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# Li et al.

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# (54) KETOL-ACID REDUCTOISOMERASE USING NADH

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- (22) Filed: Dec. 15, 2009

# **Related U.S. Application Data**

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# Publication Classification

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# (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 <b>R</b> KG <b>S</b> A <b>T</b> VAKA
16	(44)	VGL <b>R</b> SG <b>S</b> A <b>T</b> VAKA
18	(162)	IGL <b>R</b> KG <b>SNT</b> FAEA

# FIG. 2A

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

FIG. 2B



**FIG. 3** 



Cotactor consumption (340)



FIG. 5A



FIG. 5B





100

Sequence ID

51

47	(18)	KKVAIIG <b>T</b> GSQGHAHAQNLRDNGFDVVVGLRKG~KSWDKA	
48	(17)	KTVAVIG <b>X</b> SSQGHAQAQNLRDSGVEVVVGVRPG-KSFEVA	
18	(51)	FKGIKQIGVIG <b>W</b> GSQAPAQAQNLKDSLTEAKSDVVVKIGLRKGSNSFAEA	
16	(17)	KKVAIIGYSQGHAHACULKDSGVDVTVGLRSGSATVAKA	
17	(17)	KKVAIIGZSQGHAQACVLKDSGVDVTVGLRKGSATVAKA	
		101	150
47	(57)	KEDGFSVYTVAEAAKDADVVMILLPDELQPEVYEAEIAPNLQAGN	
48	(56)	KTDDFEVMSVSEAVRFAQVVQMLLPDEQQAHVYKAGVEENLRECQ	
18	(101)	RAAGFSEENGTLGDMWETISGSDLVLLLISDSAQADNYEKVFSHMK-PNF	
16	(57)	EAHQLKVADVKTAVAAADVVMILTPDEFQGRLYKEEIEPNLKKGA	
17	(57)	EAHQLKVTDVAAAVAGADLVMILTPDEFQSQLYKNEIEPNIKKGA	
		7 (* 1	000
17	(102)		200
47	(102)	MILESUCENTILECOTI PRIVOVELVAPROPORTIVARI FSEG	
40 1 8	(150)	TI CLSHOFTI CHI OSLCODERNI SVI AVCRKOBSVRRI VVOCKEVAC	
16	(102)	TLA FAHGESTHYNOVVHRADLOVIMIA PKA PCHTVR SE FVKG	
17	(102)	TLAFSHGFATHYNOVV HRADLDVIMIA PKAPGHTVR SEFVKG	
	(102)		
		201	250
47	(144)	GAVPALFAVYQDATGVATEKALSYADGIGATRAGVLETTFKEETETDLFG	
48	(143)	NGVPALVAVHQD <b>A</b> TGTALHVALAYAK <b>G</b> VGCTRAGVIETTFQEETETDLFG	
18	(200)	AGINSSFAVHQD $V$ DGRATDVALGWSI $\mathbf{A}$ LGSPFTFATTLEQEYKSDIFG	
16	(144)	GGIPDLIAIYQDASGNAKNVALSYACGVGGGRTGIIETTFKDETETDLFG	
17	(144)	GGIPDLIAIYQD <b>A</b> SGNAKNVALSYAA <b>G</b> VGGGRTGIIETTFKDETETDLFG	

FIG. 8

Sequence ID

# FIG. 9A

	70	80	90	100	110	120
	] ] ] .	!   .			1	1
17	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		KVFYI	OKDCDLS	IIQG	
43	M		KVFYI	OKDCDLS	IIQG	
44			KVFY0	OKDCDLS		
16	/M		RVFYI	OKDCDLS	IIQG	
35	M		QVYYI	OKDADLS	I I QG	
39	/M		QVYYI	OKDCDLS	IIQG	
41	/M		KVYYI	OKDCDLS	IIQS	
38	/M		NVYYI	OKDCDLS	IVQG	
15	M		KV FY I	DKDADLS	LIKG	
40	M		KVYYI	DKDADLS	LIKQ	
42	M		KVFYI	DKDCDLS-~-	IIQG- <b></b>	
37	M		QVYY	DKDCDLS~~-	IIQG	
46	M		KVEY	DKDCDLS	IIQG- <b>-</b>	
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	ANGGGSALSAQMVSAP	SINTPSATT	PDFDSSVFK	KEKVTLSGHI	DEYIVRGGRN1	FPLLPD

FIG. 9B

	130	140	150	160	170 180
		<u></u>	! !	<u>.</u>	· ·   · <u>·</u> · · · · · · · · · · · · · · · · ·
17	KKVAIIGKGB(	2GHAQACMUKDS	GV-D	/[[VGLRKGS <i>f</i>	TVAKAEAHGLK
43	KKVAIIGKGSK	2GHAQACMUKDS	GV-D	/FVGLRKGSA	TVAKAEAHGLK
44	KKVAIIGKGS(	2GHAQACNUKDS	GV-D	/TVGLRKGSA	TVAKAEAHGLK
16	KKVAIIGKGS	2GHAHACNUKDS	GV-D	/TVGLRSGSA	TVAKAEAHGLK
35	KKVAVIGKG5K	2GHAHANNUKES	GV-D	/VVGLREGSS	SAAKAQKAGLA
39	KKVAIIGYGS	26HAHANNUKDS	GV-D	ZCVGLRKGSG	SWAKAENAGLA
41	KKVAIIGYGB	2GHAHACNUKDS	GV-D	/YVGLRAGS#	SVARAEAHGLT
38	KKVAI IGYGSK	SHAHALNIQDS	NV-D	/TVGLRADSG	SWKKAENAGLK
15	KNVTIIGKG60	CHAHALNINDS	GV-K	/TVGLRKNGA	SWNKAVNAGLQ
40	RKVAIVGYG5	GHAHANNIKOS	GV-D	/TVALRPGSA	SAKRAENAGLT
42	KKVAI IGYGS	26HADACNIKDS	GV-D	TVGLRKGSA	TVARAEAHGLK
37	KKVAILGEGS	GHAHACNIKDS	GV-D	/VVGLRAGSS	SIAKAEAYGLK
46	KKVAI IGYGSK	2GHADACNUKDS	GV-D	/TIGLRKGSA	TVANAEAHGLK
34	KKIAILGYGB	2GHAHAQNURDS	GVAE	AIALRPDSA	SVKKADDAGFK
36	KKVAIIGFG56	2GHAHAMN1RDS	GV-E	7 I I GLKEGGÇ	SWARADKANFI
33	KKVAVIGYGS	2GHAHVINIRDS	GVKD	/AVALRPGSA	SIKKAEAEGLK
13	KRIAVLGYGS	2GRAWAQNURDS	GL-N	/VVGLEREGK	SWEIAKSDGIT
30	KTVAVIGYGS	2GHAHAQNUDDS	GV-D	/VVGLREDSS	SRSAAEADGLD
14	KTIAVIGKGK	2 GRADALNIRDS	GL-E	/LIGLRRGGK	SWEIATSEGFR
32	RKVAIVGYGS	2GHAHSQNURDS	GV-E	/VIGLREGSK	SAENAKEAGFE
31	KTVAVIGYG5(	2GHAHAINIKES	GV-D	/‡VGVRQGK-	SFTQAQEDGHK
47	KKVAIIGYGSK	OGHAHAQNI R DN	GF-D	/VVGLŔKGK-	SWDKAKEDGFS
48	KTVAVIGYGS	2GHADAQN1RDS	GV-E	/VVGVRPGK-	SFEVAKTDGFE
18	AFKGIKQIGVIGWGS	<u>APAQAQNI</u> KDS	LTEAKSDV-V	7KIGLRKGSN	ISFAEARAAGFSEE
	* *		* * * *	*	

GXGXX(G/A)

# FIG. 9C

Sequence ID	190	200	210	220	230	240
	· ´ [ ] [	· · · · <u> </u> · · <u>-</u> ·   ·	.	.	· <u>  </u>	1
17	VTDVAAAVAGAD	LVMILTEDEFC	SQLYKNEIER	NIKKGATLAF	SHGFAIHYN	QVVPR
43	~VTDVASAVAAAD	LVMILIPDEFC	SQLYKNEVER	NLKKGATLAF	SHGFAIHYN	QVVPR
44	VADVATAVAAAD	LVMILTPDEFC	GALYKNEIEB	NIKKGATLAF	SHGFSIHYN	QVVPR
16	VADVKTAVAAAD	VVMILTEDEFC	)GRLYKEEIEE	NLKKGATLAF	AHGESIHYN	QVVPR
35	VASIEDAAAQAD	VVMILAPDEHQ	AVIYHNQIA1	NVKPGAAIAF	AHGENIHFG	QIQPA
39	VKEVAEAVAGAD	VVMILTPDEFC	AQLYKSEIEI	NLKSGATLAF	AHGESIHYN	QIVPR
41	VKSVKDAVAAAD	VVMILTPDEFG	GRLYKDEIE	PNLKKGATLAF	AHGESIHYN	QVVPR
38	VAEVEEAVKAAD	IIMILTPDEFC	<b>XELYNDVIE</b>	NIKQGATLAF	AHGFAIHYN	QVIPR
15	VKEVAEAVKDAD	VVMILLEDEQI	ADVYKNEVHO	GNIKQGAALAF	AHGENVHYG	AVIPR
40	VKSVPEAVAGAD	LVMILTPDEF	SRLYRDEIE	NIKQGATLAF	AHGESIHYN	QVVPR
42	VTDVASAVAAAD	LVMILTPDEF	SQLYKNEVE	PNLKKGATLAF	SHGFAIHYN	QVVPR
37	TSDVASAVASAD	VVMVLTPDEFG	AQLYREEIE	PNLKQGATLAF	AHGFAIHYN	QIVPR
46	VTDVATAVAAAD	LVMILTPDEF	) GQLYKQEIE	PNIKKGATLAF	SHGFAIHYN	QVVPR
34	VLTNAEAAKWAD	ILMILAPDEH	DAAIYAEDLKI	NLRPGSAIAF	AHGLNIHFG	LIEPR
36	VKSVKEATKEAD	LIMILAPDEIG	SEIFNEEIK	PELKAGKTLAF	AHGENIHYG	QIVAP
33	VLTPAEAAAWAD	vvmilfedelg	DADLYKSELAA	ANLKPGAALVF	AHGLAIHFK	LIEAR
13	PLHTKDAVKDAD	IIIFLVPDMVG	ORTLWLESVO	YMKKGADLVF	AHGENIHYK	LIDPP
30	VATPRGAAEQAE	)LVSVL/PDFV(	PAVYE-QIE	DVLQPGDTLQF	AHGENIHYG	QIEPS
14	VYEIGEAVRKAD	VILVLI POMEG	PKVWQEQIA	PNLKEGVVVDF	AHGENVHEG	LIKPP
32	VKTTAEAAAWAD	VIMILAPDIS	DAEIFTNDIE	PNLNAGDALLF	GHGLNIHFC	LIKPA
31	VFSVKEAAAQAE	IIMVLLPDEQ	QKVYEAEIKI	DELTAGKSLVF	AHGENVHEH	QIVPP
47	VYTVAEAAKQAI	VVMILLPDEL	QPEVYEAE IAI	PNLQAGNSLVF	'AHGENVHED	QVKPP
48	VMSVSEAVRTAÇ	VVQMLLFDEQQ	QAHVYKAGVE!	ENLREGOMLLF	SHGENIHEG	QINPP
18	NGTLGDMWETISGSE	UVLLLISDSAG	DADNYE-KVF:	SHMKPNSILGL	.SHGFLLGHL	QSLGQ
	*			ł		

FIG. 9D

	250	260	270	280	290	300
	<u>.</u> . <del>.</del> <u></u>	• <u>•</u> •• <u>•</u> ••••		1	· · · <u>· ·</u> · · · <u>· ·</u> ·	
17	ADLOVIMIAPKA	PGHTVRSEFVK	GGG	IPDLIAIYQDA	SGNAKNVALS	YAAGV
43	ADLOVIMIAPKA	PGHTVRTEFVK	3GG	IPDLIAVYQDA	SGNAKNVALS	ſASGV
44	ADLOVIMIAPKA	.FGHTVRSEFVK(	GGG	IPDLIAIYQDA	SGNAKNVALS	ſASGV
16	ADLDVIMIAPKA	.PGHTVRSEFVK(	GGG	IPDLIAIYQDA	SGNAKNVALS	YACGV
35	ADLOVIMVAPKG	FGHLVRSTYVE	GGG	VPSLIAIHQDA	TGHARDIALS	YASAN
39	ADLOVIMIAPKA	FGHTVRSEFVK	G <b></b> -GG	IPDLIAIFQDA	SGSAKDLALS	YASGV
41	ADLOVIMIAPKA	PGHTVRSEFVR	G <b>-</b> GG	IPDLIAVYODA	SGNAKNLALS	YACGV
38	SDLDVIMVAPKA	.PGHTVRSEFAK	G <b></b> GG	IPDLIAIYQDA	SGCARQLALS	YAAGV
15	ADLOVIMVAPKA	. <b>F</b> GHTVRGTYAQ	GGG	VPHLIAVHODE	SGSARDIALS	YATAN
40	ADLOVIMIAPKA	FGHTVRSEFVK	G <b></b> -GG	IPDLIAIYQDA	SGKAKETALS	YASAI
42	ADLOVIMIAPKA	FGHTVRTEFVK	GGG	IPDLIAVYQDA	ASGNAKNVALS	YASGV
37	KDLCVIMVAPKA	PGHTVRTEFTK	GGG	IPDLIAIFQDA	ASGNAKNVALS	YASGI
46	ADLOVIMIAPKA	.FGHTVRSEFVK	G <b>-</b> GG	IPDLIAIYQDA	ASGNAKNVALS	YASGV
34	KDIOVEMIAPKO	PGHTVRSEYVR	GGG	VPCLVAVDQDZ	ASGNAHDIALA	YASGI
36	KGIDVIMIAPKA	FGHTVRHEFSI	GGG	TPCLIAIHQDE	sknaknlals.	YASAI
33	ADLOVEMVAPKO	PGHTVRGEYLK	G-~GG	VPCLMAVAQNY	PTGNALELALS	YASAI
13	KDSDVYMIAPKO	PGPTVREYYKA	G <b>-</b> GG	VPALVAVHODV	/SGTALHKALA	IAKGI
3,0	EDVNVIMVAPKS	PGHLVRRNYEN	DEG	TPGLIAVYQDI	PSGEAHDLGLA	YAKAI
14	KNICVIMVAPK	FGKAVREEYLA	GRG	VPALVAVYQD	(SGSALKYALA:	LAKGI
32	DDIIVGMVAPKG	FGHLVRRQFVD	G <b></b> KG	VPCLIAVIOD	PTGIAQALTLS	YAAA I
31	ADVDVFLVAPKC	FGHLVRRTYEQ	GAG	VPALFAIYQD	/TGEARDKALA	YAKGI
47	ANVCVFLVAPKO	FGHLVERTFSE	GGA	VPALFAVYQDA	ATGVATEKALS	YADGI
48	SYVDVAMVAPKS	FGHLVERVFQE	GNG	VPALVAVHOD	ATGTALHVALA	YAKGV
18	DFPKNISVIAVCPKO	MGPSVRRLYVQ	GKEVNGAG	INSSFAVHQD	/DGRATDVALG	WSIAL
	* *					

FIG. 9E

	310	320	330	340	350	360
						 :
GGGRTGIL	e'r'r F'KDETE	TDLFGEQAVL	GGGTVELVF	CAGFETLVEA	GKAPEMANFEC	LHEL
GGGRTGII	ETFFKDEFE	TDLFGEQAVL	dggtvelvr	CAGFETLVER	<b>БКАРЕМАКЕЕС</b>	LHEL
GGGRTGII	ETTEKDETE	TIDLFGEQAVL	dggTVELVF	(AGFETLVEA)	GKAPEMAKFEC	LHEL
GGGRTGII	ETTERDETE	TDLFGEQAVL	debeverve	CAGFETLVEA	GYAPEMAKFEC	LHEL
GGGRAGVI	ETSFREETE	TDLFGEQAVL	debitsLic	DAGFETLVEA	<b>ЗҮАРЕМАК FEC</b>	LHET
GGGRTGII	ettekdete	TDLFGEQAVL	<b>dGGAVELV</b> F	CAGFETLVEA	GYAPEMAKFEC	LHEL
GGGRTGII	етт ғқасте	TDLFGEQAVL	defecvervr	CAGFETLVER	<b>ЗҮАРЕМА</b> Ү FEC	LHEL
GGGRSGII	ettekdete	TDLFGEQAVI	dggavervr	MGFETLTEA	GYAPEMANFEC	LHEL
GGGRAGII	ETNEREETE	TIDLFGEQAVI	dggtvelik	CAGFETLVER	<b>З</b> КАРЕМАК FEC	LHEL
GGGRTGII	errekdere	TDLFGEQAVI	<b>dGGAVELVF</b>	CAGEDTLVEA	SYAPEMAKFEC	LHEL
GGGRTGII.	etrekdere	TDLFGEQAVL	dggTVELVF	CAGFETLVEA	GKAPEMAKFEC	LHEL
GGGRTGII	etrekdere	TDLFGEQAVL	dggavelvr	CAGFETLTER	<b>ЗКАРЕМА</b> КFEC	LHEL
GGGRTGII	ETTEKDEFE	TIDLFGEQAVL	dggTVELVF	(AGFETLVEA	GYAPEMAYFEC	LHEL
GGGRSGVI	etrrreeve	TDLFGEQAVL	debltalij	RAGFETLTER	GYAPEMAFFEC	MHEM
GGGRTGII	ettrekaere	TDLFGEQAVL	debrsario	DAGFETLVER	GKEPEMANFEC	LHEM
GGGRSGII	ETTERECE	TDLFGEQUVI	deerskri(	<b>2YGFETLVEA</b>	GYAPEMANFEC	LΗΕV
GATRAGVI	PTTFKEETE	TDLFGEQVIL	VGGIMELME	VAAFETLVED	GYQPEVANFET	INEL
GCTRAGVV.	ettrerente	TIDLFGEQAVL	dgbvtslvf	<b>TGYETLVDA</b>	GYSPEMARFEC	LNEL
GATRAGVI	etreadere	TULIGEOIVU	VGGLMELIF	KGFEVLVEM	<u> Б</u> К Q Р Е V А К F E V	LNEA
GGARAGVI	HTLFEAELV	TDLFGEQAVL	dGGTEELVF	<b>VGFEVLTEA</b>	<u>ЗКЕРЕМАК FEV</u>	LHEL
GGARAGVL.	etrekelte	TDLFGEQAVI	dgglsalvf	AGFETLTEA	з <u>кореца</u> ктес	LHEL
GATRAGVL.	etrekere	TDLFGEQAVL	dgbvtalvf	(AG FET LV DA	<b>ЗКОРЕЦАК FEC</b>	LHEL
GCTRAGVI.	ettfodere	TDLFGEQTVL	dggvtalvf	CAGFETLTEG	GKRPEIANFEC	LHEL
GSPFTE	ATTLEQEYK	SDIFGERGIL	LGAVHGIVE	CLFRRYTES	GMSEDLAYKNT	VECI

