	$\frac{1860}{-720}$
-720	-720	-720	-720	-720	-720	-720	-720	-720	
-4749	-2204	-2528	-1561	-1516	-3214	-2704	-2388	-3076	-3413
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-4340	533	848	-4689	-4265	-1145	381	490	-3215	2942
210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*
-4506	-2143	-2587	2142	3155	-3221	-2753	-2444	-2611	-3763
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
-3816	-675	-716	-4639	-3954	-1273	-796	1182	-2891	-1356
106	106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	1278
3536	-1960	-2145	-4777	-4254	-2239	-2011	-1861	-2145	-3512
399	399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	701
-4647 -381 -1115	-2447 -381 -1115	175 -381 -1115	-2692 -381 -1115	-2473 -381 -1115	-3318 -381 -1115	-2994 -381 -1115	-2692 -381 -1115	-3179 -381 -1115	-4472 -381
-4171	-359	$ \begin{array}{r} 1072 \\ 43 \\ -894 \end{array} $	-4789	-4452	-550	2368	1835	-3444	-2331
43	43		43	43	43	43	43	43	43
-894	-894		-894	-894	-894	-894	-894	-894	804
-3838	343	-1097	-5133	-4828	2329	1227	859	-3655	-3848
233	233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	0101
-2128	-2210	-2548	-2035	-1919	-3162	-2699	-2372	-1688	-3623
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	•130
677	-992	-1214	289	-2265	-1731	1097	-166	228	-2991
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8	°
275(G)	276(P)	277(R)	278(V)	279(I)	280(D)	281(E)	282(E)	283(T)	284(K)
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40200%	40300%	40400%	40500%	40600%	40700%	40800%	40900%	41000%	41100%
-1869	-1849	-3030	1245	-1891	-2720	1565	-1851	-3155	-3968
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-2552	-2488	-3044	-2885	-2577	-3118	-1846	-2532	-4024	-4467
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-1992	-1889	-1557	-2597	-2015	2342	-1269	-1968	-3473	2071
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-836	-881	-3558	-1524	72	-2046	-2345	783	-2163	-2627
117	117	117	117	117	117	117	117	117	117
106	-62	-4860	-1527	-795	-3144	-2972	-763	-1879	-4473
359	359	359	359	359	359	359	359	359	359
437	1056	-4539	1762	831	-3805	-3298	-603	2142	-4869 -5086
96	96	96	96	96	96	96	96	96	45 96
1120	-136	-4039	-472	663	-3613	-3025	889	-1139	-4869
45	45	45	45	45	45	45	45	45	45
-1960	-2019	-4838	-2554	-1975	-3957	-3782	-1949	-2782	-4862
394	394	394	394	394	394	394	394	394	394
-498	-586	-5248	-1146	1305	-3691	-3390	-492	24	-4789
275	275	275	275	275	275	275	275	275	275
-1460	-1391	4920	-1997	-1485	-1109	-476	917	-3075	-1447
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-2385	-2279	948	-2832	-2409	-1406	2855	-2362	-3852	-1495
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
$\frac{1639}{210}$	1096	-4928	2831	-134	-3870	-3485	1692	-1467	-4941
	210	210	210	210	210	210	210	210	210
	*	*	*	*	*	*	*	*	*
-2442	269	-822	-2971	-2465	1746	-944	-2414	-3962	3464
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
-526	-578	-4248	-912	-545	-3330	-2344	873	-1511	-5106
106	106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
-1863	-1928	-5421	-526	-487	-3932	-3779	-1855	-2358	-5092
399	399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	-701
-2691	-2570	-1349	-3346	-2713	-2155	-898	-2665	-4119	-2737
-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
$1690 \\ 43 \\ -894$	814	-5350	287	2205	-4122	-3889	1211	1765	-4991
	43	43	43	43	43	43	43	43	-43
	-894	-894	-894	-894	-894	-894	-894	-894	-894
732	-843	-5816	-1591	367	-4584	-4307	143	2790	-5302
233	233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
-2370	-2286	-3159	-2891	-2394	3024	-2175	-2347	-3878	-2131
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-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139
443	1871	-3656	-1646	-172	1574	-187	862	-2148	-2630
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8	-8
285(E)	286(A)	287(M)	288(K)	289(E)	290(C)	291(L)	292(K)	293(D)	294(I)
			—	—	—	—		—	

