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(54) Název přihlášky vynálezu:

Sekvence genového shluku kódující biosyntézu celesticetinu

(57) Anotace:

Sekvence shluku (clusteru) genů mikroorganismu aktinomycety Streptomyces caelestis kódujících biosyntézu, biologicky aktivní látky, celesticetinu. Použití této sekvence k přípravě hybridních látek linkosamidových antibiotik.

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Sekvence genového shluku kódující biosyntézu celesticetinu

Oblast techniky

Vynález se týká využití shluku genů pro biosyntézu antibiotika celesticetinu k výrobě hybridních látek na bázi linkosamidových antibiotik.

Dosavadní stav techniky

Linkomycin a celesticetin patří do skupiny linkosamidových antibiotik. Klinicky významnější linkomycin je produkován různými druhy aktinomycet vč. kmenu *Streptomyces lincolnensis* a je využíván k léčbě infekcí způsobených grampozitivními patogeny.

Celesticetin je druhým přirozeným linkosamidovým antibiotikem a je produkován kmenem *Streptomyces caelestis*. Byla popsána mikrobiologická a semisyntetická příprava několika derivátů celesticetinu. Celesticetin a jeho deriváty mají mnohem nižší účinnost než linkomycin a proto nejsou využívány klinicky.

V biosyntéze linkomycinu dochází k oddělené syntéze dvou prekurzorů propylprolinu (PPL) a methylthiolinkosamidu (MTL), které jsou kondenzací spojeny na N-demethyllinkomycin a ten je dále methylován na výsledný linkomycin. V případě celesticetinu je cukerná složka MTL spojena s prolinem a salicylátem.

V roce 1995 byla publikována sekvence shluku genů kódujících proteiny pro biosyntézu linkomycinu – *lmb* shluk. Tento shluk obsahuje geny pro biosyntézu obou prekurzorů, geny pro kondenzační reakci, regulační geny a geny pro rezistenci k linkomycinu. Shluk pro biosyntézu celesticetinu zatím popsán nebyl.

Podstata vynálezu

Byla popsána sekvence genového shluku pro biosyntézu celesticetinu a označena jako ccb shluk. Tato sekvence je využitelná pro přípravu nových, biologicky aktivních látek.

Pro tyto účely byla připravena kosmidová knihovna z chromozomální DNA *Streptomyces caelestis* ATCC 15084. Kosmidy nesoucí části celesticetinového shluku byly vyhledány za

použití homologních sond z linkomycinového shluku. Sekvenční analýzou kosmidů byla zjištěna přítomnost celého shluku pro biosyntézu celesticetinu.

Celesticetinový shluk (*ccb* shluk) o velikosti 27350 pb je ohraničen geny *ccr1* a *ccb5* a obsahuje celkem 24 geny. Shluk obsahuje geny pro biosyntézu cukerné složky (MTL) a salicylátu, geny pro kondenzační reakci, regulační geny a geny pro resistenci k celesticetinu. Rozdíly v biosyntéze linkomycinu a celesticetinu se odrážejí ve složení obou shluku genů. V biosyntéze celesticetinu dochází ke spojení aminocukerné jednotky MTL s proteinogenním prolinem. Proto v *ccb* shluku chybí homology linkomycinových genů zodpovědné za syntézu propylprolinu.

Dalším rozdílem je přítomnost genů *ccb1-ccb5* kódujících proteiny pro biosyntézu a připojení salicylátové jednotky, jejichž protějšky chybí v linkomycinovém shluku. Gen ccb4 se nachází pouze v celesticetinovém shluku a kóduje O-methyltransferázu nutnou pro methylaci aminocukrné jednotky v pozici 7´.

Nově získanou sekvenci genového shluku kódující biosyntézu celesticetinu a její rozdílnost vůči shluku genů kódujících biosyntézu linkomycinu, lze s výhodou využít pro navržení a přípravu nových látek na základě linkosamidových antibiotik. Zajímavou možností se zdá být příprava producenta současně nesoucího geny pro biosyntézu linkomycinu a salicylatové jednotky celesticetinu. Slibnou se jeví i možnost využití celesticetinové methyltransferázy Ccb4 k metylaci aminocukerné jednotky v molekule linkomycinu.