14410046144646000101441

Sequence ID

FIG. 9F

	370	380	390	400	410	420
					1   ,	
17	K-LIVDLMYEGGIA	NMNYSTSNNAE	YGEYVTGPEV	INAESRQAM	RNALKRIQDGE	YAKMF
43	K~LIVDLMYEGGIA	NMNYSISNNAE	YGEYVTGPEV	INAESRQAM	RNALKRIQDGE	YAKMF
44	K-LIVDLMYEGGIAI	NMNYSISNNAE	YGEYVTGPEV	INEESRKAM	RNALKRIQDGE	YAKMF
16	K-LIVDLMYEGGIA	NMNYSJ SNNAE	YGEYVTGPEV	INAESRAAM	RNALKRIQDGE	YAKMF
35	K-LIVDLLYQQGIA	NMRYSISNTAE	YGDFTRGPRV	INEESREAM	REILAEIQEGE	FAREF
39	K-LIVDLMYEGGIA	NMNYSISNNAE	YGEYVTGPEV	INDQSRAAM	RNALKRIQDGE	YAKMF
41	K-LIVDLMFEGGIA	NMNYSI SNNAE	YGEYVTGPEV	INEQSRQAM	RNALKRIQDGE	CYAKMF
38	K-LIVDLMYEGGİA	DMNYSISNNAE	YGEYVTGPEV	INEQSREAM	RNALKRIQSGE	EYAKMF
15	K-LIVDLIYEGGIGI	NMNYSISNNAE	YGEYVTGPRV	VTAETKQAM	KQCLHDIQTGE	YAKSF
40	K-LIVDLMYEQGIAI	NMNYSISNNAE	YGEYVTGVKV	INEQSRAAM	KECLANIQNG	AYAKRF
42	K-LIVDLMYEG <b>GI</b> A	NMNYSJI SNNAE	YGEYVTGPEV	INAESRQAM	RNALKRIQDGE	EYAKME
37	K-LIVDLMYEGGIA	NMNYSISNNAE	YGEYVTGPEV	INEQSREAM	RNALKRIQSGE	EYAKMF
46	K-LIVDLMYEGGIA	NMNYSLSNNAE	YGEYVTGPEV	INEESRKAM	RNALKRIQCG	EYAKMF
34	K-LIVDLIYEAGIA	NMRYSISNTAE	YGDIVSGPRV	INEESKKAM	KAILDDIQSG	REVSKE
36	K-LIVDLIYQGGIA	DMRYSVSNTAE	YGDYITGPKI	ITKETKEAM	KGVLKDIQNGE	SFAKDF
33	K-LIVDLIYEGGIA	NMRYSISNTAE	YGDYVTGSRI	ITEATKAEM	KRVLADIQSG	REVRDW
13	K-MLVDLVYEKGIS	gmlkavsdtak	YGGMTVGKFV	IDESVRKRM	KEALQRIKSGE	(FAEEW
30	K-LIVDLMYEGGÞS	EMWDSVSDTAE	YGGLTRGDRI	VDDHAREKM	EEVLEEVQNG	FAREW
14	K-LIMDLIWQRGIY	GMLNGVSDTAK	YGGLTVGPRV	IDENVKRKM	IKEAAMRVKSGE	EFAKEW
32	K-LIVDLMFEGGIS	NMNYSVSDTAE	FGGYLSGPRV	IDADTKSRM	KDILTDIQDG	FTKRL
31	K-LIVDLMYEEGLA	GMRYSISDTAQ	WGDFVSGPRV	VDAKVKESM	KEVLKDIQNG	FAKEW
47	K-LIVDLMYEGGLE	NMRYSVSDTAQ	WGDFVSGPRV	VTEDTKKAM	GTVLAEIQOG	FFARGW
48	K-LIVDLMYEG <mark>G</mark> LT	NMRHSISDTAE	FGDYVTGSRI	VTDETKKEM	KRVLTEIQQG	EFAKKW
18	TGVISKTISTKGML	ALYNSLSEEGK	K-DFQAAYSA	SYYPSMDII	YECYEDVASGS	SEIRSV

FIG. 96

Sequence ID						
	430	440	450	460	470	480
		!	] ]			1
17	ISEGATGYPSMTA	KRRNNAAHG I	E-IIGEQURS	MMPWIGANKI	IVDKAKN	
43	ITEGATGYPSMTA	KRRNNAEHG	E-VIGEKIRS	MMPWIAANKI	IVDKDKN	
44	ISEGATNYPSMTA	KRRNNAAHGI	E-IIGEQIRS	MMPWISANKI	VDKTKN	
16	ITEGAANYPSMTA	YRRNNAAHPI	E-QIGEKIR	MMPWIAANKI	VDKSKN	·
35	VLENQAGCPTLTA	RRRLAAEHEI	E-VVGERIRG	MMPWINANKI	LVDKDKN	
39	IAEGAHNYPSMTA	YRRNNAAHPI	E-QVGEKIRS	MMPWIASNKI	VDKSKN	
41	ITEGAANYPSMTA	YRRNNAAHQI	E-VVGEKIRI	MMPWIAANKI	UDKTKN	
38	I SEGATNÝPSMTA	RRRNNAEHQI	E-ITGAKUR	MMPWIGGNKI	IDKDKN	
15	LLENKAGAPTLIS	RRRLTADHQI	E-QVGAKIRA	MMPWIAKNKI	LVDQSKN	·
40	ILEGQANYPEMTA	WRRNNAAHQI	E-VVGAKIRS	MMPWIAANKI	LVDHSKN	
42	ISEGATGYPSMTA	KRRNNAAHGI	E-IIGEKURS	MMPWIAANK	EVDKDKN-+	
37	ISEGALNYPSMTA	RRRQNAAHEI	E-TYGEKURS	MMPWISANK	I VDKDKN	
46	ISEGATNYPSMTA	KRRNNAAHGI	E-IIGEQURS	MMPWISANK	LVDKTKN	·
34	VLDNRAGQPELKA	ARKRMAAHPI	E-QVGARIR	MMPWIASNKI	LVDKARN	
36	ILERRANFARMHA	ERKLMNDSLI	E-KIGREIR	MMPWISAKKI	LVDKDKN	
33	MLECKAGQPSFKA	TRRIQXEHVI	E-VYGEKIRK	MMPWISKNKI	LVDKARN	
13	VEEYGRGMPTVVN	GLSNVQNSLE	E-KIGNQURE	DLVQK	GKPKS	
30	ISENQAGRPSYKQ	LRAAEKNHDI	E-AVGEDIR	LFAW	GDD	
14	VEEYNRGAPTLRK	LMEEARTHPE	LE-KVGEEMR	KLLFGP-·		
32	IANVENGNTELEG	LRASYNNHP!	LE-ETGAKURI	LMSWVKVDAI	RAETA	
31	IVENQVNRPRFNA	INASENEHQ	E-VVGRKIRE	MMPFVKQGK	KKEAVVSVAQN	1
. 47	IAEHKAGRPNFHA	TNEKENEHE	E-VYGRKIRE	MMPFV-QPR	JKVGMK	
48	ILENQAGRPTYNA	MKKAEQNHQI	LE-KVGAELRE	MMSWIDAPK	ELVKK	
18	VLAGRRFYEKEGLPA	FPMGKIDQTH.	RMWKVĞEKVŔS	SVRPAGD-	-LGPLYPFTAC	JVYVAL

# FIG. 9H

Sequence ID						
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17						. 1
4 3						1
4 4	3 S L T P B L S F P B L S F					1
16						1
35						1
39						
41						1 5 4
38						
15		1				1 
40	*****					1
42						
37						
46						
34						
36						
33						
13						
30						1
14						1
32						1
31						1
47						F 1
48						1
18	MMAQIEILRKKGHS	YSEIINESVIE <i>F</i>	AVDSLNPEMH <i>F</i>	RGVS FMVDNC	STTARLGSRKW	VAPR

FIG. 91

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3.4 3.6 3.6 3.6 3.1 1.1.1.1 1.1.1.1 1.1.1.1 1.1.						
46 34 36 33 33 4 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1						
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18 FDY	YTT,SOOALVAVDNGAI	TNODLTSNFL	SDPVHEAT	GVCAOLRPSV	DISVTADADI	FVRPF

ence ID		17	43	44	16	35	6 E	41	38	15	40	42	37	46	34	36	33	<b>1</b> 3	30	< <b>₽</b>	32	37 37	47	48	ά	) -	ąК	5
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\*->qMfafskVYYDkDadlsGhdeylikGKkVAvIGYGSQGHAHAqNLrD

	M kV+YDkD+dls +i+GKkVA+IGYGSQGHA+A+NL+D
Sequence [D 17	1 -MKVFYDKDCDLSIIQGKKVAIIGYGSQGHAQACNLKD 37
Sequence ID 17	SGVdVvVGIRkGsaSwakAeaaGfkVktvaEAvaqADvVmiIlPDefQae SGVdV+VGIRkGsa++akAea+G+kV +va Ava+AD+VmiI+PDefQ++ 38 SGVDVTVGIRKGSATVAKAEAHGLKVTDVAAAVAGADLVMIITPDEFQSQ 87
	vYeeelepnLkpGatLaFAHGFNIHfgqIvPrafPkDiDViMVAPKgPGH
Sequence [D 17	+Y++elepn+k+GatLaF+HGF+IH++q+vPra D+DV1M+APK+PGH 88 LYKNEIEPNIKKGATLAFSHGFAIHYNOVVPRADLDVIMIAPKAPGH 134
Sequence [D 17	tVRreYvkGgGVPaLiAVyQDasGnAkdlALsYAkgiGggRAGvIETTFk tVR+e+vkGgG+P+LiA+yQDasGnAk++ALsYA+g+GggR+G+IETTFk 135 TVRSEFVKGGGIPDLIAIYQDASGNAKNVALSYAAGVGGGRTGIIETTFK 184
	effetdlfgeqavlcggvtelvkagfetlveagyapemAyfeclhelkli
Sequence ID 17	185 DETETDLFGEQAVLCGGTVELVKAGFETLVEAGYAPEMAYFECLHEIKLI 234

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  $\alpha$ -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 reductoi-somerase 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  $\alpha$ -ketoisovalerate, (pathway step c);
  - **[0021]** iv) at least one genetic construct encoding a branched-chain keto acid decarboxylase, of the conversion of  $\alpha$ -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. **5**A and **5**B—FIG. **5**A—Comparison of KARI activities of top performers from libraries E, F and G using cofactors NADH and NADPH. FIG. **5**B—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. **5**A were 30 min; in FIG. **5**B 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	CGAGGTCGGCKINAGGCACAAC	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  $\alpha$ -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  $\alpha$ -keto acid decarboxylase" refers to an enzyme that catalyzes the conversion of  $\alpha$ -ketoisovalerate to isobutyraldehyde and CO<sub>2</sub>. Preferred branched-chain  $\alpha$ -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  $\alpha$ -ketoisovalerate to isobutyryl-CoA (isobutyryl-cofactor A), using NAD<sup>+</sup> (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, 2<sup>nd</sup> 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<sub>cat</sub>=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	N
Aspartic acid	Asp	D
Cysteine	Cys	С
Glutamine	Gln	Q
Glutamic acid	Glu	Ē
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<sup>+</sup> 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<sup>++</sup> 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  $\alpha/\beta$  structure and resembles domains found in other pyridine nucleotide-dependent oxidoreductases. The C-terminal domain consists almost entirely of  $\alpha$ -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  $\alpha/\beta$  structure which is similar to that found in other pyridine nucleotide-dependent dehydrogenases. The second domain is mainly  $\alpha$ -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<sup>-1</sup> (Aulabaugh et al.; Biochemistry, 29: 2824-2830, 1990) or  $0.12 \text{ s}^{-1}$  (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. 2A. 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  $\mu$ 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<sub>M</sub> for NADH in the above mutants was still slightly high (e.g., JB1C6, SEQ ID NO: 74, has K<sub>M</sub> of 22  $\mu$ M for NADH). To further improve the NADH K<sub>M</sub> of the mutant KARIs, a "domain swapping library", which combined the nucleotide switching mutations and mutations with improved K<sub>M</sub> 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<sub>M</sub>-4-40  $\mu$ 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<sub>M</sub>-10  $\mu$ 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  $\mu$ 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<sup>+</sup>, respectively). In the presence of inorganic electron acceptors (e.g. O<sub>2</sub>, NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup>), 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  $\alpha$ -ketoisovalerate) and valine catabolism ( $\alpha$ -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  $\alpha$ -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 erythropolis* 

**[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<sub>β1</sub> 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 $\beta$ 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  $25^{\circ}$  C. to about  $40^{\circ}$  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 CO<sub>2</sub>. 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  $37^{\circ}$  C. without shaking. The archive plates were then stored at  $-80^{\circ}$  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  $\mu$ l of cells from thawed archive plates, 300  $\mu$ l of the LB medium containing 100  $\mu$ 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  $\alpha$ , $\beta$ 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<sup>-1</sup> cm<sup>-1</sup> for either NADPH or NADH. The stock solutions used were: K<sub>2</sub>HPO<sub>4</sub> (0.2 M); KH<sub>2</sub>PO<sub>4</sub> (0.2 M); EDTA (0.5 M); MgCl<sub>2</sub> (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 K<sub>2</sub>HPO<sub>4</sub>, 0.2 ml KH<sub>2</sub>PO<sub>4</sub>, 4.0 ml MgCl<sub>2</sub>, 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  $37^{\circ}$  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  $\mu$ l) prepared as described above was mixed with either NADH or NADPH (20  $\mu$ l) stock and cell extract (20  $\mu$ l) in each well of a 96-well assay plate.

The absorbance at 340 nm at  $25^{\circ}$  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 MgCl<sub>2</sub>, 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  $\mu$ 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  $\mu$ 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
C E F G H N	PF5_ilvc PF5_ilvc PF5_ilvc PF5_ilvc Good mutants from library C Good mutants from library H	47, 50, 52 and 53 47 50 52 47, 50, and 52 53 53	SEQ ID No: 1 and 2 SEQ ID No: 1 and 3 SEQ ID No: 1 and 4 SEQ ID No: 1 and 5 SEQ ID No: 1 and 6 SEQ ID NO: 20 and 21 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 ( $\Delta$ 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				
Mutant	Position 47	Position 50	Position 52	Position 53
SD2	R47Y	S50A	T52H	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	
6881E11	R47P	S50N	T52C	
6868F10	R47P		T52S	
6883G10	R47P	S50D	T52S	
6939G4	R47P	S50C	T52D	
11463D8	R47P	S50F	T52D	
9667A11	R47N	S50N	T52D	V53A
9675C8	R47Y	S50A	T52D	V53A
9650E5	R47N	S50W	T52G	V53H
9875B9	R47N	S50N	T52D	V53W
9862B9	R47D	S50W	T52G	V53W
9728G11	R47N	S50W	T52G	V53W
11461D8	R47F	S50A	T52D	V53A
11461A2	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  $\mu$ 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	$\begin{array}{l} {\rm NADH} \\ {\rm K}_{M}(\mu{\rm M}) \end{array}$
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  $\mu$ M) compared to mutants listed in Table 6 (e.g., 14-40 µM).

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TABLE 5
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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	<pre>pBAD-608-A101_090808f: CAAGAAGGGCNNKACTCTGGCCT (SEQ ID NO: 59) pBAD-631-A101_090808r: AGGCCAGAGTKNNGCCCTTCTTG (SEQ ID NO: 60)</pre>
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	$\begin{array}{c} {\rm NADH} \\ {\rm K}_{\mathcal{M}}(\mu {\rm M}) \end{array}$
ZB1	Y24F/R47Y/S50A/T52D/V53A/L61F/A156V (SEQ ID NO: 24)	40
ZF3	(SEQ ID NO: 24) Y24F/C33L/R47Y/S50A/T52D/V53A/L61F (SEQ ID NO: 25)	21
ZF2	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/A156V (SEO ID NO: 26)	17
ZB3	Y24F/C33L/R47Y/S50A/T52D/V53A/L61F/G170A (SFO UD NO: 27)	17
Z4B8	C33L/R47Y/S50A/T52D/V53A/L61F/T80I/A156V (SEQ ID NO: 28)	14

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	<u>Mutants further optimized for improved <math>K_M</math> (for NADH)</u>	
Mutant	Mutation Locations	NADH K <sub>m</sub> (µM)
3361G8	C33L/R47Y/S50A/T52D/V53A/L61F/T80I (SEO ID NO: 67)	5.5
2H10	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 <sub>m</sub> (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**.

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25 Experimentally verified 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	NP_253382.1	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 ( $\mu$  and  $\lambda$ ) 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  $\mu$  (location) and  $\lambda$  (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/ $\mu$ 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]

$10 \times reaction buffer$	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	
	•	

TI	ne	Ρ	CR	React	ion	Pro	gram	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 ( $\Delta$ 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  $\mu$ 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 <sub>340</sub> nm)	

	0.2 mM	NADH	0.2 n NAD	ıM PH
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)	9.0 µl
Template mix (5.0 ng/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  $\mu$ 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 and 42  $\mu$ 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 ( $\Delta$ 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

Mu	Mutants with improved $K_M$ (for NADH) obtained from the domain swapping library								
Mutant	Mutation Locations	NADH K <sub>M</sub> (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 NADPH			
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 MgCl<sub>2</sub>, 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  $\mu$ l of the reaction mixture was rapidly withdrawn and added to a 1.5 ml Eppendorf tube containing 10  $\mu$ 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.