41200%	41300%	41400%	41500%	41600%	41700%	41800%	41900%	42000%
-2908 -	-2340 -	-5849 ,	-249 4	2917 -	-2447 ·	-2895 4 -249	-1864 4 -249	-84 4
-3278 -	-3076 -	-4924 -	-2559 -	-601 -294	-2812 -	-3159 -294	-2545 -	4754 -294
-3060	-2508	-5862	-1999	-3546	1100	-3194	-1982	664
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-2031 117	-96	-4815 117	1488 117	-4237 117	$197 \\ 117$	$\frac{-2152}{117}$	-833 117	-2878 117
-2018	1978	-4727	829	-4078	-2387	-458	775	-3475
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-1545	1817	-5141	-536	-3532	-3103	-1693	-498	-3564
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-2513	-2012	-5970	-1472	-2894	-1089	-2487	1895	-987
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-3271	-2903	-6297	-2389	-2900	-1486	-3234	-2376	92
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1329	-621	-5765	623	-4609	-3349	2928	930	-4093
210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*
-3433	-2967	-6627	-2438	-3438	1205	-3501	-2429	-1428
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*	*	*	*	*	*	*	*	*
-1314	-914	-5028	-552	-1320	-2823	-1188	-524	-1779
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-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
-2760	-2068	3834	-1895	-4820	-3165	-3041	-537	-4315
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-1818	1712	-5092	-769	-4619	-4068	-2464	740	-4795
233	233	233	233	233	233	233	233	233
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-3134	-2895	-4203	-2374	-3577	-1603	-3363	-2361	-2553
-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-6139
346	-1354	-4435	-437	-4347	2827	-2364	-893	-2965
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295(Q)	296(S)	297(G)	298(E)	299(F)	300(A)	301(K)	302(M)	303(W)
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-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
2384	-369	-3767	30	-2019	-261	-3006	-800	-2803	-1358
-369		-369	-369	-369	-369	-369	-369	-369	-369
-2597	$\frac{-152}{117}$	-2402	-880	-868	306	-2162	-888	286	-759
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-4769	-1349	-2626	165	1528	1657	-3185	1577	-2362	698
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-4488	-915	-1339	-482	2135	-346	-2550	$\frac{1002}{45}$	-1697	232
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-4641	-1287	728	2186	584	486	2347	357	-1555	-349
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1043	-533	-3446	-816	-1484	-1011	-3017	1222	-2761	-1021
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-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-4747	-1042	-1836	-549	1211	-385	-2904	-891	-1924	2889
210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*
3052	-927	-4282	-1357	-2465	-1645	-3563	-946	-3445	-1605
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*	*	*	*	*	*	*	*	*	*
-4577 -106 -1378	-1037 106 -1378	-1686 106 -1378	935 106 -1378	-544 106 -1378	183 106 -1378	-2358 106 -1378	-935 106 -1378	-2007 106 -1378	-101 106
-4987	-2223	-2398	1352	-316	-2012	2944	-2192	-2205	-1335
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-2364 -381 -1115	-1375 -381 -1115	-4393 -381 -1115	-1738 -381 -1115	-2721 -381 -1115	-2013 -381 -1115	1262 -381 -1115	225 -381 -1115	-3738 -381 -1115	-1920 -381
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-533	233	233	233	223	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-325	2807
-2136	-1319	-4165	979	-2393	-1857	-2469	1019	-2372	-1483
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304(I)	305(L)	306(E)	307(N)	308(Q)	309(A)	310(G)	311(Y)	312(P)	313(K)
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	43100%	43200%	43300%	43400%	43500%	43600%	43700%	43800%	43900%	4400%
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	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
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	-1441	2004	-3673	472	-2110	-2474	597	-2020	-1957	-1963
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n n n	69 275	14 275) -3442) 275	1011 275	0 592 0 275	5 -1752 0 275	976	5 -575 0 275	1720 275	t 1478 0 275
	5 -1331	5 -1499	s 3030	<u>-1408</u>	7 -1610	7 1235	t 1079	5 1486	5 -1395	5 1474
	5 -720	5 -720	5 -720	-720	5 -720	5 -720	5 -720	5 -720	5 -720	5 -720
	-1919	-2415	1973	-2322	-2507	867	-644	-2366	95	-2266
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	-118 210 *	-171 210 *	-3523 210 *	$\frac{1272}{210}$	555 210 *	-444 210 *	613 210 *	1293 210 *	485 210 *	889 210 *
	-1804	-2459	-645	-647	-2529	325	-2361	-2405	-594	-2276
	-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
	*	*	*	*	*	*	*	*	*	*
	-293	-581	-2504	1714	-722	-1232	-799	-568	-525	-533
	106	106	106	106	106	106	106	106	106	106
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	-1029	151	-3822	-1863	-273	-375	-2272	-1930	121	-1871
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	-109	-701	-701	-701	-701	-701	-701	-701	-701	-701
	-2050	-2714	1726	-2618	-2797	1024	-2724	-2678	-2598	-2551
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	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
	2831 43 -894	$1086 \\ 43 \\ -894$	-3891 43 -894	517 43 -894	-367 43 -894	-337 43 -894	-724 43 -894	905 43 -894	523 43 -894	$ \begin{array}{r} 1483 \\ 43 \\ -894 \end{array} $
	521	-739	-4499	-747	-816	-2485	-1349	-833	-750	-766
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	-1695	-2382	-1920	-2314	-2397	-986	-2432	-2364	-2300	-2266
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	-5840	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139
	-766	-942	-2196	-883	2474	-154	-1311	897	505	444
	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
	-38	-8	-8	-8	-8	-8	-8	-8	-8	-8
	314(E)	315(T)	316(M)	317(H)	318(A)	319(M)	320(R)	321(R)	322(N)	323(E)
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44100%	44200%	44300%	44400%	44500%	44600%	44700%	44800%	44900%
-1595	-2540	-2601	-1830	-1805	-3370	838	-1722	-3597
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-1779	-3303	-3310	-2495	-2157	-3920	5462	-2323	-4065
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1398	-657	1283	2021	-1615	-2054	-1454	1425	-4456
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-1827	-2417	-2961	1404	-3016	-3440	-2104	-2030	-4575
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1769	2158	-1479	-518	-2160	-1898	-1648	-636	-4414
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-1133	-2254	-2812	-1391	-722	1983	-802	-1189	-1402
-720	-720	-720	-720	-720	-720	-720	-720	-720
-2121	-3124	-3528	-138	-191	-3167	-968	-2031	-1533
-466	-466	-466	-466	-466	-466	-466	-466	-466
601 210 *	-846 210 *	-1060 210 *	-131 210 *	-1327 210	-2175 210 *	-1470 210 *	2031 210 *	-4580 210 *
282	-3191	-3611	-2316	3240	-3317	-1181	-307	1586
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*	*	*	*	*	*	*	*	*
-426	-1074	4711	-537	-1663	-2317	-483	-611	-4459
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-1781	-2143	-2570	-739	-2983	-2986	-1810	-1941	-4682
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-2244	-3404	-3262	-2585	-1626	-3624	479	-2275	-2628
-381	-381	-381	-381	-381	-381	-381	-381	-381
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-204	1735	-1267	948	-86	3593	-1964	437	-4708
43	43	43	43	43	43	-13	43	-43
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-728	1756	-1245	332	-2846	-1838	-2068	-905	-5067
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1511 -149 -8	1053 -149 -8	-2064 -149 -8	-891 -149 -8	-1632 -149 -8	-2734 -149 -2336	-1530 -149 -38	-149 -8	-2445 -149 -8
324(N)	325(N)	326(H)	327(Q)	328(I)	329(E)	330(W)	331(K)	332(V)
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-4924	-2952	-2770	-3082	-4538	-2609	-3038	-1934	-2613	5644
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-5862	-2412	-2287	742	-5644	-2044	771	404	-1700	-2818
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-4815	-1280	-1133	-3436	-4832	-534	-3273	-1740	-1207	-3396
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-4727	-1191	-1073	-4963	-4989	1129	-4689	-2503	703	-3484
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-5141	619	-718	-5358	-4521	-522	-5109	-2991	-1394	-3147
275	275	275	275	275	275	275	275	275	275
-5970	-1912	-1741	1671	-5118	-1517	4369	4360	-1470	-2250
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-6297	-2792	-2637	2962	-5502	-2439	1721	441	-2235	-2389
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-5765	-445	-2826	-5138	-2789	982	-4943	-2983	-1236	$\frac{310}{210}$
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*	*	*	*	*	*	*	*	*	
-6627	-2850	-2722	-632	-5946	-2493	-576	-516	-2021	-2749
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
-5028	-884	-724	-4390	-3791	-572	-4210	-2222	-1410	-1173
106	-106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-583
3834	-2113	-2085	-5648	-4412	377	-5407	-3401	-2112	-4228
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-894	-894	-894	-894	-894	-894	-894	-894	-894	-894
-5093	-762	366	-5954	-5107	1002	-5774	-3997	-1729	-4312
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-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-2637	-2749
-4203	-2744	-2643	-3023	-4446	-2422	-2886	-1601	-1789	-3022
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
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-4435	1477	-1204	-3571	-4845	943	-3391	-1812	102	-3486
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-259	-239
333(G)	334(E)	335(K)	336(L)	337(R)	338(E)	339(M)	340(M)	341(P)	342(W)
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	46000%	46100%	46200%	46300%	46400%	46500%	46600%	46700%	46800%	46900%	47000%	47100%
	-3445 -249	-1755 -249	-1732 -249	-2662 -249	-2610 -249	-944 -249	-1344 -249	-3117 -249	-1812 -249	-1605 -249	1619 -249	-3046 *
	-3915 -294	-2439 -294	-2361 -294	-3466 -294	-2822 -294	-1340 -294	-1701 -294	-3993 -294	-2493 -294	-2188 -294	-2497 -294	-3601
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	-2216 117	-724 117	-788 117	-1645 117	-1888 117	-677 117	-1154 117	-2125 117	290 117	-74 117	$\frac{-1517}{117}$	-1362 *
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	-4559 96	-536 96	-611 96	-1607 96	1175 96	485 96	-2236 96	-2294 96	-562 96	-708 96	1469 96	-1460 -2119 * *
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	-4407 394	-1822 394	-1922 394	1624 394	-2774 394	-2134 394	-2830 394	-2733 394	531 394	34 394	-2548 394	1327
ea	-4311 275	582 275	-517 275	2847 275	-1487 275	-1202 275	-2305 275	-904 275	-447 275	-609 275	-1240 275	3429 *
9-conninued	-1008 -720	-1348 -720	-1245 -720	-2448 -720	-2178 -720	-100 -720	-236 -720	-3016 -720	-1400 -720	811 -720	-1683 -720	-2589
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IABLE	-4448 210 *	1212 210 *	828 210 *	324 210 *	3369 210 *	247 210 *	645 210 *	-1573 210 *	2441 210 *	489 210 *	2955 210 *	-1701 * 0
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	-4617 399 -1149	-553 399 -897	1040 399 -1432	-230 399 -1432	-2747 399 -1824	-2070 399 -943	-2803 399 -380	1601 399 -701	-483 399 -701	-1892 399 -701	-2531 399 -701	-1781 *
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	-4860 233 -3820	532 233 -8795	-769 233 -8991	1760 233 -3049	-2433 233 -8702	-1768 233 -8702	-3186 233 -8885	2888 233 -9088	398 233 -9088	1604 233 -8787	-1956 233 -8691	-1092 *
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LÀa	Thr	Ile	Ala 20	Val	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	Arg	Ala 30	Gln	Ser
Leu	Asn	Met 35	Lys	Asp	Ser	Gly	Leu 40	Asn	Val	Val	Val	Gly 45	Leu	Arg	Гла
Asn	Gly 50	Ala	Ser	Trp	Glu	Asn 55	Ala	Lys	Ala	Aab	Gly 60	His	Asn	Val	Met
Thr 65	Ile	Glu	Glu	Ala	Ala 70	Glu	Lys	Ala	Asp	Ile 75	Ile	His	Ile	Leu	Ile 80
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Tyr	Gly	Phe 115	Ile	Val	Pro	Pro	Lys 120	Gly	Val	Asn	Val	Val 125	Leu	Val	Ala
Pro	Lys 130	Ser	Pro	Gly	Lys	Met 135	Val	Arg	Arg	Thr	Tyr 140	Glu	Glu	Gly	Phe
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Gly	Val	Ile	Gln 180	Thr	Thr	Phe	ГЛа	Glu 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly	Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Val	Thr	Glu	Leu 205	Ile	Lys	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	Glu	Thr	Сүз	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Ile	Tyr	Gln 240
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Glu	Phe 290	Val	Leu	Glu	Lys	Gln 295	Val	Asn	His	Ala	His 300	Leu	Lys	Ala	Met
Arg 305	Arg	Ile	Glu	Gly	Asp 310	Leu	Gln	Ile	Glu	Glu 315	Val	Gly	Ala	Lys	Leu 320
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25 40 45 Ann Qiy Ala Ser Trp Aen Ann Ala Lye Ala Aep Qiy Hie Aon Val Net So So Thr Ile Clu Glu Glu Ala Ala Glu Lye Ala Aep Ile Ile Hie Ile Leu Ile Fro Aep Glu Leu Gln Ala Glu Val Tyr Glu Ser Gln Ile Lye Pro Tyr 90 Pro Aep Glu Leu Gln Ala Glu Val Tyr Glu Ser Gln Ile Lye Pro Tyr 91 92 94 95 95 96 96 97 98 98 99 99 90 90 91 91 92 94 95 95 96 96 97 98 98 99 99 90 90 91 91 92 93 94 95 95 96 96 97 98 99 99 90 91 92 93 94 95 96 <td>Lys</td> <td>Thr</td> <td>Ile</td> <td></td> <td>Val</td> <td>Ile</td> <td>Gly</td> <td>Tyr</td> <td></td> <td>Ser</td> <td>Gln</td> <td>Gly</td> <td>Arg</td> <td></td> <td>Gln</td> <td>Ser</td> <td>r</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>	Lys	Thr	Ile		Val	Ile	Gly	Tyr		Ser	Gln	Gly	Arg		Gln	Ser	r						
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65 70 75 80 Pro Asp Glu Leu Gin Ala Glu Val Tyr Glu Ser Gin Ile Lys Pro Tyr 95 Leu Lys Glu Gly Lys Thr Leu Ser Phe Ser His Gly Phe Asn Ile His 100 Tyr Gly Phe Ile Val Pro Pro Lye Gly Val Asn Val Val Leu Val Ala 115 Fro May Ger Pro Gly Lys Met Val Arg Arg Thr Tyr Glu Glu Gly DPhe 110 Gly Val Pro Gly Leu Ile Cys Ile Glu Ile Asp Ala Thr Asn Asn Ala 160 Phe Asp Ile Val Ser Ala Met Ala Lys Gly Ile Cly Leu Ser Arg Ala 175 Gly Val In Chin Thr Phe Lys Glu Glu Thr Glu Leu Ile Val Ala 186 Phe Jap Ile Val Ser Ala Met Ala Lys Gly Tyr Ala Pro Glu Met Ala Tyr 210 Gly Val In Chin Thr Phe Lys Glu Glu Thr Glu Leu Ser Arg Ala 175 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 240 Lys Glu Fhr Arg Arg Ser Arg Ile Val Thr Ala Asp Ser Lys Ala 275 Gly Gly Ile Ult Leu Arg Alu Ile Gln Asp Gly Arg Phe Thr Lys 275 Gly Gly Ile Glu Glu Glu Glu Glu Glu Glu 315 Ala Met Lys Glu Ile Glu App Leu Glu Glu Glu Glu 315 Arg Arg Leu Glu Gly Ang Leu Glu Glu Glu Glu Glu 316 310 210 Gly Glu Fhe Leu Leu Glu Glu Glu Glu Glu Glu Glu Glu Glu Gl	Asn		Ala	Ser	Trp	Asn		Ala	Lys	Ala	Asp	-	His	Asn	Val	Met	2						
Leu Lys Glu Gly Lys Thr Leu Ser Phe Ser His Gly Phe Asm Tie His 110 Tyr Gly Phe 11e Val Pro Pro Lyg Gly Val Asm Val Val Leu Val Ala 115 Tyr Gly Phe 11e Val Pro Pro Lyg Gly Val Asm Val Val Leu Val Ala 115 Pro Lyg Ser Pro Gly Lys Het Val Arg Arg Thr Tyr Glu Glu Gly Phe 130 Gly Val Pro Gly Leu He Cys I1e Glu I1e Asp Ala Thr Asm Asm Ala 160 Phe Asp 11e Val Ser Ala Met Ala Lys Gly 11e Gly Leu Ser Arg Ala 175 Gly Val I1e Gln Thr Thr Phe Lys Glu Glu Thr Glu Thr Asp Pro 180 Gly Val I1e Gln Thr Thr Phe Lys Gly Gly Val Thr Glu Leu I1e Lys Ala 180 Gly Val I1e Gln Thr Thr Phe Lys Gly Gly Val Thr Glu Leu I1e Lys Ala 180 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210 Phe Glu Thr Leu Val Glu Lu Lys Leu I1e Val Asp Leu I1e Tyr Gln 225 Gly Gly Phe Jry Asm Met Trp Asm Asp Val Ser Asm Thr Ala Glu Tyr 245 Gly Gly Ibe Lys Asm Met Trp Asm Asp Val Ser Asm Thr Ala Glu Tyr 250 Glu Phe Leu Leu Glu Lys Glu I1e Gln Asp Gly Arg Phe Thr Lys 275 Glu Fhe Leu Leu Glu Lys Glu Glu Glu Glu Asp Gly Arg Phe Thr Lys 275 Ala Met Lys Glu 11e Leu Arg Glu I1e Glu Glu Gly Arg Phe Thr Lys 270 Arg Arg Leu Glu Gly Asp Leu Gln 11e Glu Glu Val Gly Ala Lys Leu 305 Arg Arg Leu Glu Gly Asp Leu Gln 11e Glu Glu Val Gly Ala Lys Leu 305 4120 4215 420 4215 420 4215 420 4215 420 4215 420 420 420 4215 4215 4215 421 421 421 421 421 421 421 421		Ile	Glu	Glu	Ala		Glu	Lys	Ala	Asp		Ile	His	Ile	Leu		5						
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Tyr 65	Glu	Ile	Gly	Glu	Ala 70	Val	Arg	Lys	Ala	Asp 75	Val	Ile	Leu	Val	Leu 80	
			N - +-	a 1	~ 1	Deve	T	V 01	Trn	Gln	Glu	Gln	Ile	Ala	Pro	
Ile	Pro	Asp	Met	85	GIn	Pro	цув	vai	90					95		