Přehled obrázků

Příklady provedení vynálezu

Příklad 1

Chromozomální DNA *S. caelestis* ATCC 15084 byla izolována z kultury narostlé v YEME médiu (kvasniční extrakt 3 g/l, sladový extrakt 3 g/l, pepton 5 g/l, glukóza 10 g/l, pH 7,2) (50 ml) s přídavkem glycinu v konečné koncentraci 0,5% a lysozymu v konečné koncentraci 1 mg/ml. Následně chromozomální DNA byla částečně naštěpena restrikčním enzymem BamHI a ligována do kosmidového vektoru SuperCos-1 (Stratagene), který byl předem štěpen enzymy XbaI a BamHI. Výsledná ligační směs byla vnesena do lambda fágu a fág byl transfikován do kmene *E. coli* XL1-Blue MR^b podle protokolu Gigapack III XL Packaging Kit (Stratagene). Kosmidová DNA byla izolována z buněk pomocí kitu NucleoBond BAC 100 columns (Macherey-Nagel).

Příklad 2

Sondy pro hledání celesticetinových genů v kosmidové knihovně *Streptomyces caelestis* ATCC 15084 byly navrženy na základě předpokládaných konzervativních úseků genů *lmbJ*, *lmbM*, *lmbS* and *lmbZ* z *S. lincolnensis* ATCC 25466. Primery použité pro PCR amplifikaci sond jsou uvedeny v Tab.2. Sondy byly značeny digoxigeninem a použity pro hybridizaci s více než 1000 klony kosmidové knihovny.

DNA klonů dávající pozitivní signál se všemi sondami byly částečně naštěpeny restrikčním enzymem Sau3AI. Restrikční směs byla rozdělena na elektroforéze a fragmenty o velikosti 1-2 kpb byly izolovány a klonovány do vektoru pJAKO. Ligační směs byla transformována do buněk *E. coli* XL1-Blue MR^b.

Příklad 3

Knihovna DNA fragmentů celesticetinového shluku (příklad 2) byla sekvenována za použití univerzálních primerů. Výsledné sekvence byly porovnávány s databázemi NCBI na přítomnost homologů linkomycinových nebo i jiných sekundárně metabolických genů. Sekvence dvaceti pozitivních klonů byly použity k navržení primerů pro sekvenování celého celesticetinového shluku metodou primer walking.

Získané sekvence byly analyzovány pomocí programu SeqMan (DnaStar software package) a Artemis (release 9, The Sanger Institute). DNA a proteinové homology byly

vyhledávány pomocí databází na serveru BLAST (National Center for Biotechnology Information (Bethesda, MD.))

Průmyslová využitelnost

Sekvenci genového shluku kódující biosyntézu celesticetinu lze využít při vývoji nových biologicky účinných látek. Tedy ve farmacii, lékařství.

PATENTOVÉ NÁROKY

1. Sekvence genového shluku kódující biosyntézu celesticetinu označená jako ccb shluk.

2. Použití sekvence genového shluku kódující biosyntézu celesticetinu podle nároku 1 k přípravě hybridních látek linkosamidových antibiotik.

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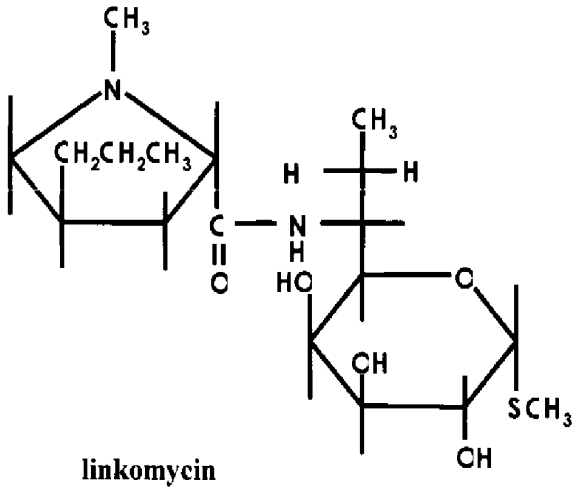
Obr. 1