HMMER2.0 [2.2e]	File format version: a unique identifier for this save file format.
NAME Functionally Verified KARIs	Name of the profile HMM
LENG 354	Mode length: the number of match states in the model.
ALPH Amino	Symbol alphabet: This determines the symbol alphabet 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.
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->J, C->T, C->C, J->B, J->I.
NULT-4-8455	The transition probability distribution for the null model (single G state).
NULLE 595-1558 85336-294-453-1158 197 249 902-1085-142-21-313 45 531 201 384-1998-644	The extreme value distribution parameters $\mu$ and lambda respectively; both floating point values.
EVD-333./12/08 0.110102	I hese values are set when the model is calibrated with hmmcalibrate. There are need to determine F volume of hit covers

Position in alignment		7100%	7200%	9600%	8700%	8800%	%0006	9100%	9200%	9300%	9400%
Y		-1030 -249	-4017 -251	1335 -249	-1421 -249	-133 -249	938 -249	-1913 -249	-3510 -219	4349 -249	4185 -249
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	1117 -369	-2040 -369	3023 -369	-3643 -369	-2436 -369
μ		-684 117	-4459 121	-1350 117	640 117	-338 118	476 117	224 117	-82 117	-4533 117	1039 117
×		-643 359	-4692 361	-1617 359	-488 369	-631 359	1715 359	829 359	-4080 359	-4313 359	-2258 359
R		96	-4823	-1798	- 383	-883 96	-1358 96	458 95	-4628 96	-4458 96	-1078 96
ď		3263	4977	-1503 45	154	-451	-1013	1146	-4417	-3835	-1513
4		-1495 394	-4790	394	-1658 394	-1705 394	-1964 394	-2010 394	-4600 394	-4920 394	-3206 394
z		1 -227 ) 275	0 -5052 2 - 276	5 -1626 ) 275	1 -252 0 275	5 -731	0 -1258	275 275	3 -442 0 275	3726 3 -3726	8 -2051 0 275
Σ		-911	5320 -722	-720	-911	-721	-720	-1502	-1318	-2838	-2098
	1	-1417 -466	-2613 -467	64 -466	-1765 -466	-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 *
->m		-1455 -626 -650	-3232 -625 *	-196 -626 *	-1686 -626 *	-167 -626 *	1279 -626 *	-2483 -626 *	2241 -626 *	-3424 -626 *	-2628 -626 *
р < -р		-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
		-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 -381 -1115
E E		-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	-136 233 -6882	-5216 232 -325	-2227 233 -6882	1125 233 -1125	1084 235 -7567	-1937 233 -7995	-803 233 -9181	-5089 233 -9181	-5210 233 -9181	-2489 233 -9181
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		1(Q)	2(M) 	3(F) 	4(A)	5(C) 	6(S)	7(K)	8(V)	9(Y) 	10(Y) —

9500%	9600%	9700%	%0066	1000%	10100%	10200%	10300%	10400%
-3541 -249	-1871 -249	-3709 -249 -2431 -249	-2349 -249	-1304 -249	-2343 -249	-1919 -249	107 -249	-1646 -249
-4550 -294	-2554 -294	-4759 -294 -2796 -294	-3086 -294	-1645 -294	-3077 -294	-1916 -294	-425 -294	-2146 -294
-3974 -369	-1993 -369	-4201 -369 1507 -369	-2518 -369	1306 -369	-2503 -369	-1550 -369	-1354 -369	-1814 -369
-2558 117	-837 117	-2742 117 -1792 117	1116 117	-1213 117	190 117	-774 117	-951 117	-973 117
-2158 359	-67 359	-2292 359 -2499 359	53 359	-232 359	2139 359	-656 359	-934 359	-728 359
-2799 96	904 96	-2987 96 -3411 96	-1186 96	-2316 96	-1164 96	-1421 96	-322 96	-1141 96
-1429 45	-68 45	-1551 45 45 -3212 45	-489 45	-2089 45	-489 45	-1202 45	-363 45	-184 45
-2961 394	-1960 394	-3046 394 -3509 -3509 394	-2294 394	-2862 394	-2289 394	-1499 394	-1675 394	-1453 394
515 275	690 275	-1073 275 -3233 -3233	1270 275	-2361 275	1860 275	-978 275	-579 275	99 275
-3681 -720	-1461 -720	-3963 -720 -1051 -720	-2023 -720	-236 -720	-2011 -720	-1591 -720	-1035 -720	-1704 -720
-4361 -466	-2387 -466	-4578 -466 -1437 -1437	-2912 -466	2299 -466	-2901 -466	-2264 -466	-1493 -466	-2269 -466
733 210 *	2294 210 *	731 210 * -3517 210	290 210 *	-2409 210 *	-628 210 *	-1317 210 *	160 210 *	-569 210 *
-4500 -626 *	-2443 -626 *	-4738 -626 * 162 -626 *	-2977 -626 *	569 -626 *	-2963 -626 *	-2091 -626 *	1552 -626 *	-2172 -626 *
-1765 -1378 -1378	-527 106 -1378	-1372 106 -1376 -1376 -2896 106	-920 -920 -1378	-1716 106 -1378	-920 106 -1378	-1211 106 -179	4297 106 -179	-433 106 -179
-2437 399 -701	-535 399 -701	-2487 399 -701 -3276 399	-2072 -2072 -399 -701	-2827 399 -701	-496 398 -701	3143 399 -3098	-1336 399 -3098	-967 399 -3098
-4581 -381 -1115	-2692 -381 -1115	-4789 -381 -1115 -1115 -2010 -381	-3202 -381 -1115	-1057 -381 -1115	-3189 -381 -1115	-2112 -381 -1115	-320 -381 -1115	-2215 -381 -1115
1042 43 -894	819 43 -894	580 43 -894 -894 -3818 43	542 43 -894	-540 43 -894	1045 43 -894	-1110 43 -894	-482 43 -894	432 43 -894
3500 233 -9181	348 233 -9181	-3700 233 -9181 -9181 -4266 233 233	2748 233 -9181	-3338 233 -9181	588 233 -325	-968 233 -6882	-545 233 -6882	3234 233 -6882
-4412 -500 -8139	-2371 -500 -8139	-4633 -500 -8139 -8139 3193 -500 -500	-2905 -600 -8139	-1113 -500 -8139	-2877 -500 -8139	-832 -500 -5840	-1313 -500 -5840	-1812 -500 -5840
-2498 -149 -8	11 -149 -8	-2663 -149 -8 -8 -149 -149	-1363 -149 -8	-1268 -149 -8	-1350 -149 -2336	-454 -149 -38	-898 -149 -38	-872 -149 -38
11(D) 	12(K) —	13(D) 	15(D) 	16(L) —	17(S) 	18(G) 	19(H) —	20(D)

	10500%	10600%	10700%	10800%	10900%	11000%	12600%	12700%	12800%	12900%
	-1505	3932	-2629	-3585	2136	-3229	-3374	-2448	-3990	-1522
	-249	-249	-249	-249	-249	-251	-249	-249	-249	-249
	-1988 -294	592 -294	-2891	-3781	2738 -294	-4130	-294	-2905	-4505 -294	-4610 -294
	-1512 -369	-932 -369	1576 -369	1435 -369	2288 -369	-3530	-3817 -369	2645 -369	3219 -369	-2957 -369
	-814 117	-1359 117	-2247 117	-2764 117	-1152	-2220 116	-2876 117	$1681 \\ 117$	-2619 117	59 117
	-653	-1443	-3411	-4493	-1109	1069	-3119	-1564	-4482	-1470
	359	359	359	359	359	358	359	359	359	359
	-527 96	-1301 96	-3812 96	-4829 96	96 96	-2362 96	-1318 96	804 96	$-5101 \\ 96$	-3937 96
	4-4	-1111	-3538	-4454 45	2257	- <u>1</u> 44	-1076	1301	-4890 46	-3694 - 45
	-1441 394	-2163 394	94 394	-4788 394	-2231 394	-2810	-3580 394	-2582 394	-4868 394	-2904 394
nca	69 69	275	3878	-4829	-778	-967	275	202	-4790	-2821
	-1331	-720	-720	-720	-1730	-3173	-2982	-2032	-1474	-3497
	-1919	-769	1990	1593	-2619	-3937	-3617	-2865	-1532	-4428
	-466	-466	-466	-466	-466	-463	-466	-466	-466	-466
	-118	-1294	-3952	-4886	2540	-1636	3681	2737	-4945	-4169
	210	210	210	210	210	210	210	210	210	210
	*	*	*	*	*	*	*	*	*	*
	-1804	-918	2306	3051	-2712	-4030	-4021	-3021	2388	-4174
	-626	-626	-626	-626	-626	-625	-626	-626	-626	-626
	*	*	*	*	*	*	*	*	*	*
	-293 106 -179	$121 \\ 106 \\ -3775$	-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
	-3098	-109	-701	-701	-701	-701	-701	-701	-701	-701
	-2050	1268	-1724	-2108	-3007	-4174	-4750	-3407	-2769	-4382
	-381	-381	-381	-381	-381	-375	-381	-381	-381	-381
	-1115	-1115	-1115	-1115	-1115	-118	-1115	-1115	-1115	-1115
	2831	-1596	-4227	-5003	-500	392	-2568	-979	-4990	-4294
	43	43	43	43	43	42	43	43	43	43
	-894	-894	-894	-894	-894	-3674	-894	-894	-894	-894
	521	-1681	-4749	-5406	306	795	-4129	-1665	-5300	-4057
	233	233	233	233	233	233	233	233	233	233
	-6882	-6882	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
	-1695	-1229	-1931	-2299	-2632	-3900	-3775	-2925	-2122	-1828
	-500	-500	-500	-500	-500	-501	-500	-500	-500	-500
	-5840	-5840	-8139	-8139	-8139	-3318	-8139	-8139	-8139	-8139
	-766	-1337	-2294	-2801	-234	-2184	-3243	-1684	-2623	3309
	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
	-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)

-continued	
ġ.	
TABLE	

13000%	13100%	13200%	13300%	13400%	13500%	1360%	13700%	13800%	13900%	14000%	14100%	14200%	14300%
-3997	-3622	-5849	4507	-5849	-3474	-4751	-4735	-2908	-4942	-295	-4211	-1278	-4731
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-4512 -294	-3838 -294	-4924 -294	2986	-4924 -294	-3927 -294	-4577 -294	-4725 -294	-3282 -294	-4724 -294	2269 -294	-4363 -294	-1678	-4650 -294
2896	1192	-5862	-3881	-5862	-2307	-5612	-3297	-3469	-3852	-3308	-276	-369	-5371
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369	-369		-369
-2621 117	-2757 117	-4815 117	-4689	-4815 117	-1885 117	-4772 117	-2005 117	-2557 117	-2762 117	-2976 117	-1660 117	-1005	-4312 117
8 -4492	0 -4506	5 -4727	0 4356	5 -4727	1 3475	6 -4704	7 -1784	6 -2614	5 -2567	8 -2990	3 217	1 -1392	2 -4115
6 359	6 359	6 359	6 359	6 359	6 359	6 359	6 369	6 359	6 359	6 359	6 359	6 - 359	6 359
<u>96 -510</u>	<u>95</u> -486	46 -538 46 -9	61 450 45 9	46 -538 45 9	<u>95</u> -354 45 -9	75 -382 45 9	71 -413 46 9	<u>65 -150</u> 45 9	77 -454 45 9	46 -142 45 9	08 -382 45 9	95  -144 45  -9	<u>55 -459</u>
873 -48	802 -44	804 -55	963 -38	804 -55	093 -33	693 45	149 -38	866 -12	728 -44	764 25	900 -36	446 28	479 -42
394	394 -	394	394 -38	394	394	394 -	394 -	394	394	394 25	394	394 28	394
4796 -4	4835 4 275 4	5141 -4 275	3723 -4	5141 -4 275	2840 -3 276	4230 -4	3009 -3 275	2112 275	3727 -3 275 -3	2454 -3 275	2817 -2 275	743 -2	4397 -4 278
-1474 -	-912 - -720	-5970 -	-3131	-5970 -	-2676 - 720	-5304 -	-3857 -	-2886 -	-4365 - -720	-2715 -	-3192 - -720	1123 -720	-5419
-1532	1175	-6297	-3040	-6297	-3373	-5564	-4749	-3553	-5025	-3071	-4024	1062	-5797
-466	-466	-466		-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-4950 210	-4899 210 *	-5765 210 *	-6134 	-5765 210 *	-3616 210	-3840 210 *	-4340 210 *	-445 210 *	-4818 210 *	-1372 210 *	-4035 210 *	-1218 210 *	-4503 210 *
2881 -626 *	3324 -626 *	-6627 -626 *	-3726 -626	-6627 -626 *	228 -626 *	-5973 -626 *	-4506 -626 *	-3782 -626 *	-4781 -626 *	-3407 -626 *	-3549 -626 *	-789 -626 *	-6022 -626 *
-5142	-4698	-5028	-1378	-5028	-3314	-4099	-3816	4738	-4271	4549	-3548	-1154	-4123
106	108	106		106	106	106	106	106	106	106	106	106	106
-1378	-1378	-1378		-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
-5111	-5170	3834	-5108	3834	-2363	-4221	3536	-3201	-2992	-3679	-2118	-2372	-3911
399	399	399	399	399	399	399	399	399	399	399	399	-299	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701
-2772 -381 -1115	-2155 -381 -1115	-5893 -381 -1115	1502 -1115	-5893 -381 -1115	-3430 -381 -1115	-5099 -381 -1115	-4647 -381 -1115	-3744 -381 -1115	-4888 -381 -1115	-783 -381 -1115	-4057 -381 -1115	1115 1236	-5073 -381 -1115
-4993	-5009	-5462	-5579	-5462	-3780	-4146	-4171	-2114	-4815	-2573	-4277	-1475	-3749
43	43	43	-43	43	43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894
-5304	-5403	-5092	5229	-5092	-3647	-3927	-3838	-2682	-4492	-2950	-4134	2044	-3389
233	233	233	233	233	233	233	233	233	233	233	233	233	233
-9181	9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	9181	-9181
-2122	-2287	-4203	3766	-4203	-2007	-4392	-2128	-3375	-2468	-3404	-1795	1950	-4117
-500	-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	-8139	-8139
-2625	-2790	-4435	-2623	-4435	-1473	-4589	677	-2667	3631	-3103	3357	-1061	-4000
-149	-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	-8	-8
31(V)	32(I) 	33(G) 	34(Y)	35(G) 	36(S) 	37(Q) 	38(G) 	39(H) 	40(A)	41(H) 	42(A)	43(Q)	44(N)

14400%	14500%	14600%	14700%	15400%	15500%	15700%	15900%	1600%	16100%	16200%	16300%
-3690	-2515	-3958	-4066	-3914	-2660	-2676	-343	-3996	-4725	-3234	-3267
-249	-249	-249	-250	-249	-249	-249	-249	-249	-249	-249	-249
-3665	-2995	-5004	-4477	-4880	-3037	-3460	-1385	-4511	-4720	-3184	-3353
-294	-294	-294	-295	-294	-294	-294	-294	-294	-294	-294	
-2629	-2730	-4517	-3259	-4157	-3323	332	2145	3088	-3275	883	-3682
-369	-369	-369	-370	-369	-369	-369	-369	-369	-369	-369	
-4399 117	-1613 117	-3007	-1962 118	-2716 117	-2716 117	-1666 117	1166 117	-2620	-1982 117	-3351 117	-2709
-5379	-1596	-2501	3508	-2284	-4081	-1483	-1630	-4488	-1761	-4757	-2894
359	359	359	360	359	359	359	359	359	359	359	359
-5002	2808	-3536	-3497	-3440	-4367	-1595	-2037	-5106	-4127	-4691	3800
96	96	96	95	96	96	96	96	96	96	96	
-4750	256	-1786	-2967	-1836	-3980	-770	-1809	-4894	-3863	-4126	-978
45	45	45	45	45	45	45	45	45	45	45	
-4997	-2603	-3196	-3026	-3116	-4518	-2505	-2810	-4871	-3132	-4820	-3439
394	394	394	393	394	394	394	394	394	394	394	-344
-5514	224	-1209	288	-53	-4472	692	-2098	-4794	-2997	-5207	-2133
275	275	275	275	275	275	275	275	275	275	275	
-1236	-2123	-4373	-3676	-4163	-611	-2427	-117	-1475	-3835	-255	-2874
-720	-720	-720	-721	-720	-720	-720	-720	-720	-720	-720	
3316	-2955	-4903	-4524	-4752	848	-3279	-757	-1533	-4732	3041	-3529
-466	-466	-466	-465	-466	-466	-466	-466	-466	-466	-466	-466
-5423	2321	-2528	-3331	-2535	-4546	92	378	-4948	-4335	-5063	804
210	210	210	211	210	211	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*	*	*
-1886	-3116	-5082	-4365	-4854	-36	-3350	1415	2623	-4486	-543	-3905
-626	-626	-626	-627	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*	*	*
-4528 106 -1378	-973 106 -1378	-2082 106 -1378	-3082 105 -1378	-2126 106 -1378	-3784 -1378 -1378	-1177 106 -1378	-1420 106 -1378	-5139 106 -1378	-3809 106 -1378	-4410 106 -1378	-1396 106
-4980	-2518	-2600	-2279	3373	-4827	-2196	-2557	-5108	3492	-5374	-3470
399	399	399	398	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	701
-2290	-3487	-5037	-4293	-4849	396	-3554	-883	-2772	-4633	-1449	-4620
-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-302	-1115	-2928	-1115	-1115	-1115	-1115	-1115	1115
-5628	-931	944	-2679	-911	-4770	2014	-2462	-4991	-4182	-5325	82
43	43	43	44	43	43	43	43	43	43	43	43
-894	-894	-894	-2405	-894	-203	-894	-894	-894	-894	-894	804
-5638	275	3855	-2363	1232	-5232	2735	-3075	-5302	-3852	-5791	-3266
233	233	233	232	233	-5232	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	01 01
-3800	-3015	-4843	-2212	-3988	-2324	-3285	366	-2122	-2107	-2938	-3724
-500	-500	-500	-500	-500	-50	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-3318	-8139	-3381	-8139	-8139	-8139	-8139	-8139	8130
-4414	-1731	-2896	-1536	-2521	-2767	-1684	369	-2624	929	-3427	3040
-149	-149	-149	-148	-149	-148	-149	-149	-149	-149	-149	-149
-8	-8	-8	-155	-8	-148	-8	-8	-8	-8	-8	•
45(L)	46(R)	47(D) 	48(S)	49(G) 	50(V)	51(D)	53(V)	54(V) 	55(G) —	<u>56(L)</u>	57(R)

continued
6
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B
T

16400%	16500%	16600%	16700%	16800%	16900%	17000%	17100%	17200%
-1915 -249	-3752 -249	-3479	-1767 -249	-4451 	-249	-1903 -249	-1836 -249	-4942 -249
-2579 -294	-4850 -294	-3910	-2441 -294	-294	4091	-2589 -294	-2455 -294	-4724 -294
-2041 -369	-4245 -369	-309	-1868 -369	-3028	983	-2025 -369	-361 -369	-3852 -369
-893 117	-2766 117	1771	-740 117	228	-972	70 117	-906 117	-2762 117
-566 359	-2297 359	2738	833 359	3517	359	96 359	-889 359	-2567 359
1079 96	-3230 96	-1902	-512 96	-3832 96	96	-659 96	-617 96	-4545 96
727 45	-1587 45	-1794 45	886 45	3606	1898 45	-100 45	692 45	-4477 45
1229 394	-3049 394	-2827 394	-1861 394	-2948 394	-2617 394	-1985 394	-2052 394	-3728 394
-556 275	421 275	-1833	1101 275	-2789	-2174	350 275	-631 275	-3727 275
-1503 -720	-4047 -720	-2945 -720	-1349 -720	-3573	-720	-1497 -720	-1353 -720	-4365 -720
-2421 -466	-4648 -466	-3793	-2265 -466	4479	-701 -466	-2419 -466	22 -466	-5025 -466
1772 210 *	-2269 210 *	1362 210 *	1599 210 *	-3996	2252	1241 210 *	2589 210 *	-4818 210 *
-2483 -626 *	-4803 -626 *	-3754 -626 *	-2309 -626 *	-4209 -626 *	560 -626 *	-2474 -626 *	-2192 -626 *	-4781 -626 *
-559 106 -1378	-1901 106 -1378	-2121 106 -1378	-435 106 -2238	1378 106	-1425 106 -1378	-556 106 -1378	-609 106 -1378	-4271 106 -1378
-1920 399 -701	-3103 399 -701	1604 399 -701	-427 399 -344	-2155 399 701	2567 399 -701	-1878 399 -701	-1963 399 -701	-2992 399 -701
-2743 -381 -1115	-4832 -381 -1115	-4005 -381 -1115	-2567 -381 -1115	-4384 -381 -1115	1926 -381 -1115	-2722 -381 -1115	-2502 -381 -1115	-4888 -381 -1115
1532 43 -894	587 43 -894	-1859 -43 -894	-98 43 -894	-4011 43 894	-2634 43 -894	1636 43 -894	770 43 -894	-4815 43 -894
-803 233 -9181	1614 233 -9181	-1932 -233 -2699	-619 233 -8943	-3742 -9181	-3261 233 -9181	212 233 -9181	-895 233 -9181	-4492 233 -9181
-2412 -500 -8139	-4661 -500 -8139	-2308 -500 -8139	-2232 -500 -7900	1604 -500 -8139	873 -500 -8139	-2404 -500 -8139	-2242 -500 -8139	-2768 -500 -8139
31 -149 -8	-2671 -149 -8	-1499 -149 -247	1362 -149 -8	1288 -149 -8	726 -149 -8	1527 -149 -8	-8 -149 -8	3631 -149 -8
58(K) 	9(G) 	0(S)		5(3)	3(W)	4(E)	5(K) 	(V)