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Pro		_						_	Luc	Glu	Glu	Ile	Glu	Pro	Asn
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	-		85				-	90					95					
Ile Ly	-	100					105			-		110						
Tyr As	115				-	120	-		-		125							
Pro Ly 13	0		-		135		-			140		-	-	-				
Gly Il 145	e Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160				
Lys As	n Val	Ala	Leu	Ser	Tyr	Ala	Ala	Gly	Val	Gly	Gly	Gly	Arg	Thr				

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Gly	Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Lys	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln
Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
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Lys	Asn														
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Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	His	Ala
Сүв	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Arg	Ser
Gly	Ser 50	Ala	Thr	Val	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Leu	Lys	Val	Ala
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ГЛа	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Суз	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
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Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Ala	1					
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Гла	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Ala 170	Val	Gly	Gly	Gly	Arg 175	Thr	:					
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Gly	Glu	Gln	Ala	Val	Leu	Суз	Gly	Gly	Thr	Val	Glu	Leu	Val	Lys	Ala	1					

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ly Ph		u I	ſhr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr	
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25			_	_	230	_	_			235	_	_			240	
ly Gl	y Il	.e A	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr	
ly Gl	u Ty		/al 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln	
la Me [.]	t Ar 27		\sn	Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys	
let Ph		.e S	Ger	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys	
rg Ar	g As	n A	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320	
rg Se	r Me	et M	/let	Pro 325	Trp	Ile	Gly	Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Ala	
ya Asi	n															
223> (400> ; tctct	SEQU	JENC	CE :	20		_	ımer	рвы) 261	>						24
210> 2 211> 2 212> 2 220> 2 220> 2 220> 2 221> 1 222> 2 222> 2	LENG TYPE ORGA FEAT OTHE FEAT NAME LOCA	TH: S: I NIS URE R I SURE VRE Z/KE	: 2 [°] DNA SM: S: UNF(S: SY: DN:	7 art: DRMA miso (26)	FION c_fea)(2	: pr ature 27)	imer e	₽F5∙								
400>	SEQU	JENC	CE :	21												
aagcc	gtgg	a do	ctto	cage	ct tạ	ggcki	nn									27
210> 3 211> 3 212> 9 213> 0 220> 3 223> 0 400> 3	LENG TYPE ORGA FEAT OTHE	TH: E: E NIS URE R I	: 23 DNA SM: E: INF(art: DRMA			-		0 866	5						
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Lys Asn

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Lys Asn

<210> SEQ ID NO 26 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: synthetic construct mutant ilcV <400> SEQUENCE: 26 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Phe Gly Ser Gln Gly His Ala Gln Ala Leu Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Tyr Lys Gly Ala Ala Asp Ala Ala Lys Ala Glu Ala His Gly Phe Lys Val Thr Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Val Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala

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<210> SEO ID NO 29 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Artificial sequence <220> FEATURE: <223> OTHER INFORMATION: synthetic construct <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (24)..(24) <223> OTHER INFORMATION: Xaa = Tyr or Phe <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (33)..(33) <223> OTHER INFORMATION: Xaa = Cys or Leu <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (47)..(47) <223> OTHER INFORMATION: Xaa = Arg or Tyr <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (50)..(50) <223> OTHER INFORMATION: Xaa = Ser or Ala <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (52)..(52) <223> OTHER INFORMATION: Xaa = Thr or Asp <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (53)..(53) <223> OTHER INFORMATION: Xaa = Val or Ala <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (61)..(61) <223> OTHER INFORMATION: Xaa = Leu or Phe <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (80)..(80) <223> OTHER INFORMATION: Xaa = Thr or Iso <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (156)..(156) <223> OTHER INFORMATION: Xaa = Ala or Val <220> FEATURE: <221> NAME/KEY: MISC_FEATURE <222> LOCATION: (170)..(170) <223> OTHER INFORMATION: Xaa = Gly or Ala <400> SEQUENCE: 29 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly 10 1 5 15 Lys Lys Val Ala Ile Ile Gly Xaa Gly Ser Gln Gly His Ala Gln Ala 2.0 25 30 Xaa Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Xaa Lys 40 35 45 Gly Xaa Ala Xaa Ala Lys Ala Glu Ala His Gly Xaa Lys Val Thr 55 50 60 Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Xaa 70 75 65 80 Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn 90 Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His 105 100 110 Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala 115 120 125 Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly 135 130 140

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Ile	His	Tyr 115		Gln	Ile	Glu	Pro 120	Ser	Glu	Aap	Val	Asn 125	Val	Thr	Met
Val	Ala 130	Pro	Lys	Ser	Pro	Gly 135	His	Leu	Val	Arg	Arg 140	Asn	Tyr	Glu	Asn
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Glu Ala His Asp Leu Gly Leu Ala Tyr Ala Lys Ala 11e Gly Cys Thr 165 165 177 185 185 185 186 186 186 186 187 186 187 186 187 186 187 186 187 186 187 186 187 186 187 186 187 187 187 187 187 187 187 187													-	con	tin	ued	
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Ala Tyr Fhe Glu Cys Leu Am Glu Leu Lys Leu He Val Asp Leu Met 235 Tyr Glu Gly Gly Am Ser Glu Met Trp Asp Ser Val Ser Asp Thr Ala 246 Tyr Glu Gly Gly Leu Thr Arg Gly Asp Asp He Val Ser Asp Thr Ala 250 Glu Tyr Gly Gly Leu Thr Arg Gly Asp Asp He Val Ser Asp Thr Ala 260 Arg Glu Lys Met Glu Glu Val Leu Glu Glu Val Gln Asm Gly Thr Fhe 275 Ala Arg Glu Trp The Ser Glu Asm Gln Ala Gly Arg Pro Ser Tyr Lys 200 Gln Leu Arg Ala Ala Glu Lys Asm His Asp The Glu Ala Val Gly Glu 305 Asp Leu Arg Ala Ala Glu Lys Asm His Asp The Glu Ala Val Gly Glu 310 2215 4215 4220 420 420 420 420 420 420 42	L	eu	Phe	-	Glu	Gln	Ala	Val		Суз	Gly	Gly	Val		Ser	Leu	Val
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145 150 155 160	P			Gly	Pro	Gly	His		Val	Arg	Arg	Thr	-	Glu	Gln	Gly	Ala
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n Leu As
n Ala Gly As
p Ala Leu Leu Phe Gly His Gly Leu As
n $% \mathbb{C} (\mathbb{R}^{n})$ Ile His Phe Asp Leu Ile Lys Pro Ala Asp Asp Ile Ile Val Gly Met Val Ala Pro Lys Gly Pro Gly His Leu Val Arg Arg Gln Phe Val Asp Gly Lys Gly Val Pro Cys Leu Ile Ala Val Asp Gln Asp Pro Thr Gly Thr Ala Gln Ala Leu Thr Leu Ser Tyr Ala Ala Ala Ile Gly Gly Ala Arg Ala Gly Val Ile Pro Thr Thr Phe Glu Ala Glu Thr Val Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Glu Glu Leu Val