17300%	17400%	17500%	1760%	17700%	17800%	18400%	18500%	1860%	18700%	18800%	18900%	19000%	19100%
-1949	-1892	-530	-4575	-249	-1851	-2354	1413	-2264	-1106	-1843	-2957	-4483	-2698
-249	-249	-249	-249		-250	-249	-249	-249	-249	-249	-249	-249	-249
-2596	-2577	-3087	-4706	1621	-2533	-2708	-1519	-2988	-1458	2212	-3790	-4580	-3091
-294	-294	-294	-294		-295	-294	-294	-294	-294	-294	-294	-294	-294
-630	-2014 -369	-2501 -369	-3996 -369	1337	-1970 -370	3269 -369	-528 -369	-2411 -369	2346 -369	-1936 -369	-3194 -369	-2894 -369	2574 -369
236	141 117	-1368 117	-2700 117	2509	687 119	-767 117	-172 117	1827 117	945 117	-829 117	-655 117	931 117	-58 117
359	666	-1245	-2451	-3277	806	-2619	-764	1451	-1695	23	-400	-1525	-2979
	359	359	359	359	359	359	359	359	359	359	359	359	359
873	-648	-1250	-4005	3524	436	-3291	-1547	-1095	-2111	421	-1976	-3919	-3700
96	96	96	96	96	95	96	96	96	96	96	96	96	96
45	-90 45	-543 45	-3376 45	-3157	672 45	-3099 45	-1174 45	-427 45	-1884 45	-73 45	-1005 45	-3730 45	-3794 45
-2052	-1977	-2329	-3545	3961	-1947	112	-2401	-2242	688	-789	-2659	-2948	-3855
394	394	394	394	394	393	394	394	394	394	394	394	394	394
-803	1161 275	-723 275	365 275	3595	-490 277	-3230 275	-1534 275	-688 275	-146 275	-510 275	-900 275	-2869 275	-3561 275
-1534	-1485 -720	-2025 -720	-4356 -720	405	-1438 -721	-897 -720	1185 -720	-1914 -720	1869 -720	-1416 -720	-2787 -720	-3471 -720	-1124 -720
-2445	-2408	-2897	-5094	1986	-2364	320	645	-2808	-792	-2331	-3605	-4333	-1435
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
1702	895	-693	-3796	3812	1721	-3369	-1445	-548	-2198	1480	-1319	-4157	-3745
210	210	210	210	210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*	*	*	*	*
-2510	-2462	-2935	-6042	505	-507	29	-637	-2863	1052	-2368	-3685	-3942	1342
-626	-626	-626	-626	-626	-627	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*	*	*	*	*
-586	-547	1397	-3456	1018	-512	-2822	-1127	1289	-1496	-527	-1391	-3680	-3240
106	106	106	106	106	105	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
-1965	-1873	-2096	3641	4135	-1854	-3417	127	-2041	-2618	-323	-120	-2153	-3763
399	399	399	399	399	399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701
-2780	-2711	-3153	-4852	3199	-2668	-1869	304	-3096	-943	-2628	-3861	-4332	-2160
-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-366	-1115	-1115	-1115	-1115	-1115	-1115	-1115	1115
1767	1234	985	-2885	4138	950	-3689	-1554	1290	-2535	612	3127	-4341	-3995
43	43	43	-3894	43	46	43	43	43	43	43	43	43	-3995
-894	-894	-894	-894	-894	-2159	-894	-894	-894	-894	-894	-894	-894	-804
-869	157	2124	-2521	-4685	338	-4149	-2131	1526	-3143	-752	1372	-4092	-4449
233	233	233	233	233	232	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-0181
-2441	-2393	-2898	-2898	2256	-2348	-1639	-1093	-2794	-957	-2321	-3540	-1868	-1721
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-8139	-3318	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8130
-1006	1489	2104	-2294	-2596	47	-1810	847	-1284	-1069	1605	-1509	3390	2003
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-155	-8	-8	-8	-8	-8	-8	-8	-8
67(Q)	68(A)	69(D)	70(G)	(DI	72(K) —	73(V)	74(K) 	75(T) —	76(V)	77(W)	78(E) —	79(A) —	80(V)

19200%	19300%	19400%	19500%	1960%	19700%	19800%	19900%	2000%	20100%	20200%	20300%	20400%	20500%
-2027	-1853	-4534	-3809	-3628	-3902	-1313	-2711	-3690	-1668	-4474	-5231	-2064	-1158
-249	-249	-249	-249	-249	-249	-249	-249	-249	249	-249	-249	-249	-249
-2652	2858	-4636	-4929	-3899	-4355	-1629	-2860	-3665	-2020	-4610	-4922	-2660	-1557
-294	-294	-294	-294	-294	-294	-294	-294	-294		-294	-294	-294	-294
-2148	-1966	-3001	-4347	2741	3019	-505	1692	-2629	1299	-3354	-5894	-1902	896
-369	-369	-369	-369	-369	-369	-369	-369	-369		-369	-369	-369	-369
-1032	-147 117	-1718 117	-2844 117	-2688 117	-2622 117	-1278 117	-2499 117	-4399 117	1598	-2068 117	-4750 117	932 117	-905 117
428	-382	706	-2353	-4427	-4487	-228	-3687	-5379	-2308	666	-4440	463	-94
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96	96	96	96	96	96	96	96	96	96	96	96	96	96
-203	1955 45	-3729 45	603 45	-4509 45	-4798 45	250 46	-3615 45	-4750 45	-2583	-3625 45	-3870 45	-554 45	1474 45
-2135	-1952	-2929	-3082	-4771	-4752	-2878	-4228	-4997	3211	3993	-4501	-2250	-2374
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-720	-720	-720	-720	-720	-720	-720	-720	-720	-720	-720	-720	-720	-720
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210	210	210	210	210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*	*	*	*	*
-2574	-2410	-4207	-4905	1963	2554	577	2791	-1888	1616	-4516	-6376	-2250	246
-626	-626	-326	-626	-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*	*	*	*	*
-654	-519	-3684	-1935	-4670	-5011	-1708	-3487	-4628	-2166	-3618	-3905	-942	$1600 \\ 106 \\ 1370$
106	106	106	106	106	106	106	106	106	-1378	106	106	106	
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	
-2043	634	-2128	-2496	-5099	-5125	-2860	-4496	-4980	-3195	-2407	-3967	-2046	-2306
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-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	-701	701
-2858 -381 -1115	-2663 -381 -1115	-4411 -381 -1115	-4912 -381 -1115	-2254 -381 -1115	-2652 -381 -1115	-968 -381 -1115	669 -381 -1115	-2290 -381 -1115	-1360 -1360	-4516 -381 -1115	-5700 -381 -1115	-2589 -381 -1115	1582 -381 1115
446	432	-4279	396	-4951	-4970	-2708	-4470	-5628	-3262	-3710	-3014	2715	-14
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-959	815	-3998	3813	-5338	-5306	-3317	-5017	-5638	-3846	-3396	4174	-983	-2012
233	233	233	233	233	233	233	233	233	-333	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	0101
-2501	-2347	-1860	-4795	-2220	-2129	-1208	-2177	-3800	-1286	-2214	-4701	-2199	-1137
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
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-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	-8	°
81(K)	82(W) 	83(A) 	84(D) —	85(V)	86(V)	87(M)	88(I) 	89(L)	90(I)	91(P) 	92(D) —	93(E) 	94(H) 

20600%	20700%	20800%	20900%	21000%	21100%	21200%	21300%	21400%	21500%	21600%
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-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-3295	-2540	-2535	-2796	3325	-2536	-2948	-2605	-4202	-2530	-3462
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
-2559	-1951	-623	2330	-3883	-1975	-2377	-1023	2240	-1954	-2849
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-2844	-874	250	-2000	-4890	238	-1185	-885	-2642	-855	-1644
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-2894	447	-760	-3105	-4357	-766	265	109	-4470	-804	-469
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4317	$\frac{711}{45}$	1017	-3351	-3864	890	-136	1767	-4705	881	-812
45		45	45	45	45	45	45	45	45	45
-3710	1217	-1947	-3870	-4964	-1952	-2143	-1994	-4828	-1983	2974
394	394	394	394	394	394	394	394	394	394	394
-2682	-567	-489	804	-3723	777	1956	-518	-4775	-529	-848
275	275	275	275	275	275	275	275	275	275	275
-2353	-1445	-1440	-826	-3132	-1443	-1886	-1515	-1251	-1436	-2420
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-2456	-2350	-2367	1460	-3041	-792	-2773	-2437	297	-2347	-3274
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-1802	232	1010	-3856	-5135	1668	-495	894	-4894	1240	-1038
210	210	210	210	210	210	210	210	210	210	210
*	*	*	*	*	*	*	*	*	*	*
-160	-2374	-2421	1986	-3727	-2422	-2836	-2493	3165	-2380	-3329
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*	*
-2460	-579	1062	-3049	-1300	888	-775	-567	-4900	876	-1216
106	106	106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-2261	-1378	-1378	-1378	-1378
-3344	-531	-1854	-3939	-5109	-1859	-1913	-436	-5119	-1887	-489
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-2790	-2645	-2671	-1846	1898	-2674	-3060	-2740	-2520	628	-3538
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-2681	-268	1381	-3939	-5581	1681	2042	2138	-4965	2003	442
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-2766	-809	1394	-4456	-5230	-734	862	863	-5316	-760	1561
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-9181	-9181	-9181	-9181	-9181	-2649	-8933	-9181	-9181	-9181	-9181
-3142	-2315	-2351	-1706	-3766	-2353	-2763	-2422	-2156	-2341	-3144
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-8	-8	-8	-8	-8	-257	-9	-8	-8	-8	-8
95(Q) 	96(A)	97(D)	98(V) 	(Y)99(Y)	100(E) 	101(E) —	102(E) 	103(I) 	104(E) 	<u>105(P)</u>

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	(				I-1-1-1			
21700%	21800%	21900%	22100%	22200%	22300%	22400%	22500%	22600%
647 -249	-3194 -249	-2622 -249 -249 -249 -249	-4521 -249	-1863 -249	-1182 -249	-3351 -249	-1442 -249	-292 -249
-2486 -294	-3071 -294	-3002 -294 -2610 -294	-4703 -294	-2540 -294	-1581 -294	-3264 -294	-1913 -294	-1356 -294
-198 -369	-369	-2858 -369 -369 -369 -369	-3974 -369	-303 -369	354 -369	506 -369	1305 -369	-2374 -369
-1120	-3292 117	-90 117 -913 117	-2674 117	-840 117	1843 117	-3236 117	-913 117	-4016 117
-1093 359	-4776 359	-1817 359 -480 359	-2418 359	790 359	334 359	-4864 359	-1111 359	-4547 359
-957 96	-4613 96	1261 96 -655 96	-3937 96	-630 96	-1501 96	-4762 96	-1135 96	-4561 96
-415 45	-4005 45	1457 45 45 1446 45	-3243 45	788 45	-1111 45	-4172 45	660 45	-3987 45
-2218 394	-4778 394	-2766 394 1941 394	-3507 394	-1966 394	-2388 394	-4857 394	-2228 394	-4871 394
3151 275	-5181 275	489 275 -552 275	1193 275	414 275	-1475 275	-5232 275	-1040 275	-4290 275
-1509 -720	2728 -720	-2208 -720 -1525 -720	-4315 -720	-1444 -720	1167 -720	-282 -720	-712 -720	-1124 -720
-2336 -466	2621 -466	-3010 -466 -2440 -466	-5053 -466	-2362 -466	-1024 -466	2935 -466	187 -466	563 -466
-479 210 *	-5022 210	3059 210 * 1139 210	-3678 210 *	927 210 *	-1275 210 *	-5103 210 *	-752 210	-5074 210 *
-2279 -626 *	1361 -626 *	-3210 -626 * -2491 -626	-5005 -626 *	-2405 -626 *	758 -626 *	1096 -626 *	-1181 -626 *	-1742 -626 *
1767 106 -1378	-4282 106 -1378	-1025 106 -1378 -589 1378	-1378 -1378 -1378	-535 106 -1378	-1111 106 -1378	-4502 106 -1378	-891 106 -1378	-2159 106 -1378
-2071 399 -701	-5488 399 -701	-2740 399 -701 -1913 399	3554 399 -701	-1868 399 -701	-2314 399 -701	-5535 399 -701	148 399 -701	-5143 399 -701
-2376 -381 -1115	-1352 -381 -1115	-3650 -381 -1115 -2747 -381 -381	-4832 -381 -1115	-2661 -381 -1115	-1155 -381 -1115	-1506 -381 -1115	-1603 -381 -1115	4216 -381 -1115
827 43 -894	-5252 43 -894	-1232 43 -894 -894 43	-2709 -2709 -894	-198 43 -894	-1461 43 -894	-5293 43 -894	-857 43 -894	-5431 43 -894
-814 233 -9181	-5826 233 -9181	-1997 233 -9181 -740 233	-2347 -2347 -9181	958 233 -9181	-14 233 -9181	-5806 233 -9181	158 233 -9181	-5436 233 -9181
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106(N) —	107(M) —	108(K)  109(P) 	110(G) 	111(A) 	112(T) —	113(L) —	114(A) —	115(F) 

22700%		22800%	22900%	23000%	23100%	23200%	23300%	23400%	23500%	23600%
-4539	647-	-3641 -249	-5849 -249	-523 -249	-2737 -249	-3791 -249	-3759 -249	3677 -249	-2021 -249	-2414
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-2983	60C-	-6022 -369	-5862 -369	-2200 -369	-2139 -369	1969 -369	-4793 -369	-3870 -369	-2133 -369	-1719
-1679		-5395 117	-4815 117	-3934 117	-1437 117	$\frac{-2653}{117}$	-3702 117	-4669 117	$\frac{-1011}{117}$	-1964
1514	600	-5391 359	-4727 359	-4839 359	413 359	-4472 359	-3508 359	-4344 359	-938 359	-2137 -359
	2	-4732	-5385	-4592 96	96	-4961 96	-3879 96	-4483	-802 96	<u>96</u>
-3656	f	-5011 + 45	-5546	- 3998	-1678	-4681	-3770		45	3585
-2896		-4960 394	-4804 394	-4880 394	394	-4824 394	-4185 394	-4955 394	-2090 394	-3153
375	717	7 -4954 0 275	) -5141	3 -4443	2 3468	-4780 275	-3481	7 -3715 275	2 1553 0 275	2187
-3523	N71-	-72(	-597(	-72(	-2052	-72(	-4811	-3127	-1622	-1121
-4469		-5786 -466	-6297 -466	1089 -466	-466	358 -466	-5304 -466	-3041 -466	-284 -466	1526
-4134 210	017 2	-4911 210 *	-5765 210 *	-5107 210 *	-1809 210 *	-4893 210 *	-3798 210 *	-5105 210 *	972 210 *	-1588
-4216 676	070-	-6314 -626 *	-6627 -626 *	-1514 -626 *	-2602 -626 *	3293 -626 *	-5496 -626 *	-3719 -626 *	-2566 -626 *	-1539 -626
-3637	-1378	5435 106 -1378	-5028 106 -1378	-2370 106 -1378	-1925 106 -1378	-4876 106 -1378	5216 106 -1378	2153 106 -1378	998 106 -1378	-2019 -106
119	-701	-4506 399 -701	3834 399 -701	-5246 399 -701	-2135 399 -701	-5123 399 -701	638 399 -701	-5097 399 -701	1844 399 -701	-2877 399
-4413 -381	-1115	-4036 -381 -1115	-5893 -381 -1115	4093 -381 -1115	-2956 -381 -1115	-2477 -381 -1115	-4166 -381 -1115	3410 -381 -1115	-2820 -381 -1115	-2068 -381
-4219 43	-894	-5009 43 -894	-5462 43 -894	-5444 43 -894	-1781 43 -894	-4969 43 -894	-3491 43 -894	-5549 43 -894	-272 43 -894	-2186
-3998	-9181	-4720 233 -9181	-5092 233 -9181	-5534 233 -9181	-2020 233 -9181	-5324 233 -9181	-3197 233 -9181	-5210 233 -9181	948 233 -9181	-2466 233
-1829	-8139	-4539 -500 -8139	-4203 -500 -8139	-3387 -500 -8139	-1899 -500 -8139	-2169 -500 -8139	-3805 -500 -8139	-3757 -500 -8139	-2519 -500 -8139	-2285 -500
3091 -140	8-	-5197 -149 -8	-4435 -149 -8	-4044 -149 -8	885 -149 -8	-2673 -149 -8	-3381 -149 -8	-4816 -149 -8	-1065 -149 -8	412 -149
116(A)		117(H) —	118(G) —	119(F) —	120(N) —	121(I) 	122(H) —	123(Y) —	124(G) 	125(Q)

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23700%	23800%	23900%	24000%	24100%	24200%	24300%	24400%	24500%	24600%	24700%	24800%	24900%	25000%
-3237	-1798	-4548	-2174	-2146	1335	-1661	-1317	637	-2441	-3098	-4190	844	-2629
-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249	-249
-3673	-2448	-4647	-2757	-2853	305	-1765	-1637	-4324	-2784	-3911	-4414	1325	-2746
-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294	-294
2003	1255	-3067	-2184	-2285	-369	-1383	-1358	-3802	1904	-3196	3796	-271	-1114
-369	-369	-369	-369	-369		-369	-369	-369	-369	-369	-369	-369	-369
-2280	-827	-1770	-1147	-1120	-1350	-866	-759	-2471	-2009	-2088	-3297	276	-2531
117	117	117	117	117	117	117	117	117	117	117	117	117	117
5 230	5 -794 5 359	-1550 359	5 51 5 359	5 297	8 -1617 5 359	2 -789	3 -786 5 359	t -2102 5 359	7 -342 5 359	5 478 5 359	8 -4013 5 359	5 -1633 5 359	5 -3242 5 359
<u>3 -4255</u> 5 96	5 -646 5 96	13 -3911 5 96	17 2195 5 96	<u>9 -936</u>	13 -1798 5 96	<u>5 -1192</u>	2 698 5 96	6 -2754 5 96	1 -3587 5 96	<u>8 -2305</u> 5 96	10 -4923 5 96	<u>5 -2086</u>	1 -3576 5 96
<u>59</u> -406	74 65	75 -359	38 124	61 -31	78 -150	<u>39 -106</u>	69 23	36 -141	73 -337	82 -124	79 -494	08 -188	94 -336
94 -406	94 4	94 4	94 4	94 4	94 4	94 4	94 4	94 -4	94 -4	94 4	94 4	94 4	94 4
44 -42; 75 39	<u>65 -19</u> 75 <u>3</u> 9	70 37	84 22. 75 39	23 -210 75 39	26 -22 <sup>7</sup> 75 -39	92 35. 75 35	49 -150 75 39	51 -290 75 -39	<u>93 -38'</u> 75 39	<u>85 -278</u> 75 39	62 -45' 75 39	<u>57</u> -260 75 39	75 -39
324 -40	337 4	598 -27	713 -8	776 -6	66 -16	416 -9	021 -3	514 21	797 -35	915 6	570 -46	-81 -21	679 -38
720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2	720 2
1515 -1 -466 -	-300 -1 -466 -	4520 -3 -466 -	2586 -1 -466 -	2680 -1 -466 -	64 -466 -	-466 -	1630 -1 -466 -	4207 -3 -466 -	- 1581 -	<u>3675 -2</u> -466 -	2626 -2 -466 -	-723 -466 -	816 4-466 -
4248	1370 210 *	4053 210 *	-289 -2 210 *	1785 -2 210 *	1891 210 *	1074	2889 -: 210 -:	1947 210 *	3697 3 210 *	1632 -3 210 *	5060 -2 210 *	2236 210 *	3877 210 *
3248 - -626 *	-93 -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 *
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-701	-701	-701	-701	-701	-3098	-3098	-109	-701	-701	-701	-701	-701	-701
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-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
-4439	1172	-3897	-637	529	-2120	-1058	-230	-651	-3983	-680	-5160	-2609	-4279
43	43	43	43	43	43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894	-894
-4813	334	-3618	-1173	1377	-2227	-997	-564	3349	-4504	3495	-5092	-3230	-4754
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-9181	-9181	-9181	-9181	-325	-6882	-6882	-6882	-9181	-9181	-9181	-9181	-9181	-9181
-1916	-2234	-1925	-2398	-2663	-1104	-937	-1483	-4159	-1713	-3444	-2888	-875	-2345
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-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-2336	-38	-38	-38	-8	-8	-8	-8	-8	-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)
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25100%	25200%	25300%	25400%	25500%	25600%	25700%	25800%	25900%	2600%
-3991	-4519	-5786	-4729	-4556	-3787	-5849	-2990	-2351	-4190
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-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
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-4483	-1643	-5166	-4529	910	-2883	-4727	-2470	567	-4013
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-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
-4869	-3052	4310	-4535	-2898	4036	-4804	1551	-2747	-4579
394	394	394	394	394	394	394	394	394	394
275	-3057	-5357	-3921	-2795	-3659	-5141	-1838	-2330	-4662 275
-1474 -720	-3532 -720	-6067 -720	-4707 -720	-3540	2095	-5970 -720	-2963 -720	-1430 -720	-2570
-1532	-4351	-6281	-5171	-4489	-3066	-6297	-3639	1634	-2626
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-4945 210 *	-4447 210 *	-5780 210 *	3994 210 *	-4146 210 *	-3912 210 *	-5765 210 *	634 210 *	-2652 210	-5060 210
2415	-3901	-6679	-5555	-4238	-3353	-6627	-3830	-1754	-905
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
-5132	-3851	-5077	-3307	-3642	-3767	-5028	4731	-2291	-4687
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-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	1370
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-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	0101
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140(V)	141(A)	142(P)	143(K)	144(G)	145(P)	146(G)	147(H)	148(T)	149(V)
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-894

-9181

-500 -8139

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	26100%	26200%	26300%	26400%	26500%	2660%	26700%	27300%	27400%	27500%
	-4993	-1919	537	4052	-1626	-1856	-4826	-2033	-4766	-3678
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	-4538	-2585	-2295	-565	-2192	-2538	-4895	-2694	-4734	-4159
	-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 117	-66	1303 117	-4679 117	-344 117	-822 117	-3532 117	-1024 117	-2095 117	825 117
	-4989	1224	-858	-4356	443	-764	-3211	-962	-1874	-4115
	359	359	359	359	359	359	359	359	359	359
	4219	2308	-736	-4500	-796	419	-4377	471	-4170	-4752
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	-3672	-128	816	-3868	695	1878	-3187	-224	-3901	-4554
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5	-4521	-560	520	-3732	-703	571	-2619	481	-3058	-4502
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	-5502	-2413	-221	-3017	-52	-2370	-5498	-2529	-4812	-1545
	-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
	-2789 210 *	-142 210 *	-259 210 *	-5127 210	498 210 *	1502 210 *	-3818 211 *	596 210 *	-4356 210 *	-4661 210 *
	-5946	-2459	-1897	-3703	-1704	-508	-5629	-2582	-4580	2349
	-626	-626	-626	-626	-656	-626	-627	-626	-626	-626
	*	*	*	*	*	*	*	*	*	*
	-3791	1031	-611	-1317	-654	-515	-3337	-672	-3843	-4593
	106	106	106	106	106	106	106	106	106	106
	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
	-4412	76	-1934	-5093	-1969	-1856	3646	2300	3627	-4797
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	-5507	-2721	-2228	3303	-2060	-2675	-5355	-2848	-4698	-2667
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	-1115	-1115	-1115	-1115	-1115	-1115	-366	-1115	-1115	-1115
	-4682 43 -894	$1012 \\ 43 \\ -894$	2078 43 -894	-5565 43 -894	821 43 -894	1156 43 -894	-2361 44 -2159	488 43 -894	-4133 43 -894	-4769 43 -894
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	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
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	-4845	-962	-902	-4820	129	576	-3239	753	-52	-2485
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	-8	-8	-8	-8	-8	-8	-155	-8	-8	-8
	150(R)	151(R)	152(E)	153(Y)	154(V)	155(Q)	156(G)	157(G)	158(G)	159(V)
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27600%	27700%	27800%	27900%	-28000%	28100%	28200%	28300%	28400%	28500%
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-2665 359	872 359	460 359	-3916 359	-2567 359	-4483 359	-2318 359	-4704 359	-2522 359	-1056
-3757 96	-1093 96	-3567 96	-4320 96	-4545 96	-5102 96	-2362 96	-3826 96	-3575 96	-1013 96
-3244 45	-545 45	-3433 45	-4023 45	-4477 45	-4891 45	-1815 45	4575 45	-1872 45	-538 45
4031 394	-2199 394	-3626 394	-4439 394	-3728 394	-4869 394	-3330 394	-4693 394	-3235 394	1212 394
1199 275	-891 275	-3360 275	-4323 275	-3727 275	-4791 275	-2047 275	-4230 276	428 275	-943
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-3527 210 *	-617 210 *	-3689 210 *	-4413 210 *	-4818 210 *	-4949 210 *	-2056 210 *	-3840 210 *	-2604 210 *	150 210 *
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-2991 399 -701	-219 399 -701	-3121 399 -701	-4674 399 -701	-2992 399 -701	-5102 399 -701	-3242 399 -701	-4221 399 -701	-2633 399 -701	-2002 
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-2753 43 -894	-656 43 -894	-3997 43 -894	-4613 43 -894	-4815 43 -894	-4990 43 -894	-2051 43 -894	-4146 43 -894	-902 43 -894	138 43 894
-2413 233 -9181	1357 233 -9181	-3995 233 -9181	-5072 233 -9181	-4492 233 -9181	-5301 233 -9181	903 233 -9181	-3927 233 -9181	3943 233 -9181	-1274 233 -0181
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160(P) —	161(C) —	162(L) —	163(I) —	164(A) —	165(V) 	166(H) 	167(Q) —	168(D) —	169(A)

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(600%	100%	800%	%006	%0000	100%	200%	300%	400%	500%	600%	1700%	800%
<u>9</u> 28	6 58	9 28	9 28	9 25	9 25	<u>9</u> 29	9 25	9 25	<u>3</u> 9 26	9 29	9 25	96
-356 -24	-474 -24	-185	-494 -24	-216	-208	-24	-24	-24	-24	434 -24	-24	-172 -24
-4143	-4622	-2534	-4724	-2690	-2793	-1425	-4599	-3665	-4623	2928	-4636	-2330
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2313 117	-3317 117	1230 117	-2762 117	1094 117	-1058	-311 117	409 117	-4399 117	-1675 117	-3536	-1718 117	$\frac{14}{117}$
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394	394	394	394	394	394	394	394	394	394	394	394	394
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275	275	275	275	275	275	275	275	275	275	275	275	275
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-3938	-5188	-2363	-5025	851	-2624	1388	-4412	3316	-4462	-250	-4490	-2040
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-3839	-5283	-2415	-4781	-2603	-2684	1266	-4129	-1886	-4213	233	-4207	324
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*	*	*	*	*	*	*	*	*	*	*	*	*
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1001	-2978	-736	-4492	-1358	2320	-3279	-4064	-5638	-3961	-4918	-3998	113
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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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0056	%00	%00	%00	%00	%00	%00
538	300	302	303	304	305	306
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-3070	-4099 394 -4804 394	-2890 394	-986 394	-4263 394	-2734 394	-4606 394
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-4246 43 -894	-4153 43 -894 -5462 43	-894 -4536 -894 -894	-3769 43 -894	-3846 43 -894	-3873 43 -894	-5222 43 -894
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183(G)	184(I) — — 		.87(G) —	188(R) 	89(A)	190(G)

30700%	30800% 3000%	30900%	31100%	31200%	31300%	31400%	31500%	31600%	31700%	31800%
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629 117	-2703 117 -2007	-2097 117 4033 117	3742 117	-4016 117	-1432 117	-2041 117	-4728 117	3305 117	-1902 117	3756
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-4783 96	-4400 96	-2430 96 -4545 96	-3387 96	-4561 96	2054 96	-2087 96	-4570 96	-2526 96	-1064 96	-3797
-4584 45	-4008 45 1533	-4580 -4580 -4580	-2988 45	-3987 45	767 45	1281 45	-3838 45	-2309 45	-923 45	-3588 45
-4668 394	-4535 394 -1208	-1208 394 398 394	-2901 394	-4871 394	-2475 394	-2713 394	-4513 394	-2816 394	-2749 394	-2947 394
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-4788 43 -894	-4785 43 -894	52/9 43 -894 -894 -4810 43 -894	-2978 43 -894	-5431 43 -894	$1220 \\ 43 \\ -894$	2880 43 -894	3919 43 -894	-2982 43 -894	3293 43 -894	-4016 43
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191(V) —	192(I) — — 03/F)	93(E) 	95(T) 	196(F)	97(K) 	198(R) —	(99(E)	200(T)	201(E) 	202(T)

-8139 -9181 -894 -1115 -701- -1378

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-4750	-3571	-3740	-4815	-4726	-2944	191	-2619	-4399	-2306
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-5744	-146	-1314	-5970	-5604	-3189	-928	-1466	-1236	-930
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-4478 210 *	-5292 210	-4916 210 *	-5765 210 *	-4238 210 *	-443 210 *	-2577 210 *	-4932 210 *	-5423 210	-4138 210 *
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-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*	*
-3905 106 -1378	-4569 106 -1378	-2120 106 -1378	-5028 106 -1378	-3886 106 -1378	-1763 106 -1378	-2072 106 -1378	-5100 106 -1378	-4628 106 -1378	-3598 106
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-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8	°
203(D) —	204(L) 	205(F) 	206(G) —	207(E) 	208(Q) —	209(A)	210(V) —	211(L) —	

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-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
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-4727	-1784	-2089	773	545	-5215	-4362	-1509	-1483	-1606
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1 -480- 5 39-	9 -3149 5 394	7 -301	3 -224	<u>5 39</u>	4 -496	<u>3 -477</u> 5 39.	8 -254 5 39	7 -2498	<u>3 -297</u> 5 39.
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-5765	-4340	-2734	-821	328	$\frac{-5292}{210}$	-4810	2925	-32	-3625
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213(G)	214(G)	215(V)	216(M)	217(E)	218(L)	219(V)	220(K)	221(A)	222(G)
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33900%	3400%	34100% 34200%	34300%	34400%	34500%	34600%	34700%	34800%
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-4650 117	-2441 117	3354 117 -3729 117	2510 117	-2968	-1158	-4815 117	-4068	-923 117
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-4496 96	874 96	920 96 -4314 96	-4011 96	-3454 96	-1425 96	-5385 96	-4111 96	345 96
-3874	-1336	-1995 $-3864$ $-3864$	-3879	-1761	-905	-5546	-3735	2086 45
-4949 394	2951 394	-2936 394 -4756 394	-3600 394	- 3182 394	-2355	-4804 394	-4707 394	-2035
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-5070 399 -701	-2485 399 -701	-2483 399 -701 -5068 399	-701 -3037 -309 -701	-2594 399 -701	-292 399 -701	3834 399 -701	-4705 399 -701	-1925 399 -701
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223(F) 	224(E) 	225(T) 	227(V) 	228(E) —	229(A) 	230(G) —	231(Y) —	232(Q) 

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34900%
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-4636
-249
-4763
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-4817
-369
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0 -3320 5 359
39 -3760
45 90
945 -290 394 -
564 40
275 3
4771 -2 -720
-5281 -466
-3337
210
*
-5423
-626
*
-3173
106
-1378
-3359
399
-701
-5220
-381
-1115
817
43
-894
-1922
233
-9181
-4071
-500
-8139
-3403
-149
-8
233(P)

36300%	-36400%	36500%	36600%	36700%	36800%	36900%	37000%	37100%	37200%
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-632	3728	-227	-4998	-1038	2587	-3544	-3622	-2921	-6627
-626	-626	-626	-626	-626	-626	-626	-626	-626	-626
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-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
-5375	-5109	-3732	-1039	-4633	-4861	-5401	3135	201	-5462
43	43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894	-894
-5954	-5473	-4095	3864	-4842	-5342	-5142	-568	-1215	-5092
233	233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
-3023	-2484	-1668	-4569	-2715	-2356	-3630	-3457	-2818	-4203
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139
-3571	-2980	-1685	-2963	-2768	-2822	-4562	-1959	-347	-4435
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8	-8
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	-3009	2521	-3902	-4325
-369	-369	-369	-369	-369	-369	-369	-369	-369	-369
-1980	162	-2131	-3558	-1535	-1509	-1692	-2636	-2664	-2821
117	117	117	117	117	117	117	117	117	117
-2884	1037	-1845	-4860	-1789	-1389	3391	-4466	-3681	-2334
359	359	359	359	359	359	359	359	359	359
-3221	-993	-2243	-4539	2408	-1264	-3936	-4978	-4355	-3311
96	96	96	96	96	96	96	96	96	96
-2958	-498	-1148	-4039	-1215	-659	-3648	-4713	-4203	-1612
45	45	45	45	45	45	45	45	45	45
-3653	-2155	-2763	-4838	-2814	-2446	-2903	-4827	-3638	-3069
394	394	394	394	394	394	394	394	394	397
194	-905	3219	-5248	2239	1645	-2782	-4770	-3492	3045
275	275	275	275	275	275	275	275	275	275
1999	-817	-3055	4920	-1133	-2110	-3561	-1267	-4364	-4140
-720	-720	-720	-720	-720	-720	-720	-720	-720	-720
1017	-49	-3838	-948	380	-2941	-4511	332	-5102	-4718
-466	-466	-466	-466	-466	-466	-466	-466	-466	-466
-3389 210 *	-577 210	-1535 210 *	-4928 210 *	-1089 210 *	441 210 *	-4132 210 *	-4891 210 *	-4527 210 *	-2320 210 *
3134 -626 *	-1355 -626	-3930 -626 *	-822 -626 *	-1596 -626 *	-2986 -626 *	-4260 -626 *	2957 -626 *	-4988 -626 *	-4885 -626 *
-2668	-793	-1518	-4248	-1089	1172	-3634	-4905	-4045	-1922
106	106	106	106	106	106	106	106	106	106
-1378	-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
-381	-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
-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
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
-1769	-1640	-3809	-3159	-1949	-2973	-1844	-2149	-2711	-4778
-500	-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139	-8139
-2042	1914	365	-3656	-1614	-1548	279	-2853	-2212	-2725
-149	-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8	-8
256(I)	257(A)	258(N)	259(M)	260(R)	261(Y)	262(S)	263(I)	264(S)	265(N)
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ed	
-continu	
9	
ABLE	
H	

38300%	38400%	38500%	38600%	38700%	38800%	38900%	39000%	39100%
-3271	-4687	-2917	4211	-5635	-3791	3508	-1553	-2006
-249	-249	-249	-249	-249	-249	-249	-249	-249
-4055	-4697	-3455	2997	-4754	-4894	-1615	-1907	-2454
-294	-294	-294	-294	-294	-294	-294	-294	-294
-3351	-3194	-3199	-3637	-5613	-4301	-1282	2578	822
-369	-369	-369	-369	-369	-369	-369	-369	-369
3157 117	-1908 117	-2032 117	-4391 117	-4560 117	$\frac{-2809}{117}$	-2410 117	$1701 \\ 117$	2345 117
-1897	-1690	-1923	-4199	-4461	-2330	-3181	-2129	1341
359	359	359	359	359	359	359	359	359
-2277	-4099	-773	-4299	-5178	-3297	-3468	-2514	887
96	96	96	96	96	96	96	96	96
-1369 45	-3858 45	1323 45	-3741	-5312 45	-1621 45	-3109 45	942 45	-1149 45
-2850	-3080	-2827	-4847	-4606	-3073	-3886	-3060	-2421
394	394	394	394	394	394	394	394	394
2125	-2978	-1209	-3649	-4896	-1084	-3537	-2568	-1442
275	275	275	275	275	275	275	275	275
-3103	-3754	-2636	-2965	-5741	-4115	1558	-429	-1265
-720	-720	-720	-720	-720	-720	-720	-720	-720
-3859	-4660	-3417	-2951	-6087	-4703	892	-1	-2016
-466	-466	-466	-466	-466	-466	-466	-466	-466
-1685 210 *	-4332 210 *	1878 210 *	634 210 *	-5533 210 *	-2320 210 *	-3766 210 *	-2570 210	-1234 210 *
-3874	-4400	-3596	-3522	-6386	-4863	255	691	-1769
-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*
-1713	-3798	-1284	-1299	-4823	-1932	-2215	-1955	-1387
106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1980	-1378	-1378	-1378	-1378
-2367	659	-2588	-4972	3828	1758	-4046	-3035	-2112
399	399	399	399	399	399	399	-3035	399
-701	-701	-701	-701	-422	-701	-701	-701	-701
-4071	-4573	-3935	1910	-5686	-4880	2447	-1283	-2170
-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
903	-4290	2964	-5310	-5222	1828	-4137	-2894	-1401
43	43	43	43	43	43	43	43	43
-894	-894	-894	-894	-894	-894	-894	-894	-894
-596	-3979	-1036	-5100	-4855	3025	-4651	-3480	-1918
233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-3345	-9034	-9181	-9181	-9181	-9181
-3396	-2035	-3488	-3618	-3995	-4705	-2175	-1250	-1643
-500	-500	-500	-500	-500	-500	-500	-500	-500
-8139	-8139	-8139	-8139	-7992	-8139	-8139	-8139	-6139
-2061	3410	-2118	-4524	-4176	-2710	-2497	-1425	516
-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-155	-8	-8	-8	-8	-8
266(T)	267(A)	268(E)	269(Y)	270(G)	271(D)	272(Y)	273(V)	274(T)
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ABLE 9-6	continued
ABLE	9
Η	TABLE

39200%	39300%	39400% 39500%	39600%	39700%	39800%	39900%	4000%	40100%
-4735 -249	1093 -249	-2084 -249 -249 -249	-3250 -249	1327 -249	1152 -249	-1871 -249	-3270 -249	-3189 -249
-4725 -294	-2467 -294	-2671 -294 -294 -294	-3688 -294	-3366 -294	-2885 -294	-2555 -294	-3549 -294	-3267 -294
-3297 -369	-873 -369	-2191 -369 -369 -369 -369	2182 -369	-2804 -369	-2310 -369	-1994 -369	1397 -369	-3556 -369
-2005 117	-939 117	-1133 117 -2487 117	-2288 117	1851 117	-1162 117	-837 117	2366 117	-2650 117
-1784 359	465 359	417 359 -4102 359	226 359	-1561 359	-1065 359	116 359	2251 359	-2874 359
-4137 96	-802 96	2862 96 -4784 96	-4274 96	-1742 96	-992 96	-620 96	-3214 96	2705 96
-3871 45	-260	-273 -57 -45 -4585 -4585	-4082	-899	-358	1356	-2916	-937 -45
-3149 394	2813 394	-2228 394 -4649 394	394	-2573 394	-2190 394	478 394	-2821 394	-3430 394
-3009 275	-651 275	-795 275 275 275	-4066 275	2295 275	-640 275	590 275	-2557 275	-2188 275
-3857 -720	-1351 -720	-1653 -720 -1443 -720	-1326 -720	-2403 -720	-1808 -720	-1462 -720	-2328 -720	1860 -720
-4749 -466	-2204 -466	-2528 -466 -1561 -1561 -466	-1516 -466	-3214 -466	-2704 -466	-2388 -466	-3076 -466	-3413 -466
-4340 210 *	533 210 *	848 210 * -4689 210	* -4265 210 *	-1145 210 *	381 210 *	490 210 *	-3215 210 *	2942 210 *
-4506 -626 *	-2143 -626 *	-2587 -626 * 2142 -626	* 3155 -626 *	-3221 -626 *	-2753 -626 *	-2444 -626 *	-2611 -626 *	-3763 -626 *
-3816 106 -1378	-675 106 -1378	-716 106 -1378 -4639 106	-1378 -3954 -1378 -1378	-1273 106 -1378	-796 -1378 -1378	1182 106 -1378	-2891 106 -1378	-1356 106 -1378
3536 399 -701	-1960 399 -701	-2145 399 -701 -4777 399	-701 -4254 399 -701	-2239 399 -701	-2011 399 -701	-1861 399 -701	-2145 399 -701	-3512 399 -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 -1115
-4171 43 -894	-359 43 -894	1072 43 -894 -4789 43	-894 -4452 -894 -894	-550 43 -894	2368 43 -894	1835 43 -894	-3444 43 -894	-2331 43 -894
-3838 233 -9181	343 233 -9181	-1097 233 -9181 -5133 233	-9181 -4828 233 -9181	2329 233 -9181	1227 233 -9181	859 233 -9181	-3655 233 -9181	-3848 233 -9181
-2128 -500 -8139	-2210 -500 -8139	-2548 -500 -8139 -8139 -2035 -2035	-8139 -1919 -500 -8139	-3162 -500 -8139	-2699 -500 -8139	-2372 -500 -8139	-1688 -500 -8139	-3623 -500 -8139
677 -149 -8	-992 -149 -8	-1214 -149 -8 -8 -149	-8 -2265 -149 -8	-1731 -149 -8	1097 -149 -8	-166 -149 -8	228 -149 -8	-2991 -149 -8
275(G) 	276(P) —	277(R) 	279(I) 	280(D) —	281(E) —	282(E) —	283(T) —	284(K) 