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(Ju	Gly	Gly	Ile	Ala 245	Asn	Met	Arg	Tyr	Ser 250	Ile	Ser	Asn	Thr	Ala 255	Glu	
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i	\la	Glu	Met 275		Arg	Val	Leu	Ala 280		Ile	Gln	Ser	Gly 285		Phe	Val	
i	٩rg			Met	Leu	Glu			Ala	Gly	Gln			Phe	Lys	Ala	
		290 Arg	Arg	Ile	Gln		295 Glu	His	Val	Ile		300 Val	Val	Gly	Glu	-	
	305 Leu	Arg	Gly	Met	Met	310 Pro	Trp	Ile	Ser	Lys	315 Asn	Lys	Leu	Val	Asp	320 Lys	
;	Ala	Arq	Asn		325					330					335		
-		- 5															
•	211 212	-> LE 2> TY	EQ II ENGTH (PE : RGANI	H: 3 PRT		omona	as mo	obili	ls								
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	let L	Lys	Val	Tyr	Tyr 5	Asp	Ser	Asp	Ala	Asp 10	Leu	Gly	Leu	Ile	Lys 15	Ser	
1	JÀa	Lys	Ile	Ala 20	Ile	Leu	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	His	Ala	
(3ln	Asn	Leu 35	Arg	Asp	Ser	Gly	Val 40	Ala	Glu	Val	Ala	Ile 45	Ala	Leu	Arg	
I	Pro	Asp 50	Ser	Ala	Ser	Val	Lys 55	Lys	Ala	Gln	Aap	Ala 60	Gly	Phe	Lys	Val	
	leu 55	Thr	Asn	Ala	Glu	Ala 70	Ala	Lys	Trp	Ala	Asp 75	Ile	Leu	Met	Ile	Leu 80	
i	Ala	Pro	Asp	Glu	His 85	Gln	Ala	Ala	Ile	Tyr 90	Ala	Glu	Asp	Leu	Lys 95	Asp	
i	\sn	Leu	Arg	Pro 100	Gly	Ser	Ala	Ile	Ala 105	Phe	Ala	His	Gly	Leu 110	Asn	Ile	
1	lis	Phe	Gly 115	Leu	Ile	Glu	Pro	Arg 120		Asp	Ile	Asp	Val 125	Phe	Met	Ile	
j	Ala	Pro 130		Gly	Pro	Gly	His 135	Thr	Val	Arg	Ser	Glu 140	Tyr	Val	Arg	Gly	
	31y 145		Val	Pro	Суз	Leu 150		Ala	Val	Asp	Gln 155		Ala	Ser	Gly	Asn 160	
						100							a 1	a 1			
	Ala	His	Asp	Ile	Ala 165	Leu	Ala	Tyr	Ala		Gly	IIe	GIY	GIY	_		
			-	Ile	Ala 165 Glu			-	Arg	170	-		-	Thr	175	-	
:	Ser	Gly	Val	Ile 180	165	Thr	Thr	Phe	Arg 185	170 Glu	Glu	Val	Glu	Thr 190	175 Asp	Leu	

												2011	C 111	aca	
Ala	Gly 210	Phe	Glu	Thr	Leu	Thr 215	Glu	Ala	Gly	Tyr	Ala 220	Pro	Glu	Met	Ala
Phe 225	Phe	Glu	Суз	Met	His 230	Glu	Met	ГЛа	Leu	Ile 235		Asp	Leu	Ile	Tyr 240
Glu	Ala	Gly	Ile	Ala 245	Asn	Met	Arg	Tyr	Ser 250	Ile	Ser	Asn	Thr	Ala 255	Glu
Tyr	Gly	Aap	Ile 260	Val	Ser	Gly	Pro	Arg 265	Val	Ile	Asn	Glu	Glu 270	Ser	Lys
Lya	Ala	Met 275	ГЛа	Ala	Ile	Leu	Asp 280	Asp	Ile	Gln	Ser	Gly 285	Arg	Phe	Val
Ser	Lys 290	Phe	Val	Leu	Asp	Asn 295	Arg	Ala	Gly	Gln	Pro 300	Glu	Leu	Lys	Ala
Ala 305	Arg	Lys	Arg	Met	Ala 310	Ala	His	Pro	Ile	Glu 315		Val	Gly	Ala	Arg 320
	Arg	Lys	Met	Met 325		Trp	Ile	Ala	Ser 330			Leu	Val	Asp 335	
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Asn	Asn	Leu 35	Lys	Glu	Ser	Gly	Val 40	Asp	Val	Val	Val	Gly 45	Leu	Arg	Glu
Gly	Ser 50	Ser	Ser	Ala	Ala	Lys 55	Ala	Gln	Lys	Ala	Gly 60	Leu	Ala	Val	Ala
Ser 65	Ile	Glu	Asp	Ala	Ala 70	Ala	Gln	Ala	Asp	Val 75	Val	Met	Ile	Leu	Ala 80
Pro	Asp	Glu	His	Gln 85	Ala	Val	Ile	Tyr	His 90	Asn	Gln	Ile	Ala	Pro 95	Asn
Val	Lys	Pro	Gly 100	Ala	Ala	Ile	Ala	Phe 105	Ala	His	Gly	Phe	Asn 110	Ile	His
Phe	Gly	Gln 115	Ile	Gln	Pro	Ala	Ala 120	Asp	Leu	Asp	Val	Ile 125	Met	Val	Ala
Pro	Lys 130	Gly	Pro	Gly	His	Leu 135	Val	Arg	Ser	Thr	Tyr 140		Glu	Gly	Gly
Gly 145	Val	Pro	Ser	Leu	Ile 150	Ala	Ile	His	Gln	Asp 155		Thr	Gly	Lys	Ala 160
Lys	Asp	Ile	Ala	Leu 165	Ser	Tyr	Ala	Ser	Ala 170	Asn	Gly	Gly	Gly	Arg 175	
Gly	Val	Ile	Glu 180	Thr	Ser	Phe	Arg	Glu 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly	Glu	Gln 195	Ala	Val	Leu	Сүз	Gly 200		Ile	Thr	Ser	Leu 205	Ile	Gln	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220		Met	Ala	Tyr

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Phe G 225	lu	Суз	Leu	His	Glu 230	Thr	Lys	Leu	Ile	Val 235	Asp	Leu	Leu	Tyr	Gln 240
Gly G	ly	Ile	Ala	Asn 245	Met	Arg	Tyr	Ser	Ile 250	Ser	Asn	Thr	Ala	Glu 255	Tyr
Gly A	ab	Phe	Thr 260	Arg	Gly	Pro	Arg	Val 265	Ile	Asn	Glu	Glu	Ser 270	Arg	Glu
Ala M		Arg 275	Glu	Ile	Leu	Ala	Glu 280		Gln	Glu	Gly	Glu 285	Phe	Ala	Arg
Glu P 2	he 90	Val	Leu	Glu	Asn	Gln 295	Ala	Gly	Cys	Pro	Thr 300	Leu	Thr	Ala	Arg
Arg A 305	rg	Leu	Ala	Ala	Glu 310	His	Glu	Ile	Glu	Val 315	Val	Gly	Glu	Arg	Leu 320
Arg G	ly	Met	Met	Pro 325	Trp	Ile	Asn	Ala	Asn 330	Lys	Leu	Val	Asp	Lys 335	Asp
Lys A	sn														
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His A		Met 35	Asn	Leu	Arg	Asp	Ser 40	Gly	Val	Glu	Val	Ile 45	Ile	Gly	Leu
Lys G 5		Gly	Gly	Gln	Ser	Trp 55	Ala	Lys	Ala	Gln	Lys 60	Ala	Asn	Phe	Ile
Val L 65	Уa	Ser	Val	Lys	Glu 70	Ala	Thr	Lys	Glu	Ala 75	Asp	Leu	Ile	Met	Ile 80
Leu A	la	Pro	Asp	Glu 85	Ile	Gln	Ser	Glu	Ile 90	Phe	Asn	Glu	Glu	Ile 95	ГЛа
Pro G	lu	Leu	Lys 100	Ala	Gly	Lys	Thr	Leu 105	Ala	Phe	Ala	His	Gly 110	Phe	Asn
Ile H		Tyr 115	Gly	Gln	Ile	Val	Ala 120	Pro	Lys	Gly	Ile	Asp 125	Val	Ile	Met
Ile A 1	la 30	Pro	Гла	Ala	Pro	Gly 135	His	Thr	Val	Arg	His 140	Glu	Phe	Ser	Ile
Gly G 145	ly	Gly	Thr	Pro	Cys 150	Leu	Ile	Ala	Ile	His 155	Gln	Asp	Glu	Ser	Lys 160
Asn A	la	Lys	Asn	Leu 165	Ala	Leu	Ser	Tyr	Ala 170	Ser	Ala	Ile	Gly	Gly 175	Gly
Arg T	hr	Gly	Ile 180	Ile	Glu	Thr	Thr	Phe 185	Lys	Ala	Glu	Thr	Glu 190	Thr	Asp
Leu P		Gly 195	Glu	Gln	Ala	Val	Leu 200		Gly	Gly	Leu	Ser 205	Ala	Leu	Ile
Gln A 2	la 10	Gly	Phe	Glu	Thr	Leu 215	Val	Glu	Ala	Gly	Tyr 220	Glu	Pro	Glu	Met
Ala T 225	yr	Phe	Glu	СЛа	Leu 230		Glu	Met	Lys	Leu 235	Ile	Val	Asp	Leu	Ile 240