40200%	40300%	40400% 40500%	40600%	40700%	40800%	40900%	41000%	41100%
-1869 -249	-1849 -249	-3030 -249 1245 -249	-1891 -249	-2720 -249	1565 -249	-1851 -249	-3155 -249	-3968 -249
-2552 -294	-2488 -294	-3044 -294 -2885 -294	-2577 -294	-3118 -294	-1846 -294	-2532 -294	-4024 -294	-4467 -294
-1992 -369	-1889 -369	-1557 -369 -369 -369 -369	-2015 -369	2342 -369	-1269 -369	-1968 -369	-3473 -369	2071 -369
-836	-881 117	-3558 117 -1524 117	$\frac{72}{117}$	-2046 117	-2345 117	783 117	-2163 117	-2627 117
106 359	-62 359	-4860 359 -1527 359	-795 359	-3144 359	-2972 359	-763 359	-1879 359	-4473 359
437 96	1056 96	-4539 96 1762 96	831 96	-3805 96	-3298 96	-603 96	2142 96	-5086 96
$\frac{1120}{45}$	-136 45	-4039 45 45 45 45	663 45	-3613 45	-3025 45	889 45	-1139 45	-4869 45
-1960 394	-2019 394	-4838 394 -2554 394	-1975 394	-3957 394	-3782 394	-1949 394	-2782 394	-4862 394
-498 275	-586 275	-5248 275 275 275 -1146 275	1305 275	-3691 275	-3390 275	-492 275	24 275	-4789 275
-1460 -720	-1391 -720	4920 -720 -1997 -720	-1485 -720	-1109 -720	-476 -720	917 -720	-3075 -720	-1447 -720
-2385 -466	-2279 -466	948 -466 -2832 -466	-2409 -466	-1406 -466	2855 -466	-2362 -466	-3852 -466	-1495 -466
$\frac{1639}{210}$	$\frac{1096}{210}$	-4928 210 * * 2831 210	-134 210 *	-3870 210 *	-3485 210 *	$   \frac{1692}{210} $	-1467 210 *	-4941 210 *
-2442 -626 *	269 -626 *	-822 -626 * * -2971 -626	-2465 -626 *	1746 -626 *	-944 -626 *	-2414 -626 *	-3962 -626 *	3464 -626 *
-526 106 -1378	-578 106 -1378	-4248 106 -1378 -912 106 -1378	-545 -545 -1378	-3330 106 -1378	-2344 106 -1378	873 106 -1378	-1511 106 -1378	-5106 106 -1378
-1863 399 -701	-1928 399 -701	-5421 399 -701 -526 399 -701	-487 399 -701	-3932 399 -701	-3779 399 -701	-1855 399 -701	-2358 399 -701	-5092 399 -701
-2691 -381 -1115	-2570 -381 -1115	-1349 -381 -1115 -3346 -3346 -381 -1115	-2713 -381 -1115	-2155 -381 -1115	-898 -381 -1115	-2665 -381 -1115	-4119 -381 -1115	-2737 -381 -1115
1690     43     -894	814 43 -894	-5350 -5350 -894 -894 -894	2205 43 -894	-4122 43 -894	-3889 -3889 -3894 -894	1211 43 -894	1765 43 -894	-4991 43 -894
732 233 -9181	-843 233 -9181	-5816 233 233 -9181 -1591 233 233 -9181	367 233 -9181	-4584 233 -9181	-4307 233 -9181	143 233 -9181	2790 233 -9181	-5302 233 -9181
-2370 -500 -8139	-2286 -500 -8139	-3159 -500 -500 -8139 -2891 -2891 -500 -8139	-2394 -500 -8139	3024 -500 -8139	-2175 -500 -8139	-2347 -500 -8139	-3878 -500 -8139	-2131 -500 -8139
443 -149 -8	1871 -149 -8	-3656 -149 -8 -8 -1646 -149 -8	-172 -149 -8	1574 -149 -8	-187 -149 -8	862 -149 -8	-2148 -149 -8	-2630 -149 -8
285(E) —	286(A) —	287(M) 	289(E) 	290(C) 	291(L) —	292(K) —	293(D) —	294(I) 

41200%	41300%	41400%	41500%	41600%	41700%	41800%	41900%	42000%
-2908	-2340	-5849	-1890	2917	-2447	-2895	-1864	-84
-249	-249	-249	-249	-249	-249	-249	-249	-249
-3278	-3076	-4924	-2559	-601	-2812	-3159	-2545	4754
-294	-294	-294	-294	-294	-294	-294	-294	-294
-3060	-2508	-5862	-1999	-3546	1100	-3194	-1982	664
-369	-369	-369	-369	-369	-369	-369	-369	-369
-2031	-96	-4815	1488	-4237	197	-2152	-833	-2878
117	117	117	117	117	117	117	117	117
-2018	1978	4727	829	-4078	-2387	-458	775	-3475
359	359	359	359	359	359	359	359	359
-430	-1177	-5385	96	-4209	-3298	2488	722	-3743
96	96	96		96	96	96	96	96
3817 45	724 45	-5546 -45	-97 45	-3628 -45	-3073 45	-758 45	-66 45	-3330
-2936	-2288	-4804	-1991	-4750	-3432	-3047	-1958	-4179
394	394	394	394	394	394	394	394	394
-1545	1817	-5141	-536	-3532	-3103	-1693	-498	-3564
275	275	275	275	275	275	275	275	275
-2513	-2012	-5970	-1472	-2894	-1089	-2487	1895	-987
-720	-720	-720	-720	-720	-720	-720	-720	-720
-3271	-2903	-6297	-2389	-2900	-1486	-3234	-2376	92
-466	-466	-466	-466	-466	-466	-466	-466	-466
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
-626	-626	-626	-626	-626	-626	-626	-626	-626
*	*	*	*	*	*	*	*	*
-1314	-914	-5028	-552	-1320	-2823	-1188	-524	-1779
106	106	106	106	106	106	106	106	106
-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378	-1378
-2760	-2068	3834	-1895	-4820	-3165	-3041	-537	-4315
399	399	399	399	399	399	399	399	399
-701	-701	-701	-701	-701	-701	-701	-701	-701
-3862	-3192	-5893	-2697	3858	-2047	-4038	-2680	3045
-381	-381	-381	-381	-381	-381	-381	-381	-381
-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115	-1115
-1401	354	-5462	2013	543	-3628	163	$1780 \\ 43 \\ -894$	-4482
43	43	43	43	43	43	43		43
-894	-894	-894	-894	-894	-894	-894		-894
-1818	1712	-5092	-769	-4619	-4068	-2464	740	-4795
233	233	233	233	233	233	233	233	233
-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181	-9181
-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
-149	-149	-149	-149	-149	-149	-149	-149	-149
-8	-8	-8	-8	-8	-8	-8	-8	-8
295(Q)	296(S)	297(G)	298(E)	299(F)	300(A)	301(K)	302(M)	303(W)
		—						

				<u></u>						
42100%		42200%	42300%	42400%	42500%	42600%	42700%	42800%	42900%	43000%
-3596	-249	-1329 -249	-3401 -249	1446 -249	-1895 -249	-1624 -249	-1874 -249	2446 -249	-3277 -249	-1317 -249
-3934	-294	-1756 -294	-4362 -294	-1990 -294	-2566 -294	-2169 -294	-2814 -294	-1730 -294	-3832 -294	-1637 -294
2384	-369	84 -369	-3767 -369	-369	-2019 -369	-261 -369	-3006 -369	-800 -369	-2803 -369	-1358 -369
-2597	117	-152 117	-2402	-880	-868 117	306 117	-2162 117	-888 117	286 117	-759 117
-4301	359	767 359	-2045 359	-1022 359	-812 359	-953 359	-1986 359	-1172 359	-1566 359	-786 359
-4769	96	-1349 96	-2626 96	165 96	1528 96	1657 96	-3185 96	1577 96	-2362 96	698 96
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continued
9
Ц
В
E

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-1713	-2727	-3161	-561	-560	-3234	-1218	1337	3227
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continued
9
ĽE
AB

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1 -440 5 39	2 -182 5 39	<u>7 -192</u>	7 162 5 39	7 -277 5 39	2 -213	5 -283 5 39	4 -273 5 39	7 53 5 39	<u>66</u>	0 -254 5 39	9 132
08 -431 20 27	48 58 20 27 45 51	1 <u>c-</u> 20 277	48 284 20 27	78 -148 20 27	00 -120 20 27	36 -230 20 27	16 -90 20 27	00 -44 20 27	11 -60 20 27	83 -124 20 27	<b>89</b> 342 *
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SEQUENCE LISTING

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Leu Asn Met Lys Asj 35	p Ser Gly I 4	Leu Asn Val 40	Val Val Gly 45	Leu Arg Lys
Asn Gly Ala Ser Trj 50	p Glu Asn A 55	Ala Lys Ala	Asp Gly His 60	Asn Val Met
Thr Ile Glu Glu Al 65	a Ala Glu I 70	Lys Ala Asp	Ile Ile His 75	Ile Leu Ile 80
Pro Asp Glu Leu Gli 85	n Ala Glu N	Val Tyr Glu 90	Ser Gln Ile	Lys Pro Tyr 95
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Tyr Gly Phe Ile Va 115	l Pro Pro I 1	Lys Gly Val 120	Asn Val Val 125	Leu Val Ala
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Phe	210 Glu	Thr	Суз	His	Glu	215 Leu	Lys	Leu	Ile	Val	220 Asp	Leu	Ile	Tyr	Gln
225 Lys	Gly	Phe	Lys	Asn	230 Met	Trp	Asn	Asp	Val	235 Ser	Asn	Thr	Ala	Glu	240 Tyr
Glv	Glv	Leu	- Thr	245 Arq	Arq	- Ser	Arq	- Ile	250 Val	Thr	Ala	Asp	Ser	255 Lvs	Ala
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AT4	Dhe	275	Jan		Jeu	ALY	280		UII	م م	ULY	285	Ine	1111	шүр
GIU	290	ьeu	ьeu	GIU	гда	GIN 295	vai	ser	Tyr	AIA	н1s 300	ьeu	гуа	ser	met
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Phe	Glu	Thr	Val	Ser 165	Ala	Met	Ala	Lys	Gly 170	Ile	Gly	Leu	Ser	Arg 175	Ala
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Pro Ly 13	s Al O	a Pro	⊃ Gly	' His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
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Gly	7 Glu	. Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Ala
Ala	. Met	Arg 275	Asn	Ala	Leu	Lys	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met	: Phe 290	Ile	Thr	Glu	Gly	Ala 295	Ala	Asn	Tyr	Pro	Ser 300	Met	Thr	Ala	Tyr
Arç 305	J Arg	Asn	Asn	Ala	Ala 310	His	Pro	Ile	Glu	Gln 315	Ile	Gly	Glu	Lys	Leu 320
Arç	, J Ala	Met	Met	Pro	Trp	Ile	Ala	Ala	Asn	Lys	Ile	Val	Asp	Lys	Ser
Lys	Asn	L		325					330					335	
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Met 1	Lys	Val	Phe	Tyr 5	Asp	Lys	Asp	Суз	Asp 10	Leu	Ser	Ile	Ile	Gln 15	Gly
Lys	a ràa	Val	Ala 20	Ile	Ile	Gly	Phe	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Cys	s Asn	Leu 35	Lys	Aap	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Tyr	Lys
Gl}	7 Ala 50	Ala	Asp	Ala	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Phe	Lys	Val	Thr
Asr 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	e Lys	Lys	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Туг	: Asn	Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Asp	Val	Ile 125	Met	Ile	Ala
Pro	Lys	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe	Val	Lys	Gly	Gly
Gly 145	7 Ile	Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys	Asn	Val	Ala	Leu	Ser	Tyr	Ala	Ala	Ala	Val	Gly	Gly	Gly	Arg	Thr
Gly	/ Ile	Ile	Glu	Thr	Thr	Phe	Lys	Asp	Glu	Thr	Glu	Thr	Asp	Leu	Phe
Gla	Glu	Gln	180 21a	Val	Leu	Cva	Glv	182	Thr	Val	Glu	Leu	7.20 7.20	Ive	719

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Phe Glu Cys 225	Leu His Glu Leu Lys Leu Ile 230	Val Asp Leu Met Tyr Glu 235 240	
Gly Gly Ile	Ala Asn Met Asn Tyr Ser Ile 245 250	Ser Asn Asn Ala Glu Tyr 255	
Gly Glu Tyr	Val Thr Gly Pro Glu Val Ile 260 265	Asn Ala Glu Ser Arg Gln 270	
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Met Phe Ile 290	Ser Glu Gly Ala Thr Gly Tyr 295	Pro Ser Met Thr Ala Lys 300	
Arg Arg Asn 305	Asn Ala Ala His Gly Ile Glu 310	Ile Ile Gly Glu Gln Leu 315 320	
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Lys Asn			
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Lys Asn

<210> SEQ ID NO 25 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: synthetic construct mutant ilcV <400> SEQUENCE: 25 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 20 25 30 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 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 

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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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Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Xaa 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	Сув	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Lys	Ala
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Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Aab	Leu	Met	Tyr	Glu 240
Gly	Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
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Ala	Met	Arg	∠60 Asn	Ala	Leu	Lys	Arg	∠₀5 Ile	Gln	Asp	Gly	Glu	∠70 Tyr	Ala	Lys
Met	Phe	275 Ile	Ser	Glu	Gly	Ala	280 Thr	Gly	Tyr	Pro	Ser	285 Met	Thr	Ala	Lys
7.20	290 Arc	Acr	Age	<u>م</u> ۲ م	<u>م</u> ا م	295 uic	G1	T <sup>1</sup> C	G1	T10	300 Tlc	C1	c1	d r	Leu
Arg 305	Arg	ASU	ASN	лта	лта 310	nis	σтλ	тте	ыų	315	тте	сту	GIU	GTU	цец 320
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Ala	His	Ala 35	Gln	Asn	Leu	Asp	Asp 40	Ser	Gly	Val	Asp	Val 45	Val	Val	Gly
Leu	Arg 50	Glu	Asp	Ser	Ser	Ser 55	Arg	Ser	Ala	Ala	Glu 60	Ala	Asp	Gly	Leu
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Val	Leu	Val	Pro	Asp 85	Thr	Val	Gln	Pro	Ala 90	Val	Tyr	Glu	Gln	Ile 95	Glu
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Ile	His	Tyr	Gly	Gln	Ile	Glu	Pro	Ser	Glu	Asp	Val	Asn	Val	Thr	Met
		116					120					1 つ 戸			
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Val Asp	Ala 130 Glu	115 Pro Gly	Lys Thr	Ser Pro	Pro Gly	Gly 135 Leu	120 His Leu	Leu Ala	Val Val	Arg Tyr	Arg 140 Gln	125 Asn Asp	Tyr Pro	Glu Ser	Asn Gly

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Leu	Phe	Gly	Glu	Gln	Ala	Val	Leu	Сув	Gly	Gly	Val	Thr	Ser	Leu	Val
Lys	Thr	Gly	Tyr	Glu	Thr	Leu	Val	Asp	Ala	Gly	Tyr	Ser	Pro	Glu	Met
Ala	∠10 Tyr	Phe	Glu	Сув	Leu	∠15 Asn	Glu	Leu	Lys	Leu	∠20 Ile	Val	Asp	Leu	Met
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Glu	Tvr	Glv	Glv	245 Leu	Thr	Ara	Glv	Asp	250 Ara	Tle	Val	Asp	Asn	255 His	Ala
-	- 1 y 1		260		~ 7			265				-	270		
Arg	Glu	Lys 275	Met	Glu	Glu	Val	Leu 280	Glu	Glu	Val	Gln	Asn 285	Gly	Thr	Phe
Ala	Arg 290	Glu	Trp	Ile	Ser	Glu 295	Asn	Gln	Ala	Gly	Arg 300	Pro	Ser	Tyr	Гла
Gln 305	Leu	Arg	Ala	Ala	Glu 310	ГЛЗ	Asn	His	Asp	Ile 315	Glu	Ala	Val	Gly	Glu 320
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Ala	Leu	Asn 35	Leu	ГЛа	Glu	Ser	Gly 40	Val	Asp	Val	Ile	Val 45	Gly	Val	Arg
Gln	Gly 50	Lys	Ser	Phe	Thr	Gln 55	Ala	Gln	Glu	Asp	Gly 60	His	Lys	Val	Phe
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Pro	Asp	Glu	Gln	Gln 85	Gln	Гла	Val	Tyr	Glu 90	Ala	Glu	Ile	Lys	Asp 95	Glu
Leu	Thr	Ala	Gly	ГЛа	Ser	Leu	Val	Phe	Ala	His	Gly	Phe	Asn	Val	His
Phe	His	Gln	100 Ile	Val	Pro	Pro	Ala	105 Asp	Val	Asp	Val	Phe	110 Leu	Val	Ala
Pro	Terr	115 Clw	Pro	Gly	uia	Len	120 Val	Ara	Ara	Thr	Tvr	125 Glu	Gln	Glv	Ale
	100				<b>m</b> • • •		س ال ال	· - 4	· A	****	- 1 4 0	oru	<b>3111</b>	CTY	1 11 Cl
	130	- GIY		-	птэ 	135		_		_	140				
Gly 145	130 Val	Pro	Ala	Leu	Phe 150	135 Ala	Ile	Tyr	Gln	Asp 155	Val	Thr	Gly	Glu	Ala 160
Gly 145 Arg	Lys 130 Val Asp	Pro Lys	Ala Ala	Leu Leu 165	Phe 150 Ala	135 Ala Tyr	Ile Ala	Tyr Lys	Gln Gly 170	Asp 155 Ile	Val Gly	Thr Gly	Gly Ala	Glu Arg 175	Ala 160 Ala

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Gly Glu Gln Ala Val Leu Cys Gly Gly Leu Ser Ala Leu Val Lys Ala Gly Phe Glu Thr Leu Thr Glu Ala Gly Tyr Gln Pro Glu Leu Ala Tyr Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu Glu Gly Leu Ala Gly Met Arg Tyr Ser Ile Ser Asp Thr Ala Gln Trp Gly Asp Phe Val Ser Gly Pro Arg Val Val Asp Ala Lys Val Lys Glu Ser Met Lys Glu Val Leu Lys Asp Ile Gln Asn Gly Thr Phe Ala Lys Glu Trp Ile Val Glu Asn Gln Val Asn Arg Pro Arg Phe Asn Ala Ile Asn Ala Ser Glu Asn Glu His Gln Ile Glu Val Val Gly Arg Lys Leu Arg Glu Met Met Pro Phe Val Lys Gln Gly Lys Lys Glu Ala Val Val Ser Val Ala Gln Asn <210> SEQ ID NO 32 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: Corynebacterium glutamicun <400> SEQUENCE: 32 Met Ala Ile Glu Leu Leu Tyr Asp Ala Asp Ala Asp Leu Ser Leu Ile Gln Gly Arg Lys Val Ala Ile Val Gly Tyr Gly Ser Gln Gly His Ala His Ser Gln Asn Leu Arg Asp Ser Gly Val Glu Val Val Ile Gly Leu Arg Glu Gly Ser Lys Ser Ala Glu Lys Ala Lys Glu Ala Gly Phe Glu Val Lys Thr Thr Ala Glu Ala Ala Ala Trp Ala Asp Val Ile Met Leu Leu Ala Pro Asp Thr Ser Gln Ala Glu Ile Phe Thr Asn Asp Ile Glu Pro As<br/>n Leu As<br/>n Ala Gly As<br/>p Ala Leu Leu Phe Gly His Gly Leu As<br/>n $% \mathbb{C} = \mathbb{C} \left( \mathbb{C} \right)$ 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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Ala	Tyr	Phe	Glu	Val	Leu	His	Glu	Leu	Lys	Leu	Ile	Val	Asp	Leu	et	
225 Dha	<i>a</i> 1	<b>a</b> 1	<b>a</b> 1	<b>T</b> ] •	230	7	Met	7	The same	235	Vel	Com	7.000	mla za	40	
Pile	GIU	GIY	GIY	245	ser	ASII	Met	ASII	250	ser	vai	ser	Авр	255	Id	
Glu	Phe	Gly	Gly 260	Tyr	Leu	Ser	Gly	Pro 265	Arg	Val	Ile	Asp	Ala 270	Asp	hr	
Lys	Ser	Arg	Met	Lys	Asp	Ile	Leu 280	Thr	Asp	Ile	Gln	Asp	Gly	Thr	he	
Thr	Lys	Arg	Leu	Ile	Ala	Asn	Val	Glu	Asn	Gly	Asn	Thr	Glu	Leu	lu	
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Thr	Ala															
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Lys	Lys								τU					15	et.	
Leu		Val	Ala 20	Val	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	15 His	al	
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Pro Leu 65 Thr Asn His Ala	Asn Gly 50 Thr Pro Leu Phe Pro 130	Val Leu 35 Ser Pro Asp Lys Lys 115 Lys	Ala 20 Arg Ala Ala Glu Pro 100 Leu Gly	Val Asp Ser Glu Leu 85 Gly Ile Pro	Ile Ser Ile Ala 70 Gln Ala Glu Gly	Gly Gly 55 Ala Ala Ala Ala His 135	Tyr Val Lys Ala Asp Leu Arg 120 Thr	Gly 25 Lys Ala Trp Leu Val 105 Ala Val	Ser Asp Glu Ala Tyr 90 Phe Asp	Gln Val Ala Asp 75 Lys Ala Leu Gly	Gly Ala Glu Glu Val Ser His Asp Glu 140	His Val Gly Val Glu Gly Val 125 Tyr	Ala 30 Ala Leu Met Leu 110 Phe Leu	15 His Leu Lys Ile Ala 95 Ala Met Lys	al rg al eu o la le al	
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Asp 290	пр	Met	цец	Giù	295	цув	AIA	GIY	GIII	300	ser	Pile	цув	AIa	
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- ui ~	7 ~~~	т <b>л</b> -	<u>،</u>	150	77-		71-	-	155	т Т 1 -	C1	C1	- -	160	
uts	чар	тте	лта 165	ьeu	лта	ıyr	лта	3er 170	σтλ	тте	сту	σтλ	цту 175	чīд	
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of the set o	31yGluGlnValValLeuCysGlyGlyLeuSerLysLeuIle210PheGluThrLeuValGluAlaGlyTyrAlaProGluMet210PheGluThrLeuValLysLeuIleValAspLeuIle210CysLeuHisGluValLysLeuIleValAspLeuIle210CysLeuHisGluValLysLeuIleThrAspLeuIle230CysLysAspMetArgTyrSerIleThrGluAlaThr245CysArgValLeuAlaAspIleGluAspThrCluAsp270SizArgValLeuAlaAspIleGluAspAspAspAsp270TheLeuGluYasAspIleGluG	
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Lys Ser	Lys	Lys 20	Val	Ala	Ile	Ile	Gly 25	Phe	Gly	Ser	Gln	Gly 30	His	Ala
His Ala	Met 35	Asn	Leu	Arg	Asp	Ser 40	Gly	Val	Glu	Val	Ile 45	Ile	Gly	Leu
Lys Glu 50	Gly	Gly	Gln	Ser	Trp 55	Ala	Lys	Ala	Gln	Lys 60	Ala	Asn	Phe	Ile
Val Lys 65	Ser	Val	Гла	Glu 70	Ala	Thr	Lys	Glu	Ala 75	Aap	Leu	Ile	Met	Ile 80
Leu Ala	Pro	Asp	Glu 85	Ile	Gln	Ser	Glu	Ile 90	Phe	Asn	Glu	Glu	Ile 95	Lys
Pro Glu	Leu	Lys 100	Ala	Gly	Lys	Thr	Leu 105	Ala	Phe	Ala	His	Gly 110	Phe	Asn
Ile His	Tyr 115	Gly	Gln	Ile	Val	Ala 120	Pro	Гуз	Gly	Ile	Asp 125	Val	Ile	Met
Ile Ala 130	Pro	Lys	Ala	Pro	Gly 135	His	Thr	Val	Arg	His 140	Glu	Phe	Ser	Ile
Gly Gly 145	Gly	Thr	Pro	Cys 150	Leu	Ile	Ala	Ile	His 155	Gln	Asp	Glu	Ser	Lys 160
Asn Ala	Гла	Asn	Leu 165	Ala	Leu	Ser	Tyr	Ala 170	Ser	Ala	Ile	Gly	Gly 175	Gly
Arg Thr	Gly	Ile 180	Ile	Glu	Thr	Thr	Phe 185	Lys	Ala	Glu	Thr	Glu 190	Thr	Asp
Leu Phe	Gly 195	Glu	Gln	Ala	Val	Leu 200	Суз	Gly	Gly	Leu	Ser 205	Ala	Leu	Ile
Gln Ala	Gly	Phe	Glu	Thr	Leu 215	Val	Glu	Ala	Gly	Tyr 220	Glu	Pro	Glu	Met
Ala Tyr	Phe	Glu	Суз	Leu	His	Glu	Met	Гла	Leu	Ile	Val	Asp	Leu	Ile
225				230					235					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<br/>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<br/>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 

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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<br/>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<br/>n Gly His Ala Gl<br/>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 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 

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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<br/>n Gly His Ala Gl<br/>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

<212> TYPE: P <213> ORGANIS	RT M: Pseudo	monas e	ntomoph	nila						
<400> SEQUENC	E: 45									
Met Lys Val P 1	he Tyr As 5	p Lys A	ар Суа	Asp 10	Leu	Ser	Ile	Ile	Gln 15	Gly
Lys Lys Val A 2	la Ile Il 0	e Gly T	yr Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Cys Asn Leu L 35	ya Aap Se	r Gly V 4	al Asp 0	Val	Thr	Ile	Gly 45	Leu	Arg	Lys
Gly Ser Ala T 50	hr Val Al	a Lys A 55	la Glu	Ala	His	Gly 60	Leu	Lys	Val	Thr
Asp Val Ala T 65	hr Ala Va 70	l Ala A	la Ala	Asb	Leu 75	Val	Met	Ile	Leu	Thr 80
Pro Asp Glu P	he Gln Gl <sup>.</sup> 85	y Gln L	eu Tyr	Lys 90	Gln	Glu	Ile	Glu	Pro 95	Asn
Ile Lys Lys G 1	ly Ala Th 00	r Leu A	la Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Tyr Asn Gln V 115	al Val Pr	o Arg A 1	la Asp 20	Leu	Asp	Val	Ile 125	Met	Ile	Ala
Pro Lys Ala P 130	ro Gly Hi	s Thr V 135	al Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
Gly Ile Pro A 145	sp Leu Il 15	e Ala I 0	le Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys Asn Val A	la Leu Se 165	r Tyr A	la Ser	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
Gly Ile Ile G 1	lu Thr Th 80	r Phe L	ys Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly Glu Gln A 195	la Val Le	u Cys G 2	ly Gly 00	Thr	Val	Glu	Leu 205	Val	ГЛЗ	Ala
Gly Phe Glu T 210	hr Leu Va	l Glu A 215	la Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe Glu Cys L 225	eu His Gl 23	u Leu L 0	ys Leu	Ile	Val 235	Aap	Leu	Met	Tyr	Glu 240
Gly Gly Ile A	la Asn Me 245	t Asn T	yr Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Gly Glu Tyr V 2	al Thr Gl <sup>.</sup> 60	y Pro G	lu Val 265	Ile	Asn	Glu	Glu	Ser 270	Arg	Lys
Ala Met Arg A 275	sn Ala Le	u Lys A 2	rg Ile 80	Gln	Asp	Gly	Glu 285	Tyr	Ala	Lys
Met Phe Ile S 290	er Glu Gl	y Ala T 295	hr Asn	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg Arg Asn A 305	sn Ala Al 31	a His G O	ly Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
Arg Ser Met M	et Pro Tr 325	p Ile S	er Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Thr
Lys Asn										

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

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<400	)> SH	EQUEI	ICE :	46											
Met 1	ГÀа	Val	Tyr	Tyr 5	Asp	Гла	Asp	Суа	Asp 10	Leu	Ser	Ile	Ile	Gln 15	Gly
Lys	Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
СЛа	Asn	Leu 35	Lys	Aap	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	Aab	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
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Thr	Glu	Phe 140	Val	Гла	Gly	Gly
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
Gly	Glu	Gln 195	Ala	Val	Leu	Сүз	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	Сув	Leu	His	Glu 230	Leu	ГЛа	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	Val 315	Ile	Gly	Glu	Gln	Leu 320
Arg	Ala	Met	Met	Pro 325	Trp	Ile	Ala	Ala	Asn 330	Lys	Ile	Val	Asp	Lys 335	Thr
Lys	Asn														

<210> SEQ ID NO 47 <211> LENGTH: 336 <212> TYPE: PRT <213> ORGANISM: Bacillus cereus

<400> SEQUENCE: 47

Met Ala Lys Val Tyr Tyr Glu Lys Asp Val Thr Val Asn Val Leu Lys

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1			5					10					15	
Glu Lys I	γya	Val 20	Ala	Ile	Ile	Gly	Tyr 25	Gly	Ser	Gln	Gly	His 30	Ala	His
Ala Gln A 3	Asn 35	Leu	Arg	Asp	Asn	Gly 40	Phe	Asp	Val	Val	Val 45	Gly	Leu	Arg
Lys Gly I 50	ŢĀa	Ser	Trp	Asp	Lуз 55	Ala	Lys	Glu	Asp	Gly 60	Phe	Ser	Val	Tyr
Thr Val A 65	Ala	Glu	Ala	Ala 70	Lys	Gln	Ala	Aab	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 A	Ala	Gly 100	Asn	Ser	Leu	Val	Phe 105	Ala	His	Gly	Phe	Asn 110	Val	His
Phe Asp G	31n 115	Val	Гла	Pro	Pro	Ala 120	Asn	Val	Asp	Val	Phe 125	Leu	Val	Ala
Pro Lys G 130	Gly	Pro	Gly	His	Leu 135	Val	Arg	Arg	Thr	Phe 140	Ser	Glu	Gly	Gly
Ala Val F 145	?ro	Ala	Leu	Phe 150	Ala	Val	Tyr	Gln	Asp 155	Ala	Thr	Gly	Val	Ala 160
Thr Glu I	jàa	Ala	Leu 165	Ser	Tyr	Ala	Asp	Gly 170	Ile	Gly	Ala	Thr	Arg 175	Ala
Gly Val I	Leu	Glu 180	Thr	Thr	Phe	Lys	Glu 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly Glu G 1	31n 195	Ala	Val	Leu	Суз	Gly 200	Gly	Val	Thr	Ala	Leu 205	Val	Lys	Ala
Gly Phe G	Glu	Thr	Leu	Val	Asp 215	Ala	Gly	Tyr	Gln	Pro 220	Glu	Leu	Ala	Tyr
Phe Glu C	Cya	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Gly Gly I	Leu	Glu	Asn 245	Met	Arg	Tyr	Ser	Val 250	Ser	Asp	Thr	Ala	Gln 255	Trp
Gly Asp H	?he	Val 260	Ser	Gly	Pro	Arg	Val 265	Val	Thr	Glu	Asp	Thr	Lys	Lys
Ala Met G	Gly	Thr	Val	Leu	Ala	Glu	Ile	Gln	Aap	Gly	Thr	Phe	Ala	Arg
Gly Trp I	Ile	Ala	Glu	His	Lys	Ala	Gly	Arg	Pro	Asn	285 Phe	His	Ala	Thr
Asn Glu I	jys	Glu	Asn	Glu	∠95 His	Glu	Ile	Glu	Val	300 Val	Gly	Arg	Lys	Leu
305 Arg Glu M	Met	Met	Pro	310 Phe	Val	Gln	Pro	Arg	315 Val	Lys	Val	Gly	Met	320 Lys
			325					330					335	
<210> SEQ <211> LEN <212> TYP <213> ORG	Q ID NGTH PE: GANI	NO 1: 33 PRT SM:	48 35 Bac:	illu	s ce:	reus								
<400> SEQ	QUEN	ICE :	48											
Met Lys 1 1	Ihr	Tyr	Tyr 5	Glu	ГЛа	Asp	Ala	Asn 10	Val	Glu	Leu	Leu	Lys 15	Gly
Lys Thr V	/al	Ala 20	Val	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala

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Gln	Asn	Leu 35	Arg	Asp	Ser	Gly	Val 40	Glu	Val	Val	Val	Gly 45	Val	Arg	Pro
Gly	Lys 50	Ser	Phe	Glu	Val	Ala 55	Lys	Thr	Asp	Gly	Phe 60	Glu	Val	Met	Ser
Val 65	Ser	Glu	Ala	Val	Arg 70	Thr	Ala	Gln	Val	Val 75	Gln	Met	Leu	Leu	Pro 80
Asp	Glu	Gln	Gln	Ala 85	His	Val	Tyr	Lys	Ala 90	Gly	Val	Glu	Glu	Asn 95	Leu
Arg	Glu	Gly	Gln 100	Met	Leu	Leu	Phe	Ser 105	His	Gly	Phe	Asn	Ile 110	His	Phe
Gly	Gln	Ile 115	Asn	Pro	Pro	Ser	Tyr 120	Val	Asp	Val	Ala	Met 125	Val	Ala	Pro
Lys	Ser 130	Pro	Gly	His	Leu	Val 135	Arg	Arg	Val	Phe	Gln 140	Glu	Gly	Asn	Gly
Val 145	Pro	Ala	Leu	Val	Ala 150	Val	His	Gln	Asp	Ala 155	Thr	Gly	Thr	Ala	Leu 160
His	Val	Ala	Leu	Ala 165	Tyr	Ala	Lys	Gly	Val 170	Gly	Суз	Thr	Arg	Ala 175	Gly
Val	Ile	Glu	Thr 180	Thr	Phe	Gln	Glu	Glu 185	Thr	Glu	Thr	Asp	Leu 190	Phe	Gly
Glu	Gln	Thr 195	Val	Leu	Суз	Gly	Gly 200	Val	Thr	Ala	Leu	Val 205	Гла	Ala	Gly
Phe	Glu 210	Thr	Leu	Thr	Glu	Gly 215	Gly	Tyr	Arg	Pro	Glu 220	Ile	Ala	Tyr	Phe
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Lys 305	Ala	Glu	Gln	Asn	His 310	Gln	Leu	Glu	Lys	Val 315	Gly	Ala	Glu	Leu	Arg 320
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			35					40					45				 	 		 
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L	Уa	Pro	Phe 435	Met	Ala	Glu	Leu	Gln 440	Pro	Gly	Asp	Leu	Gly 445	Lys	Ala	Ile				

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Va	1 GI 37	u Leu '0	Ala	Phe	Glu	Thr 375	Met	Val	Ala	Ser	Gly 380	Ile	Ile	Glu	Glu
Se 38	r A] 5	.a Tyr	Tyr	Glu	Ser 390	Leu	His	Glu	Thr	Pro 395	Leu	Ile	Ala	Asn	Cys 400
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Gl	n Al	a His 435	Ala	Ser	Ser	Leu	Thr 440	Leu	Glu	Glu	Leu	Gly 445	Gly	Gly	Leu
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As 46	p A] 5	a Ile	Arg	Asp	His 470	Asp	Val	Glu	Ile	Ile 475	Gly	His	Glu	Leu	Arg 480
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Су	s Gl		20	Суз	Arg	Phe	Met	Leu Asp 25	Ser 10 Ala	Met Ser	Arg Glu	Glu Phe	Lys Ala 30	Leu 15 Gly	Asp Gly
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G1: Se 65	n G] 5( r Ty	u Tyr 35 y Leu y Thr	20 Ala Asn Leu	Cys Lys Gln Arg	Arg Gly Gly Lys 70	Phe Lys Leu 55 Glu	Met Lys 40 Asn Ala	Leu Asp 25 Ile Met Ile	Ser 10 Ala Val Arg Ala	Met Ser Ile Asp Glu 75	Arg Glu Val Ser 60 Lys	Glu Phe Gly 45 Gly Arg	Lys Ala 30 Cys Leu Gln	Leu 15 Gly Gly Asp Ser	Asp Gly Ala Val Tyr 80
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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	Сүв	Leu	His	Glu 230	Leu	Гла	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240	
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Gly	Glu	Tyr	Val	Thr	Gly	Pro	Glu	Val	Ile	Asn	Ala	Glu	Ser	Arg	Gln	
Ala	Met	Arg	∠60 Asn	Ala	Leu	Lys	Arg	∠₀5 Ile	Gln	Asp	Gly	Glu	∠70 Tyr	Ala	Гла	
Met	Phe	275 Ile	Ser	Glu	Gly	Ala	280 Thr	Gly	Tyr	Pro	Ser	285 Met	Thr	Ala	Lys	
Ara	290 Arg	Agn	Agn	۵la	- 21a	295 Hig	Glv	- Tle	Glu	TIA	300 Tle	Glv	Glu	Gln	- I.eu	
305		11911			310		Сту ~-		-	315		Сту 	-	-	320	
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Lys	Asn															
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Pro	Asp	Glu	Phe	Gln	Ser	Gln	Leu	Tyr	Lys	Asn	Glu	Ile	Glu	Pro	Asn	
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Tyr	Asn	Gln	100 Val	Val	Pro	Arg	Ala	105 Asp	Leu	Asp	Val	Ile	110 Met	Ile	Ala	
Pro	Ive	115 Ale	Pro	Glv	Hig	Thr	120 Val	- Ara	Ser	Glu	Phe	125 Val	Ive	Glv	Glv	
~ 1	130					135					140	I	-10	y		
GLY 145	Шe	Pro	Asp	Leu	11e 150	Ala	цТе	Tyr	GIn	Asp 155	val	Ser	GΤΆ	Asn	A1a 160	
Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr	
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Gly	Phe	Glu	Thr	Leu	Val	Glu	Ala	Gly	Tyr	Ala	Pro	Glu	Met	Ala	Tyr	