Tyr Gln Gly Gly Ile Ala Asp Met Arg Tyr Ser Val Ser Asn Thr Ala Glu Tyr Gly Asp Tyr Ile Thr Gly Pro Lys Ile Ile Thr Lys Glu Thr Lys Glu Ala Met Lys Gly Val Leu Lys Asp Ile Gln Asn Gly Ser Phe Ala Lys Asp Phe Ile Leu Glu Arg Arg Ala Asn Phe Ala Arg Met His Ala Glu Arg Lys Leu Met Asn Asp Ser Leu Ile Glu Lys Thr Gly Arg Glu Leu Arg Ala Met Met Pro Trp Ile Ser Ala Lys Lys Leu Val Asp Lys Asp Lys Asn <210> SEQ ID NO 37 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Marinobacter aquaeolei <400> SEQUENCE: 37 Met Gln Val Tyr Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Leu Gly Phe Gly Ser Gl
n Gly His Ala His Ala 20\$25\$30 Cys Asn Leu Lys Asp Ser Gly Val Asp Val Val Val Gly Leu Arg Ala Gly Ser Ser Ser Ile Ala Lys Ala Glu Ala Tyr Gly Leu Lys Thr Ser
 Asp Val Ala Ser Ala Val Ala Ser Ala Asp Val Val Met Val Leu Thr

 65
 70
 75
 80
 Pro Asp Glu Phe Gln Ala Gln Leu Tyr Arg Glu Glu Ile Glu Pro Asn Leu Lys Gln Gly Ala Thr Leu Ala Phe Ala His Gly Phe Ala Ile His Tyr Asn Gln Ile Val Pro Arg Lys Asp Leu Asp Val Ile Met Val Ala Pro Lys Ala Pro Gly His Thr Val Arg Thr Glu Phe Thr Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Phe Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ser Gly Ile Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Ala Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Thr Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr

Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Glu Gln Ser Arg Glu Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Ser Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Leu Asn Tyr Pro Ser Met Thr Ala Arg Arg Arg Gln Asn Ala Ala His Glu Ile Glu Thr Val Gly Glu Lys Leu Arg Ser Met Met Pro Trp Ile Ser Ala Asn Lys Ile Val Asp Lys Asp Lys Asn <210> SEQ ID NO 38 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Psychrobacter arcticus <400> SEQUENCE: 38 Met Asn Val Tyr Tyr Asp Lys Asp Cys Asp Leu Ser Ile Val Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gl
n Gly His Ala His Ala Leu Asn Leu Gln Asp Ser Asn Val Asp Val Thr Val Gly Leu Arg Ala Asp Ser Gly Ser Trp Lys Lys Ala Glu Asn Ala Gly Leu Lys Val Ala Glu Val Glu Ala Val Lys Ala Ala Asp Ile Ile Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Lys Glu Leu Tyr Asn Asp Val Ile Glu Pro Asn Ile Lys Gln Gly Ala Thr Leu Ala Phe Ala His Gly Phe Ala Ile His Tyr Asn Gln Val Ile Pro Arg Ser Asp Leu Asp Val Ile Met Val Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Ala Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Ala Ser Gly Gln Ala Lys Gln Leu Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Ser Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Ala Val Glu Leu Val Lys Met Gly Phe Glu Thr Leu Thr Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asp Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Glu Gln Ser Arg Glu

Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Ser Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Asn Tyr Pro Ser Met Thr Ala Arg Arg Arg Asn Asn Ala Glu His Gln Ile Glu Ile Thr Gly Ala Lys Leu Arg Gly Met Met Pro Trp Ile Gly Gly Asn Lys Ile Ile Asp Lys Asp Lys Asn <210> SEQ ID NO 39 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Hahella chejuensis <400> SEQUENCE: 39 Met Gln Val Tyr Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala His Ala Asn Asn Leu Lys Asp Ser Gly Val Asp Val Cys Val Gly Leu Arg Lys 35 40 45 Gly Ser Gly Ser Trp Ala Lys Ala Glu Asn Ala Gly Leu Ala Val Lys 50 55 60 Glu Val Ala Glu Ala Val Ala Gly Ala Asp Val Val Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Ala Gln Leu Tyr Lys Ser Glu Ile Glu Pro Asn Leu Lys Ser Gly Ala Thr Leu Ala Phe Ala His Gly Phe Ser Ile His Tyr Asn Gln Ile Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Phe Gln Asp Ala Ser Gly Ser Ala Lys Asp Leu Ala Leu Ser Tyr Ala Ser Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Ala Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Asp Gln Ser Arg Ala - 265 Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys

Arg Arg Asn Asn Ala Ala His Pro Ile Glu Gln Val Gly Glu Lys Leu Arg Ser Met Met Pro Trp Ile Ala Ser Asn Lys Ile Val Asp Lys Ser Lys Asn <210> SEQ ID NO 40 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Thiobacillus denitrificans <400> SEQUENCE: 40 Met Lys Val Tyr Tyr Asp Lys Asp Ala Asp Leu Ser Leu Ile Lys Gln Arg Lys Val Ala Ile Val Gly Tyr Gly Ser Gln Gly His Ala His Ala Asn Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Ala Leu Arg Pro Gly Ser Ala Ser Ala Lys Lys Ala Glu Asn Ala Gly Leu Thr Val Lys Ser Val Pro Glu Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Thr65707580 Pro Asp Glu Phe Gln Ser Arg Leu Tyr Arg Asp Glu Ile Glu Pro Asn Ile Lys Gl
n Gly Ala Thr Leu Ala Phe Ala His Gly Phe Ser Ile His $% \left({{{\left[{{{\left[{{{c_{1}}} \right]}}} \right]}} \right)$ Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Ala Ser Gly Lys Ala Lys Glu Thr Ala Leu Ser Tyr Ala Ser Ala Ile Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Ala Val Glu Leu Val Lys Ala Gly Phe Asp Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Val Lys Val Ile Asn Glu Gln Ser Arg Ala Ala Met Lys Glu Cys Leu Ala Asn Ile Gln Asn Gly Ala Tyr Ala Lys Arg Phe Ile Leu Glu Gly Gln Ala Asn Tyr Pro Glu Met Thr Ala Trp

Met Phe Ile Ala Glu Gly Ala His Asn Tyr Pro Ser Met Thr Ala Tyr

Arg Ser Met Met Pro Trp Ile Ala Ala Asn Lys Leu Val Asp His Ser Lys Asn <210> SEQ ID NO 41 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Azotobacter vinelandii <400> SEQUENCE: 41 Met Lys Val Tyr Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Ser Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala His Ala Cys Asn Leu Lys Asp Ser Gly Val Asp Val Tyr Val Gly Leu Arg Ala Gly Ser Ala Ser Val Ala Lys Ala Glu Ala His Gly Leu Thr Val Lys Ser Val Lys Asp Ala Val Ala Ala Ala Asp Val Val Met Ile Leu Thr Pro Asp Glu Phe Gln Gly Arg Leu Tyr Lys Asp Glu Ile Glu Pro Asn Leu Lys Lys Gly Ala Thr Leu Ala Phe Ala His Gly Phe Ser Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Arg Gly Gly Gly Ile Pro Asp Leu Ile Ala Val Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Leu Ala Leu Ser Tyr Ala Cys Gly Val Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Cys Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Phe Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Glu Gln Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Thr Glu Gly Ala Ala Asn Tyr Pro Ser Met Thr Ala Tyr Arg Arg Asn Asn Ala Ala His Gln Ile Glu Val Val Gly Glu Lys Leu

Arg Arg Asn Asn Ala Ala His Gln Ile Glu Val Val Gly Ala Lys Leu

Arg Thr Met Met Pro Trp Ile Ala Ala Asn Lys Ile Val Asp Lys Thr Lys Asn <210> SEQ ID NO 42 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Pseudomonas syringae <400> SEOUENCE: 42 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Arg Lys Gly Ser Ala Thr Val Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr Asp Val Ala Ser Ala Val Ala Ala Ala Asp Leu Val Met Ile Leu Thr Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Val Glu Pro Asn Leu Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Thr Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Val Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ser Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Lys Leu Arg Ser Met Met Pro Trp Ile Ala Ala Asn Lys Ile Val Asp Lys Asp

Lys Asn

<210> SEQ ID NO 43 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Pseudomonas syringae <400> SEOUENCE: 43 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gl
n Gly His Ala Gl
n Ala $\ensuremath{\mathsf{Gln}}$ 2.0 Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Arg Lys Gly Ser Ala Thr Val Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr Asp Val Ala Ser Ala Val Ala Ala Ala Asp Leu Val Met Ile Leu Thr Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Val Glu Pro Asn Leu Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Thr Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Val Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ser Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala 2.05 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Thr Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Glu His Gly Ile Glu Val Ile Gly Glu Lys Leu Arg Ser Met Met Pro Trp Ile Ala Ala Asn Lys Ile Val Asp Lys Asp

Lys Asn

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<210> SEO ID NO 44 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Pseudomonas putida <400> SEOUENCE: 44 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gl
n Gly His Ala Gl
n Ala $\ensuremath{\mathsf{S}}$ 2.0 Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Arg Lys Gly Ser Ala Thr Val Ala Lys Ala Glu Ala His Gly Leu Lys Val Ala Asp Val Ala Thr Ala Val Ala Ala Ala Asp Leu Val Met Ile Leu Thr Pro Asp Glu Phe Gln Gly Ala Leu Tyr Lys Asn Glu Ile Glu Pro Asn Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ser Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ser Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Glu Glu Ser Arg Lys Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Asn Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Ser Ala Asn Lys Ile Val Asp Lys Thr Lys Asn

<210> SEQ ID NO 45 <211> LENGTH: 338

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	2 > T 3 > O			Pse	udom	onas	ento	omopl	nila						
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Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Суз	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Ile	Gly 45	Leu	Arg	Lys
Gly	Ser 50	Ala	Thr	Val	Ala	Lуз 55	Ala	Glu	Ala	His	Gly 60	Leu	Lys	Val	Thr
Asp 65	Val	Ala	Thr	Ala	Val 70	Ala	Ala	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Thr 80
Pro	Asp	Glu	Phe	Gln 85	Gly	Gln	Leu	Tyr	Lys 90	Gln	Glu	Ile	Glu	Pro 95	Asn
Ile	Lys	Lys	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Aap	Val	Ile 125	Met	Ile	Ala
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
Gly 145	Ile	Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ser	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
Gly	Ile	Ile	Glu 180	Thr	Thr	Phe	ГЛа	Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly	Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Гла	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	Glu	СЛа	Leu	His	Glu 230	Leu	ГЛа	Leu	Ile	Val 235	Aap	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Glu	Glu	Ser 270	Arg	Lys
Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Asn	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
Arg	Ser	Met	Met	Pro 325		Ile	Ser	Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Thr
гла	Asn														

<210> SEQ ID NO 46 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Pseudomonas mendocina

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Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Суз	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Ile	Gly 45	Leu	Arg	Lys
Gly	Ser 50	Ala	Thr	Val	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Leu	Lys	Val	Thr
Asp 65	Val	Ala	Ser	Ala	Val 70	Ala	Ala	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Thr 80
Pro	Asp	Glu	Phe	Gln 85	Gly	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn
Ile	Lys	Lys	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Asp	Val	Ile 125	Met	Ile	Ala
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Gly 145	Ile	Pro	Asp	Leu	Ile 150	Ala	Val	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ser	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
Gly	Ile	Ile	Glu 180	Thr	Thr	Phe	Lys	Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
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Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln
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Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Val 315	Ile	Gly	Glu	Gln	Leu 320
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ГЛа	Gly 50	ГÀа	Ser	Trp	Asp	Lys 55	Ala	Lya	Glu	Aap	Gly 60	Phe	Ser	Val	Tyr
Thr 65	Val	Ala	Glu	Ala	Ala 70	ГЛа	Gln	Ala	Asp	Val 75	Val	Met	Ile	Leu	Leu 80
Pro	Asp	Glu	Leu	Gln 85	Pro	Glu	Val	Tyr	Glu 90	Ala	Glu	Ile	Ala	Pro 95	Asn
Leu	Gln	Ala	Gly 100	Asn	Ser	Leu	Val	Phe 105	Ala	His	Gly	Phe	Asn 110	Val	His
Phe	Asb	Gln 115	Val	Lys	Pro	Pro	Ala 120	Asn	Val	Asp	Val	Phe 125	Leu	Val	Ala
Pro	Lys 130	Gly	Pro	Gly	His	Leu 135	Val	Arg	Arg	Thr	Phe 140	Ser	Glu	Gly	Gly
Ala 145	Val	Pro	Ala	Leu	Phe 150	Ala	Val	Tyr	Gln	Asp 155	Ala	Thr	Gly	Val	Ala 160
Thr	Glu	Lys	Ala	Leu 165	Ser	Tyr	Ala	Asp	Gly 170	Ile	Gly	Ala	Thr	Arg 175	Ala
Gly	Val	Leu	Glu 180	Thr	Thr	Phe	Lys	Glu 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly	Glu	Gln 195	Ala	Val	Leu	Сүз	Gly 200	Gly	Val	Thr	Ala	Leu 205	Val	Lys	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Asp 215	Ala	Gly	Tyr	Gln	Pro 220	Glu	Leu	Ala	Tyr
Phe 225	Glu	Сүз	Leu	His	Glu 230	Leu	ГÀа	Leu	Ile	Val 235	Aap	Leu	Met	Tyr	Glu 240
Gly	Gly	Leu	Glu	Asn 245	Met	Arg	Tyr	Ser	Val 250	Ser	Aap	Thr	Ala	Gln 255	Trp
Gly	Asp	Phe	Val 260	Ser	Gly	Pro	Arg	Val 265	Val	Thr	Glu	Asp	Thr 270	Lys	Lys
Ala	Met	Gly 275	Thr	Val	Leu	Ala	Glu 280	Ile	Gln	Asp	Gly	Thr 285	Phe	Ala	Arg
Gly	Trp 290	Ile	Ala	Glu	His	Lys 295	Ala	Gly	Arg	Pro	Asn 300	Phe	His	Ala	Thr
Asn 305	Glu	Lys	Glu	Asn	Glu 310	His	Glu	Ile	Glu	Val 315	Val	Gly	Arg	Lys	Leu 320
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Lys	Ala	Thr	Glu	Asn 85	Gly	Phe	Lys	Val	Gly 90	Thr	Tyr	Glu	Glu	Leu 95	Ile
Pro	Gln	Ala	Asp 100	Leu	Val	Ile	Asn	Leu 105	Thr	Pro	Asp	ГЛа	Gln 110	His	Ser
Asp	Val	Val 115	Arg	Thr	Val	Gln	Pro 120	Leu	Met	Lys	Asp	Gly 125	Ala	Ala	Leu
Gly	Tyr 130	Ser	His	Gly	Phe	Asn 135	Ile	Val	Glu	Val	Gly 140	Glu	Gln	Ile	Arg
Lys 145	Asp	Ile	Thr	Val	Val 150	Met	Val	Ala	Pro	Lys 155	Суз	Pro	Gly	Thr	Glu 160
Val	Arg	Glu	Glu	Tyr 165	Lys	Arg	Gly	Phe	Gly 170	Val	Pro	Thr	Leu	Ile 175	Ala
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Ser	Phe 210	Val	Ala	Glu	Val	Lys 215	Ser	Asp	Leu	Met	Gly 220	Glu	Gln	Thr	Ile
Leu 225	Cys	Gly	Met	Leu	Gln 230	Ala	Gly	Ser	Leu	Leu 235	Сүз	Phe	Asp	Lys	Leu 240
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Ala	Asn	Asp	Asp	Lys 325	ГЛЗ	Leu	Leu	Thr	Trp 330	Arg	Glu	Glu	Thr	Gly 335	Lys
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Val	Glu 370	Leu	Ala	Phe	Glu	Thr 375	Met	Val	Asp	Ser	Gly 380	Ile	Ile	Glu	Glu
Ser 385	Ala	Tyr	Tyr	Glu	Ser 390	Leu	His	Glu	Leu	Pro 395	Leu	Ile	Ala	Asn	Thr 400
Ile	Ala	Arg	Lys	Arg 405	Leu	Tyr	Glu	Met	Asn 410	Val	Val	Ile	Ser	Asp 415	Thr
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Lys	Pro	Phe 435	Met	Ala	Glu	Leu	Gln 440	Pro	Gly	Asp	Leu	Gly 445	Lys	Ala	Ile

Pro Glu Gly Ala Val Asp Asn Gly Gln Leu Arg Asp Val Asn Glu Ala Ile Arg Ser His Ala Ile Glu Gl
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n Thr Ile Leu Cys Gly Met Leu Gl
n Thr Gly Ala Val Leu Gly His Gl
n Gl
n Leu Ile Asn Leu Gly Val Asp Ala Ala Tyr Ala Arg Lys Leu Ile Gln Tyr Gly Trp Glu Thr Val Thr Glu Gly Leu Lys His Gly Gly Ile Thr Asn Met Met Asp Arg Leu Ser Asn Pro Ala Lys Ile Lys Ala Phe Asp Met Ser Glu Glu Leu Lys Val Thr Leu Arg Pro Leu Phe Glu Lys His Met Asp Asp Ile Ile Glu Gly Glu Phe Ser His Thr Met Met Ile Asp Trp

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Ser 385	Ala	Tyr	Tyr	Glu	Ser 390	Leu	His	Glu	Thr	Pro 395	Leu	Ile	Ala	Asn	Сув 400
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Glu	Val	Arg	Ala	Glu 165	Tyr	Gln	Arg	Gly	Phe 170	Gly	Val	Pro	Thr	Leu 175	Ile

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-	со	nt	11	nu	ed

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LYa	Asp		420 Met	Ala	Ser	Val		425 Thr	Glu	Val	Ile		430 Lys	Gly	Leu	J								
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Ala	450 Asp	Ile	Arg	Ser	His	455 Tyr	Ile	Glu	Glu	Ile	460 Gly	Glu	Glu	Leu	Arg	Э								
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Ser	Phe	Ser 35		Leu	Ser	Pro	Gln 40		Ile	Arg	Ala	Arg 45	Arg	Ser	Ile	Э								

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	С	со	con	cont	conti	contin	continu	continue

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Met 1	Lys	val	Pne	Tyr 5	Asb	гла	Aab	сув	Asp 10	Leu	Ser	IIe	IIe	GIN 15	GIŸ	
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- COIIC	TITUEO	

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-	Asn Ile			165		-			170		-	Thr	-		Phe
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Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys 290 295 300
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Leu Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu His Lys 35 40 45	
Gly Asp Ala Tyr Tyr Ala Lys Ala Glu Ala His Gly Phe Lys Val Thr 50 55 60	
Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Ile 65 70 75 80	
65 /0 /5 80	
Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn	
Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn 85 90 95 Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His	
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85 90 95 Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His 100 105 Phi Gly Phe Ala Ile His 110 Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met 112 110 Phe Ala 125 Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly	
85 90 95 Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His 100 Ile His 100 Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met 11e Ala 115 Ile Ala 120 Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly 130 Ile Arg Lus Ile Ala Ile Tyr Gln Asp Val Ser Gly Asn Ala	

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Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys 290 295 300
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Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn 85 90 95
Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His 100 105 110
Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala 115 120 125
Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly 130 135 140
Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Val Ser Gly Asn Ala 145 150 155 160
Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr 165 170 175
Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe
180 185 190

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Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr	r	
Phe 225	Glu	Сув	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Aap	Leu	Met	Tyr	Glu 240		
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr	r	
Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln	n	
Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys	8	
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys	s	
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320		
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ГЛа	Lys	Val	Ala 20	Ile	Ile	Gly	Phe	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala	a	
Leu	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Phe	Lys	8	
Gly	Ala 50	Ala	Asp	Ala	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Phe	Lys	Val	Thr	r	
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Pro	Asp	Glu	Phe	Gln 85	Ser	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn	n	
Ile	Lys	Lys	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His	s	
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Asp	Val	Ile 125	Met	Ile	Ala	a	
Pro	Lys 130		Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	ГЛа	Gly	Gly	У	
Gly 145		Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155		Ser	Gly	Asn	Ala 160		
		Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170			Gly	Gly	Arg 175			
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Gly	Glu	Gln 195		Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205		Lys	Ala	a	
Gly	Phe		Thr	Leu	Val	Glu			Tyr	Ala	Pro		Met	Ala	Tyr	r	