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 	בבי	L	-	11	u	-	u.
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Phe 225	Glu	Cys	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
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	ГЛа	Arg 280	Ile	Gln	Asp	Gly	Glu 285	Tyr	Ala	Гла
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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Met 1	ГЛЗ	Val	Phe	Tyr 5	Asp	гла	Aab	Cys	Asp 10	Leu	Ser	IIe	lle	GIn 15	GIY
Lys	ГЛЗ	Val	Ala 20	Ile	Ile	Gly	Phe	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Сүз	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asb	Val	Thr	Val	Gly 45	Leu	Tyr	Lys
Gly	Ala 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	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
Pro	Lys 130	Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe 140	Val	Lys	Gly	Gly
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Lys	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	Lys	Ala
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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 70 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: KARI mutant 3F12 <400> SEQUENCE: 70 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<br/>n Gly His Ala Gl<br/>n Ala  $\ensuremath{\mathsf{Gln}}$ 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 Ile 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 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 225 230 235 240
Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr 245 250 255
Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln 260 265 270
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Lys Asn
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Leu Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu His Lys 35 40 45 45 Gly Asp Ala Tyr Tyr Ala Lys Ala Glu Ala His Gly Phe Lys Val Thr 50 55 60	
Leu       Asn       Leu       Lys       Asn       Ser       Gly       Asn       Asn       Val       Thr       Val       Gly       Leu       His       Lys         Gly       Asp       Asn       Tyr       Tyr       Ala       Lys       Ala       Glu       Ala       His       Gly       Gly       Lys       Val       Thr         So       Asp       Asp       Tyr       Ala       Lys       Ala       Glu       Ala       His       Gly       For       Lys       Val       Thr         Asp       So       Asp       Ala       Ala       Glu       Ala       Ala       Ala       Glu       Ala       Ala       Ala       So       The       Lys       Val       Thr         Asp       Val       Ala       Ala       Ala       Ala       Glu       Ala       Ala<	
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Leu       Asn       Leu       Asn       Asn       Yal       Asn       Yal       Asn       Yal       Yal       Asn       Yal       Yal       Asn       Yal       Y	
Ieu       Asu       Ieu       Asu       Asu       Asu       Asu       Yal       Asu       A	
IeuAsnIeuSupAspGuYalAspValTurValGlyIeuHisIysG1AppAppAppTurAppAppGlyAppGlyGlyGlyGlyFunFunAppValAppAppAppAppAppGlyAppFunGlyFunFunAppValAppAppAppAppAppAppAppFunFunFunFunAppValAppAppAppAppAppAppAppAppFunFunFunFunAppValAppAppAppAppAppAppAppAppFunFunFunFunFunAppValAppAppAppAppAppAppAppAppAppFunFunFunFunAppA	

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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		
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Leu	Asn	Leu 35	Lys	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Thr	Lys		
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Phe Glu 225	Суа	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
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Gly Ala 50	Ala	Asp	Ala	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Phe	ГÀа	Val	Thr
Asp Val 65	Ala	Ala	Ala	Val 70	Ala	Gly	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Ile 80
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Arg	290 Arg	Asn	Asn	Ala	Ala	295 His	Gly	Ile	Glu	Ile	300 Ile	Gly	Glu	Gln	Leu
305	Sor	Mot	Mot	Bro	310 Trp	TIO	Clw	71-	Agn	315	TIO	Vol	Agr	Iva	320
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Lys	Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
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Phe 225	Glu	Cys	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240	u 0	
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ore s	- vai	20	e		GTY	1110	25 25	Net				30	5111 m	ma
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Gl	ly I 2	Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
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	Lys Asr	n													
Met   Lys   Val   Pro   Ty   Asp   Lys   Asp   Leu   Ser   I.e   I.	<210> S <211> I <212> 7 <213> C <220> F <223> C <400> S	SEQ II LENGTI TYPE: DRGANI FEATUI DTHER SEQUEI	D NO H: 3 PRT ISM: RE: INF NCE:	87 38 art: ORMA	ific: TION	ial : : mu <sup>.</sup>	seque tant	ence C4D:	12						
Lys     Val     Ala     Ile     Ile     Gly     Tyr     Gly     Ser     Gln     Gly     His     Ala     Gln     Ala     Gln     Ala     Gln     Ala     Gln     Ala     Gln     Ala     Gln     Gln     His     Ala     Gln     Ala     Mas     Gln     Mas     Gln     Fund     Mas     Fund     Mas     Gln     Fund     Mas     Gln     Fund     Mas     Gln     Fund     Gln     Fund     Gln     Fund     Mas     Gln     Fund     Mas     Gln     Mas     Gln     Mas     Mas     Mas     Mas     Mas	Met Lys 1	s Val	Phe	Tyr 5	Asp	ГЛа	Asp	Суз	Asp 10	Leu	Ser	Ile	Ile	Gln 15	Gly
CysAsnLeuLysAspSerGlyValAspValThrValGlyLeuCysLysGlyTrpAlaGlyTrpAlaLysAlaGluAlaHisGlyLeuLysValThrGlyTrpAlaGlyTrpAlaLysAlaGlyAlaGlyLeuValThrGlyTrpAlaAlaAlaValAlaGlyAlaAlspLeuValMetIleLeuThr65ValAlaAlaAlaValAlaGlyAlaAspLeuValMetIleLeuThr65ValAlaAlaAlaValAlaGlyAlaAspLeuValMetIleLeuThr65ValAlaAlaAlaAlaProAspLeuThrLeuMetIleLeuThr65ValSerGlyAspGlyAspLeuAspLeuValMetIleHasSer70AspGlyAlaThrLeuAlaPheSerHisGlyPheAlaIleAlaIle71AspAspLeuAspLeuAspLeuAspAspValIleAspAspIleAspIleAspIleAspIleAspIle<	rya rya	s Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Giv Trp Ala Giv Trp Ala $Civ$ Trp Ala $Viv$ Ala $Viv$ Ala Giv Ala Ala $Civ$ Ala $Civ$ $Civ$ $Viv$ $Civ$ $Viv$ $Vi$	Cys Asr	n Leu 35	Гуз	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Суз	Lys
Asp   Val   Ala   Ala   Val   Ala   Gly   Ala   Asp   Leu   Tur   Lys   Asp   Lue   Tur   So     Pro   Asp   Glu   Phe   Gln   Ser   Gln   Leu   Tyr   Lys   Asp   Glu   Ile   Glu   Pro   Asp     Ile   Lys   Gly   Ala   Thr   Leu   Ala   Pro   Ser   His   Gly   Pro   Asp   Asp   Pro   Asp   Asp   Pro   Asp   Asp   Pro   Pro   Asp   Pro   Pro   Pro   Pro   Pro   Asp   Pro   Pro   Pro   Pro   Pro   Pro   Pro   Pro   Pro   P	Gly Trp 50	p Ala	Gly	Trp	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Leu	Lys	Val	Thr
ProAspGluPheGlnSerGlnLeuTyrLysAsnGluIleGluProAsn $11e$ LysLysGlyAlaThrLeuAlaPheSerHisGlyPheAlaIleHis $11r$ AsnGlnValValProArgAlaAspLeuAspValIleAlaIleAla $11r$ AsnGlnValValProArgAlaAspLeuAspValIleAlaIleAla $11s$ ValValValProArgAlaAspLeuAspValIleAlaIleAla $11s$ ValValValProArgAlaArgSerGluPheAlaIleAla $11s$ ValValProArgAlaArgSerGluPheAlaLysGlyGly $11s$ ProAspLeuIleAlaIleTyrGlnAspAlaSerGlyGlyGlyGlyAspAlaIleAsp $11s$ ProAspLeuIleAlaLeuTyrAlaGlyAspAlaGlyGlyAspAlaIleAspIleAlaIleAspIleAlaIleAspIleAlaIleAspIleAlaIleAspIle<	Asp Val	l Ala	Ala	Ala	Val 70	Ala	Gly	Ala	Asp	Leu 75	Val	Met	Ile	Leu	Thr 80
IleLysGlyAlaThrLeuAlaPheSerHisGlyPheAlaIleHisTyrAsnGlnValValProArgAlaAspLeuAspValIleMetIleAlaProLysAlaProGlyHisThrValArgSerGluPheValLysGlyGlyGlyGlyIleProGlyHisThrValArgSerGluPheValLysGlyGlyGlyGlyIleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAspAlaIcoGlyIleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAspAlaIcoIcoLysAsnValAlaLeuSerTyrAlaAlaGlyValGlyGlyGlyAspIco <td>Pro Asp</td> <td>ọ Glu</td> <td>Phe</td> <td>Gln 85</td> <td>Ser</td> <td>Gln</td> <td>Leu</td> <td>Tyr</td> <td>Lys 90</td> <td>Asn</td> <td>Glu</td> <td>Ile</td> <td>Glu</td> <td>Pro 95</td> <td>Asn</td>	Pro Asp	ọ Glu	Phe	Gln 85	Ser	Gln	Leu	Tyr	Lys 90	Asn	Glu	Ile	Glu	Pro 95	Asn
Tyr Asn $\lim_{115}$ Val Val Pro Arg $\lim_{120}$ Asp $\lim_{120}$ Asp Val $\lim_{125}$ Met Ile Ala $\lim_{130}$ 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 $\lim_{145}$ Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Arg Thr $\lim_{160}$ Asn Val Ala Leu Ser Tyr Ala Ala Gly Thr Glu Thr Asp Leu Phe $\lim_{180}$ Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe $\lim_{190}$ Gly Glu Gln Ala Val Leu Cys Gly Gly Gly Thr Val Glu Leu Val Lys Ala $\lim_{195}$ Che Val Ala Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr $\lim_{210}$ Phe Glu Thr Leu Val Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu $\lim_{225}$ Glu Gly Ile Ala Asm Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr	Ile Lys	a Lya	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
ProLysAlaProGlyHisThrValArgSerGluPheValLysGlyGlyGlyGlyIleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAsnAla145IleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAsnAla145IleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAsnAla145IleGluThrThrPheAlaGlyGlyValGlyGlyGlyArgThr145IleGluThrThrThrPheLysAspGluThrGluGlyArgThr145IleGluThrThrPheLysAspGluThrGluAspLeuPhe140GluGluThrThrThrPheLysAspGluThrGluAlaPhe140GluGluAlaValLeuCysGlyGlyThrValGluLeuValAla140GluGluThrLeuValGlyAlaGlyThrValGluLusLusAla140GluGluThrLeuValGlyLusLus <td>Tyr Asr</td> <td>n Gln 115</td> <td>Val</td> <td>Val</td> <td>Pro</td> <td>Arg</td> <td>Ala 120</td> <td>Asp</td> <td>Leu</td> <td>Aap</td> <td>Val</td> <td>Ile 125</td> <td>Met</td> <td>Ile</td> <td>Ala</td>	Tyr Asr	n Gln 115	Val	Val	Pro	Arg	Ala 120	Asp	Leu	Aap	Val	Ile 125	Met	Ile	Ala
GlyIleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAsnAla145IleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAsnAla145IleProAspLeuIleAlaIleTyrGlnAspAlaSerGlyAspAla145IleProAspLeuSerTyrAlaAlaGlyValGlyGlyAspAspAla145IleGluAlaLeuSerTyrAlaAlaGlyValGlyGlyAspLeuPhe146IleGluThrThrThrPheLysAspGluThrGluAspLeuPhe146GluGluGluThrThrThrPheIleSerGluThrAspLeuPhe146GluGluGluThrThrThrPheIleSerGluThrAspLeuPhe146GluGluGluThrLeuValGluGluThrNuGluLueValAsp147PheGluGluThrLeuValGluAspLeuNuTyrAspLeuAspLueAsp147PheGluGluThr <t< td=""><td>Pro Lys</td><td>s Ala</td><td>Pro</td><td>Gly</td><td>His</td><td>Thr 135</td><td>Val</td><td>Arg</td><td>Ser</td><td>Glu</td><td>Phe</td><td>Val</td><td>Lys</td><td>Gly</td><td>Gly</td></t<>	Pro Lys	s Ala	Pro	Gly	His	Thr 135	Val	Arg	Ser	Glu	Phe	Val	Lys	Gly	Gly
Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val Gly Gly Gly Gly Arg Thr 165Gly Ile Ile Glu Thr Thr Phe Lys Asp Glu Thr Glu Thr Asp Leu Phe 180Gly Glu Gln Ala Val Leu Cys Gly Gly Gly Thr Val Glu Leu Val Lys Ala 200Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 230Gly Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr	Gly Ile	e Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Gly IleIleGlu Thr Thr PheLysAspGlu Thr Glu Thr AspLeuPheGly GluGln AlaValLeuCysGlyGlyThr ValGluLeuValLysAlaGly PheGluThr LeuValGluAlaGlyThr ValGluLeuValLysAlaGly PheGluThr LeuValGluAlaGlyTyrAlaProGluMetAlaTyrPheGluCysLeuHisGluLeuLysLeuIleValAspLeuMetAlaTyrGly GlyIleAlaAsnMetAsnTyrSerIleSerAsnAlaGluTyrGly GlyIleAlaAsnMetAsnTyrSerIleSerAsnAlaGluTyrGlyGlyIleAlaAsnMetAsnTyrSerAsnAsnAlaGluTyrGlyGlyIleAlaAsnMetAsnTyrSerAsnAsnAlaGluTyrGlyGlyIleAlaAsnMetAsnTyrSerAsnAsnAlaGluTyrGlyGlyIleAsnMetAsnTyrSerSerAsnAlaGluTyrGlyGlyGlyIleSerSerAsnAsn <td< td=""><td>Lys Asr</td><td>n Val</td><td>Ala</td><td>Leu 165</td><td>Ser</td><td>Tyr</td><td>Ala</td><td>Ala</td><td>Gly</td><td>Val</td><td>Gly</td><td>Gly</td><td>Gly</td><td>Arg</td><td>Thr</td></td<>	Lys Asr	n Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly	Val	Gly	Gly	Gly	Arg	Thr
Gly Glu Gln Ala Val Leu Cys Gly Gly Gly Thr Val Glu Leu Val Lys Ala 200Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 230Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr	Gly Ile	e Ile	Glu 180	Thr	Thr	Phe	Lys	Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210     Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 230     Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr 100	Gly Glu	ı Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Гуз	Ala
Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu   225   230   Cly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr	Gly Phe	e Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro	Glu	Met	Ala	Tyr
Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr	Phe Glu	r CÀa	Leu	His	Glu 230	Leu	Гуа	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
246 960 975	Gly Gly	y Ile	Ala	Asn	Met	Asn	Tyr	Ser	Ile	Ser	Asn	Asn	Ala	Glu	240 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<br/>n Gly His Ala Gl<br/>n Ala  $\ensuremath{\mathsf{Gln}}$ Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Ala Lys Gly Trp Ala Gly Trp 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 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 

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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<br/>n Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Pro Lys 35<br/> 40 45Gly Glu Ala Ala Trp 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

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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	Lys
Met	: Phe 290	Ile	Ser	Glu	Gly	Ala 295	Thr	Gly	Tyr	Pro	Ser 300	Met	Thr	Ala	Lys
Arg 305	g Arg	Asn	Asn	Ala	Ala 310	His	Gly	Ile	Glu	Ile 315	Ile	Gly	Glu	Gln	Leu 320
Arç	g Ser	Met	Met	Pro 325	Trp	Ile	Gly	Ala	Asn 330	Lya	Ile	Val	Asp	Lys 335	Ala
Lys	s Asn														
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<40	00> S	equei	ICE :	90											
Met 1	Lys	Val	Phe	Tyr 5	Asp	ГЛа	Asp	Суз	Asp 10	Leu	Ser	Ile	Ile	Gln 15	Gly
Lys	s Lys	Val	Ala 20	Ile	Ile	Gly	Tyr	Gly 25	Ser	Gln	Gly	His	Ala 30	Gln	Ala
Суа	Asn	Leu 35	ГЛа	Asp	Ser	Gly	Val 40	Asp	Val	Thr	Val	Gly 45	Leu	Gly	Lys
Glγ	7 Trp 50	Ala	Gly	Trp	Ala	Lys 55	Ala	Glu	Ala	His	Gly 60	Leu	Lys	Val	Thr
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	e Lys	Lys	Gly 100	Ala	Thr	Leu	Ala	Phe 105	Ser	His	Gly	Phe	Ala 110	Ile	His
Туз	: 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
Glչ 145	7 Ile	Pro	Asp	Leu	Ile 150	Ala	Ile	Tyr	Gln	Asp 155	Ala	Ser	Gly	Asn	Ala 160
Lys	s Asn	Val	Ala	Leu 165	Ser	Tyr	Ala	Ala	Gly 170	Val	Gly	Gly	Gly	Arg 175	Thr
Glγ	7 Ile	Ile	Glu 180	Thr	Thr	Phe	Lys	Asp 185	Glu	Thr	Glu	Thr	Asp 190	Leu	Phe
Glγ	7 Glu	Gln 195	Ala	Val	Leu	Суз	Gly 200	Gly	Thr	Val	Glu	Leu 205	Val	Lys	Ala
Glγ	7 Phe 210	Glu	Thr	Leu	Val	Glu 215	Ala	Gly	Tyr	Ala	Pro 220	Glu	Met	Ala	Tyr
Phe 225	e Glu	Суз	Leu	His	Glu 230	Leu	Lys	Leu	Ile	Val 235	Asp	Leu	Met	Tyr	Glu 240
Glγ	7 Gly	Ile	Ala	Asn 245	Met	Asn	Tyr	Ser	Ile 250	Ser	Asn	Asn	Ala	Glu 255	Tyr
Glγ	/ Glu	Tyr	Val 260	Thr	Gly	Pro	Glu	Val 265	Ile	Asn	Ala	Glu	Ser 270	Arg	Gln

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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 

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 94 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 9862B9 <400> SEQUENCE: 94 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<br/>n Gly His Ala Gl<br/>n Ala  $\ensuremath{\mathsf{Gln}}$ Cys Asn Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu Asp Lys 35 40 45 Gly Trp Ala Gly Trp 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 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 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<br/>n Leu Lys Asp Ser Gly Val Asp Val Thr Val Gly Leu As<br/>n Lys 35 $\phantom{100}$ 40 $\phantom{100}$ 45 Gly Asn Ala Asp Trp Ala Lys Ala Glu Ala His Gly Leu Lys Val Thr 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 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 120 115 125 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 Ala Ser Gly Asn Ala 155 145 150 160 Lys Asn Val Ala Leu Ser Tyr Ala Ala Gly Val 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 Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Val Glu Leu Val Lys Ala 200 195 205 Gly Phe Glu Thr Leu Val Glu Ala Gly Tyr Ala Pro Glu Met Ala Tyr 210 215 220 Phe Glu Cys Leu His Glu Leu Lys Leu Ile Val Asp Leu Met Tyr Glu 235 225 230 240 Gly Gly Ile Ala Asn Met Asn Tyr Ser Ile Ser Asn Asn Ala Glu Tyr 245 250 255 Gly Glu Tyr Val Thr Gly Pro Glu Val Ile Asn Ala Glu Ser Arg Gln 260 265 270 Ala Met Arg Asn Ala Leu Lys Arg Ile Gln Asp Gly Glu Tyr Ala Lys 280 275 285 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 96 <211> LENGTH: 338 <212> TYPE: PRT <213> ORGANISM: artificial sequence <220> FEATURE: <223> OTHER INFORMATION: mutant 11461D8 <400> SEQUENCE: 96 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 Phe Lys Gly Ala 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 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 

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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

-continued

305					310					315					320
Arg	Ser	Met	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  $\mu$ 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.

\* \* \* \* \*