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Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln
Ala	Met	Arg 275		Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
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Leu	Asn	Leu 35	Lys	Aap	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Asn	Lys
Gly	Asn 50	Ala	Asp	Ala	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Phe	Lys	Val	Thr
Asp 65	Val	Ala	Ala	Ala	Val 70	Ala	Gly	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Ile 80
Pro	Asp	Glu	Phe	Gln 85	Ser	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn
Ile	Lys	Lys	Gly 100		Thr					His	Gly	Phe	Ala 110	Ile	His
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Asp	Val	Ile 125	Met	Ile	Ala
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
Gly 145	Ile	Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Val	Ser	Gly	Asn	Ala 160
ГЛЗ	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
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Gly	Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Гла	Ala
Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr

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Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 82 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant JEA7 <400> SEQUENCE: 82 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Phe Gly Ser Gl
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Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
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Gly	Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln
Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Aap	Gly	Glu 285	Tyr	Ala	Lys
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Phe	Glu	Cys	Leu	His	Glu	Leu	Lys	Leu	Ile	Val	Asp	Leu	Met	Tyr	Glu

120

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Суз	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Tyr	Гла
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GLY GLY ILe Als Am Net Am TYP Ser Tie Ger Am An Als Glu Syr GLY GLU TYF Val Thr GLY Pro GLU Val ILe Am Als GLU Ser Arg GLU Als Met Arg 200 Als Met Arg Am Als Leu Lyb Arg ILe GLU Arg GLU GLU Var Als Lyb 200 Als Met Arg Am An Als Leu Lyb Arg ILe GLU Arg GLU												-	con	τın	uea			
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Met Phe 11e Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys 290 Arg Asg Aen Aen Ala Ala His Gly Tile Glu Tile Gly Glu Gln Leu 315 310 310 310 310 310 310 320 Arg Ser Met Met Pro Tp Ile Gly Ala Aen Lys Ile Val Asp Lys Ala 325 Typ Aen <pre> </pre> (110 ESRO ID NO 86 <pre> <pre> Arg Ser Met Met Pro Tp Ile Gly Ala Aen Lys Ile Val Asp Lys Ala 320 </pre> <pre> <pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre></pre>	Gly	Glu	Tyr		Thr	Gly	Pro	Glu		Ile	Asn	Ala	Glu		Arg	Gln		
290 295 300 300 Arg Arg An An Ala Ala Min Gly Ile Glu Ile Ile Gly Glu Glu Eu 315 315 315 Arg Ser Met Met Pro Trp Ile Gly Ala An Lyø Ile Val Aøp Lyø Ala 315 313 313 310 310 310 315 Lyø Am 210 > 550 JD N0 86 211 > LEMCOTH: 338 $211 > LEMCOTH: 338211 > CHCOTH: 338212 > CHCOTH: 13811 = 110 CHT The Ja Lyø Ja CH Ja Ja CH Ja Ja GH Ja Ang Leu Ja CH Lie Leu Thr 609511 = Lyø Lyø CHJ Ala Thr Leu Ala Phe Ser His CHJ He Lie Thr 809010 = 10010 =$	Ala	Met		Asn	Ala	Leu	Lys		Ile	Gln	Asp	Gly		Tyr	Ala	Lys		
363 310 315 320 Arg Ser Net Mer Pro Tz lle Gly Ala Ann lys lle Val Ap Lys Ala Jys Ala 1ys Ann 330 330 10 10 2110 SEO ID NO 96 330 330 10 10 2121 FYER SEO ID NO 96 330 10 10 2121 FYER SEO ID NO 96 330 10 10 2121 FYER SEO GEOMENE: 330 10 10 11 2020 FEANWER: SEOUENE: 30 10 11 10 11 2000 SEQUENCE: 86 Met Joo An Jao 11 10 11 11 10 <td< td=""><td>Met</td><td></td><td>Ile</td><td>Ser</td><td>Glu</td><td>Gly</td><td></td><td>Thr</td><td>Gly</td><td>Tyr</td><td>Pro</td><td></td><td>Met</td><td>Thr</td><td>Ala</td><td>Lys</td><td></td><td></td></td<>	Met		Ile	Ser	Glu	Gly		Thr	Gly	Tyr	Pro		Met	Thr	Ala	Lys		
1 225 330 1 335 Lyg Am *210 > SEQ ID NO 06 ************************************			Asn	Asn	Ala		His	Gly	Ile	Glu		Ile	Gly	Glu	Gln			
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CyaAsLeuLysAsSerGlyValAsValThValGlyLeuPheLysGlyAlaAsPTrValAsValThValGlyLeuPheLysGlyAlaAlaAsProProAsProProAsAla	1				5	_	-	_	-	10					15	_		
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65 70 75 80 Pro Asp Gl Pre Sin Gln Lev Ty Lys Asn Glu Pre Asn Ile Lys Lys Gly Ala Pre Ala Ile Glu Pre Ala Pre Ala Pre Ala Ile His Tyr Asn Glu Val Val Pre Ala Ile Mat Ile Ala Ile Asn Ile Mat Ile Ala Ile Asn Ile Ala Ile Asn Ile Ile Ala Ile Asn Ile Ile Ala Ile Asn Ile Ile Asn Ile Ile Ile Ile Ile Ile Ile Ile <td>Gly</td> <td></td> <td>Ala</td> <td>Asp</td> <td>Trp</td> <td>Ala</td> <td></td> <td>Ala</td> <td>Glu</td> <td>Ala</td> <td>His</td> <td></td> <td>Leu</td> <td>Lys</td> <td>Val</td> <td>Thr</td> <td></td> <td></td>	Gly		Ala	Asp	Trp	Ala		Ala	Glu	Ala	His		Leu	Lys	Val	Thr		
35 90 95 Ile Lys Lys Gly Ala Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Val Pho Arg Ala Asp Leu Asp Val Ile Met Ile Ala Tyr Asn Gln Val Val Pro Gly Ala Pho Ser His Gly Phe Ala Ile Met Ile Ala Tyr Asn Gln Val Val Pro Gly Ala Pho Ser Glu Pho Pho Ala Ile Met Ile Ala Tyr Asn Gln Val Val Pro Gly Fin Pho Arg Ala Asp Leu Asp Val Ile Met Ile Ala Tyr Asn Gly Ala Pro Gly Fin Pho Arg Ala Asp Con Pho			Ala	Ala	Ala		Ala	Gly	Ala	Asp		Val	Met	Ile	Leu			
100 105 110 Tyr Asn Gln Val Val Arg Ala Asp Leu Asp Val 126 Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Gly Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ala Ile Yat Ala Ser Gly Asn Ala Ile Yat Ala Ser Gly Asn Ala Ile Yat Ala Ser Gly Asn Ala Ile Yat Ala Ile Ala Ile Yat Ala Ile Yat Ala Ile Yat Ala Ile Ile	Prc	Asp	Glu	Phe		Ser	Gln	Leu	Tyr	-	Asn	Glu	Ile	Glu		Asn		
115 120 125 Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Gly Ile Pro Ass Leu Ile Ala Pro Ala Ala Pro Ala Ala Leu Fro Ala Leu Ile Tro Gly Ala Ala Ala Gly Ala Ala Fro Ala Leu Fro Ala Leu Fro Ala Ile Fro Ala Ile Fro Ala Ile Fro Ser Gly Ala Ala Fro Fro Ala Ile Fro Fro Ala Ile Fro F	Ile	Lys	Lys	-	Ala	Thr	Leu	Ala		Ser	His	Gly	Phe		Ile	His		
130 140 Gly II Ser III III III III III III III III III IIII IIII IIIII IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	Tyr	Asn		Val	Val	Pro	Arg		Asp	Leu	Asp	Val		Met	Ile	Ala		
145 150 160 Lys Asn Val Ala Lee Tyr Ala Alg Alg Alg Gly Val Gly Gly Gly Alg Alg Gly Ile Ile Slo Tyr Ala Ala Alg Gly Gly Gly Gly Gly Alg Frag Gly Ile Slo Tyr Frag Gly Slo Tyr Slo Frag Slo Frag Gly Gly Slo Slo Tyr Slo Slo Slo Slo Slo Slo Slo Gly Gly Slo Gly Glo Slo Slo Slo Slo Slo Slo Slo Slo Slo Glo Slo Glo Slo Slo Slo Slo Slo Slo Slo Slo Slo	Pro		Ala	Pro	Gly	His		Val	Arg	Ser	Glu		Val	Lys	Gly	Gly		
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180 185 190 Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala 200 110 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210 111 Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 235 240	ГЛЭ	Asn	Val	Ala		Ser	Tyr	Ala	Ala	-	Val	Gly	Gly	Gly	-	Thr		
195200205Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210215Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 235235225230235	Gly	Ile	Ile		Thr	Thr	Phe	Lys	-	Glu	Thr	Glu	Thr	-	Leu	Phe		
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225 230 235 240	Gly			Thr	Leu	Val			Gly	Tyr	Ala		Glu	Met	Ala	Tyr		
Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr			Cys	Leu	His			Lys	Leu	Ile		Asp	Leu	Met	Tyr			
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				245					250					255	
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Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280		Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Гла
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
Arg	Ser	Met	Met	Pro 325	Trp	Ile	Gly	Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Ala
Lys	Asn														
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Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Суа	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Aap	Val	Thr	Val	Gly 45	Leu	Суз	Lys
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Asp 65	Val	Ala	Ala	Ala	Val 70	Ala	Gly	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Thr 80
Pro	Asp	Glu	Phe	Gln 85	Ser	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn
Ile	Гла	Гла	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Aap	Leu	Asp	Val	Ile 125	Met	Ile	Ala
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
Gly 145	Ile	Pro	Asp	Leu	Ile 150		Ile	Tyr	Gln	Asp 155		Ser	Gly	Asn	Ala 160
Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
Gly	Ile	Ile	Glu 180	Thr	Thr	Phe	Lys	Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly	Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200		Thr	Val	Glu	Leu 205	Val	Lys	Ala
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Phe 225	Glu	Сүз	Leu	His	Glu 230		Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr

Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 88 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant SE <400> SEQUENCE: 88 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gl
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Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 89 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant SE2 <400> SEQUENCE: 89 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala Cys As
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			260					265					270		
Ala	Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Aap	Gly	Glu 285	Tyr	Ala	ГÀа
Met	Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg 305	Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
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Lys	Asn														
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1 1	-12	vai	1116	1 y 1 5	чар	- Цур	чар	сув	Азр 10	Jeu	DGT	116	116	15	Сту
Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Суз	Asn	Leu 35	ГЛа	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Gly	ГЛЗ
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Pro .	Asp	Glu	Phe	Gln 85	Ser	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn
Ile	Lys	Lys	Gly 100		Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Tyr	Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Aap	Val	Ile 125	Met	Ile	Ala
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
Gly 145	Ile	Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys	Asn	Val	Ala	Leu 165		Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
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Gly	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245		Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
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Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 92 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 9650E5 <400> SEQUENCE: 92 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala 20 25 30 Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Asn Lys Gly Trp Ala Gly His Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr 50 55 60 Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys

Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 93 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 9667A11 <400> SEQUENCE: 93 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Asn Lys Gly Asn Ala Gly His Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr 50 55 60 Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Ala Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys

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Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys 290 295 300 Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu 305 310 315 320 Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala 325 330 335 Lys Asn <210> SEQ ID NO 95 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 9875B9 <400> SEQUENCE: 95 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly 10 Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala 20 25 30 Cys As
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Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu

Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu Arg Ser Met Met Pro Trp Ile Gly Ala Asn Lys Ile Val Asp Lys Ala Lys Asn <210> SEQ ID NO 98 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 1151B4 <400> SEQUENCE: 98 Met Lys Val Phe Tyr Asp Lys Asp Cys Asp Leu Ser Ile Ile Gln Gly Lys Lys Val Ala Ile Ile Gly Tyr Gly Ser Gln Gly His Ala Gln Ala Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Asn Lys 35 40 45 Gly Asn Ala Asp Ala Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr Asp Val Ala Ala Ala Val Ala Gly Ala Asp Leu Val Met Ile Leu Thr 65 70 75 80 Pro Asp Glu Phe Gln Ser Gln Leu Tyr Lys Asn Glu Ile Glu Pro Asn Ile Lys Lys Gly Ala Thr Leu Ala Phe Ser His Gly Phe Ala Ile His Tyr Asn Gln Val Val Pro Arg Ala Asp Leu Asp Val Ile Met Ile Ala Pro Lys Ala Pro Gly His Thr Val Arg Ser Glu Phe Val Lys Gly Gly Gly Ile Pro Asp Leu Ile Ala Ile Tyr Gln Asp Val Ser Gly Asn Ala Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys Met Phe Ile Ser Glu Gly Ala Thr Gly Tyr Pro Ser Met Thr Ala Lys Arg Arg Asn Asn Ala Ala His Gly Ile Glu Ile Ile Gly Glu Gln Leu

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305			310					315					320
Arg Ser Met	t Met	Pro 325	Trp	Ile	Gly	Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Ala
Lys Asn													

What is claimed is:

1. A mutant ketol-acid reductoisomerase enzyme comprising the amino acid sequence as set forth in SEQ ID NO: 29.

2. A nucleic acid molecule encoding the mutant ketol-acid reductoisomerase enzyme of claim **1**.

3. A nucleic acid molecule encoding a mutant ketol-acid reductoisomerase enzyme having the amino acid sequence as set forth in SEQ ID NO:19.

4. A mutant ketol-acid reductoisomerase enzyme as set for in SEQ ID NO:19

5. A recombinant cell comprising the mutant ketol-acid reductoisomerase enzyme of claim **1**.

6. A mutant ketol-acid reductoisomerase enzyme as set forth in SEQ ID NO:17 comprising at least one mutation at a residue selected from the group consisting of 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, 165, and 170.

7. A mutant ketol-acid reductoisomerase enzyme according to claim **6** wherein:

- a) the residue at position 47 has an amino acid substation selected from the group consisting of A, C, D, F, G, I, L, N, P, H, T, E and Y;
- b) the residue at position 50 has an amino acid substitution selected from the group consisting of A, C, D, E, F, G, M, N, V, W and I;
- c) the residue at position 52 has an amino acid substitution selected from the group consisting of A, C, D, G, H, N, Y, and S;
- d) the residue at position 53 has an amino acid substitution selected from the group consisting of A, H, I, W, Y, G, and R;
- e) the residue at position 156 has an amino acid substitution of V;
- f) the residue at position 165 has an amino acid substitution of M;
- g) the residue at position 61 has an amino acid substitution of F;
- h) the residue at position 170 has an amino acid substitution of A;
- i) the residue at position 24 has an amino acid substitution of F;
- j) the residue at position 33 has an amino acid substitution of L;
- k) the residue at position 80 has an amino acid substitution of I; and
- l) the residue at position 115 has an amino acid substitution of L.

8. A nucleic acid molecule encoding the mutant ketol-acid reductoisomerase enzyme of claim **6**.

9. A method for the evolution of an NADPH binding ketolacid reductoisomerase enzyme to an NADH using form comprising:

 a) providing a ketol-acid reductoisomerase enzyme which uses NADPH having a specific native amino acid sequence;

- b) identifying the cofactor switching residues in the enzyme of (a) based on the amino acid sequence of the *Pseudomonas fluorescens* ketol-acid reductoisomerase enzyme as set for the in SEQ ID NO:17 wherein the cofactor switching residues are at positions selected from the group consisting of: 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, 165, and 170; and
- c) creating mutations in at least one of the cofactor switching residues of (b) to create a mutant enzyme wherein said mutant enzyme binds NADH.
- **10**. The method of claim **9** wherein:
- a) the residue at position 47 has an amino acid substitution selected from the group consisting of A, C, D, F, G, I, L, N, P, H, T, E and Y;
- b) the residue at position 50 has an amino acid substitution selected from the group consisting of A, C, D, E, F, G, M, N, V, W and I;
- c) the residue at position 52 has an amino acid substitution selected from the group consisting of A, C, D, G, H, N, Y, and S;
- d) the residue at position 53 has an amino acid substitution selected from the group consisting of A, H, I, W, Y, G, and R;
- e) the residue at position 156 has an amino acid substitution of V;
- f) the residue at position 165 has an amino acid substitution of M;
- g) the residue at position 61 has an amino acid substitution of F;
- h) the residue at position 170 has an amino acid substitution of A;
- i) the residue at position 24 has an amino acid substitution of F;
- j) the residue at position 33 has an amino acid substitution of L;
- k) the residue at position 80 has an amino acid substitution of I; and
- l) the residue at position 115 has an amino acid substitution of L.

11. The method of claim 9 wherein the ketol-acid reductoisomerase enzyme has the amino acid sequence as set forth in SEQ ID NO: 29.

12. A method for the production of isobutanol comprising:

- a) providing a recombinant microbial host cell comprising the following genetic constructs:
 - i) at least one genetic construct encoding an acetolactate synthase enzyme for the conversion of pyruvate to acetolactate;
 - ii) at least one genetic construct encoding a ketol-acid reductoisomerase enzyme of either of claim 1 or 6;
 - iii) at least one genetic construct encoding an acetohydroxy acid dehydratase for the conversion of 2,3dihydroxyisovalerate to α-ketoisovalerate, (pathway step c);

- iv) at least one genetic construct encoding a branchedchain keto acid decarboxylase, of the conversion of α-ketoisovalerate to isobutyraldehyde, (pathway step d);
- v) at least one genetic construct encoding a branchedchain alcohol dehydrogenase for the conversion of isobutyraldehyde to isobutanol (pathway step e); and
- b) growing the host cell of (a) under conditions where iso-butanol is produced.

13. A method for the evolution and identification of an NADPH binding ketol-acid reductoisomerase enzyme to an NADH using form comprising:

- a) providing a ketol-acid reductoisomerase enzyme which uses NADPH having a specific native amino acid sequence;
- b) identifying the amino acid residues in the native amino acid sequence whose side chains are in close proximity to the adenosyl 2'-phosphate of NADPH as mutagenesis targets;
- c) creating a library of mutant ketol-acid reductoisomerase enzymes from the class I ketol-acid reductoisomerase enzyme of step (a), having at least one mutation in at least one of the mutagenesis target sites of step (b); and
- d) screening the library of mutant ketol-acid reductoisomerase enzymes of step (c) to identify NADH binding mutant of ketol-acid reductoisomerase enzyme.

14. A mutant ketol-acid reductoisomerase enzyme having the amino acid sequence selected from the group consisting of SEQ ID NO: 24, 25, 26, 27, 28, 67, 68, 70, 75, 79, 80, 81 and 82.

15. A method for evolution of an NADPH specific ketolacid reductoisomerase enzyme to an NADH using form comprising:

a) providing a mutant enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 28, 67, 68, 69, 70, and 84;

- b) constructing a site-saturation library targeting amino acid positions 47, 50, 52 and 53 of the mutant enzyme of (a); and
- c) screening the site-saturation library of (b) to identify mutants which accept NADH instead of NADPH as cofactor.

16. A method for evolution of an NADPH specific ketolacid reductoisomerase enzyme to an NADH using form comprising:

- a) providing a DNA fragment encoding a mutant enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, and 98 containing mutations in cofactor specificity domain;
- b) producing a DNA fragment cofactor specificity domain of (a);
- c) providing a DNA fragment encoding a mutant enzyme having mutations in cofactor binding affinity domain selected from the group consisting of SEQ ID NOs: 28, 67, 68, 69, 70, 84 and 86;
- d) incorporating mutations of step (b) into mutants of step (c); and
- e) screening mutants of step (d) for mutant enzymes having a ratio of NADH/NADPH utilization is greater than one.

16. The method of claim 15 wherein the K_M for NADH is less than 15 μ M.

17. A mutant ketol-acid reductoisomerase enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOS: 75, 76, 77 and 78.

18. A mutant ketol-acid reductoisomerase enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOS: 79, 80, 81, 82, and 83.

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