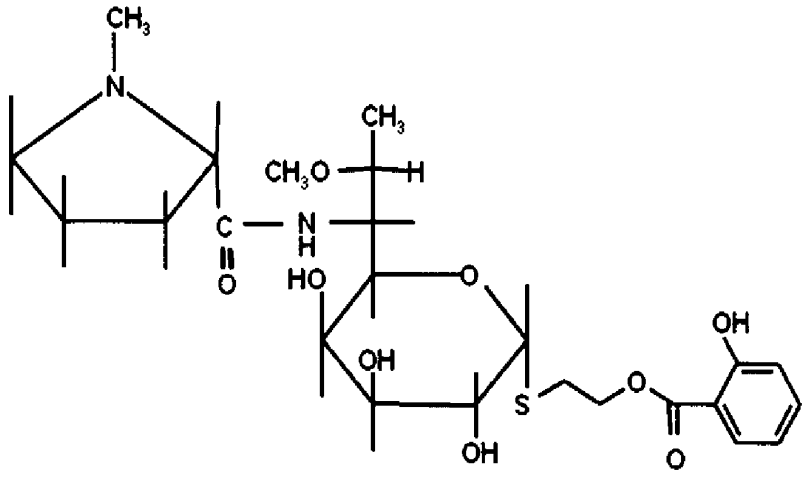
PPL MTL



linkomycin

Obr. 2

Prolin MTL Salicylát



celesticetin

Obr. 3

primer	sekvence
lmbJs	5'-CCGGAATTCCCTCCGTTTCGAGGCAGT-3'
lmbJa	5'-AACCCAAGCTTCGAACTCGGCGTGGGTA-3'
lmbMs	5'-CCGGAATTCTGAACCCGTCCAGGTGG-3'
lmbMa	5'-AACCCAAGCTTGTCGTGCACCTGGCCTC-3'
mbSs	5'-CCGGAATTCACCGCCTTGGTGCGTTC-3'
lmbSa	5'-AACCCAAGCTTGCCGAGGCGCAATCCTT-3'
lmbZs	5'-CCGGAATTCCGCGACCCGGCACATCT-3'
lmbZa	5'-AACCCAAGCTTGTGCGGGCCCTGATCGA-3'

04.05.06

Obr.4

Aminokyselinové sekvence proteinů, kódovaných ccb shlukem genů a jeho okolím

1-5267 - okolí ccb shluku
5268..6293
Ccr1 - 23S rRNA metyltransferáza, resistence k celesticetinu
MTEWGPTHRKGPEQGKKSTGYGRSEAQRGPGFRTRGASAERGRT
GGGPAAPVTADERRRVWGQNFFKSKAAVRRFTAQIGAVEGLPTVEIGPGSGMITKELA
KSGEPLTVVEIDDQWARLLAEELPSHVTMVNEDFLSWGPETECFRMVGNLPFGASTEI
LRTCLGYGPDRFLEGVFLVQLEFARKRAGAWGGNLFNAQWSPWFTFHMGHEFPRHCFR
PVPKTDTATLFVDPRRDPLLPWRERVAYQELVSAVFNTGQLTVGEAAQRVNVRKPADW
LRRAEVFPETRVKDLDAEDWSALFATHQPGRPRTGAGGRPSAGRRQGGRRGMPPPGGG
PRHQRRR
6819..8273
CcbIH - neznámá funkce, podobnost s LmbIH z lmb shluku
MKGAEPLAGAAFSTAARLGADRTVVQLVRTTVEDTVVGADSAPG
HARSDRFEIAVRVHRDGAVGVADGPLLDRHSVLDLTERAAELARASGAGRSVRPAQRP
APGNPQERVYRSPVAIDPFSLSPGERAEPLREARARALAVAGCTHAVVRASAYRRESM
LLSSDGDRHHLESTQTGGWLTAAADGPSGPATRSYPDRTGRHDMGGWEHVHGLDLPGG
AERAAREAVLLSRAKPCPSGTGPAVIDSSQLTLQIHESIGHPLELDRILGWELDYAGG
SFVGLTDVGHLAYASDQVTVVADPVSGRGVGSMPWDDEGTPTAVTPLIDRGLLVGLLS
STTSTAAAGLPSAPGSARTEGAGLPLVRMTNINLQPGSGSLGDLLSDMGDGLLISGNQ
SFSIDDDRDRFHFSCELAWRVRGGRLVEPLRAPRYHGRTLDFWRSCQRVAGAEEWAMH
SVTFCMKGNPIQTARVGHGAAPALFSEVSYGSWE
8270..9040
CcbJ - N-demethylcelesticetin methyltransferáza, podobnost s LmbJ z lmb shluku
MRNYDETTYGDQIADVYDEWPGDAGPPPDGREAALFVAALAAAR
PVLELGVGTGRVAFPLADLGVEVHGVESSEPMLDKLREKAAAHPNGNLVVPVLGNFAK
LDLGEQRYSVVFAAFNTLFCLLGQDEQIDCMRQARELLEPGGTFVVQCLNPAGQRLAT
GNTFGTVELEDTAVHLEASKHDPLAQTLSAHHIVLSEGGGIRLFPYRLRYAYPAELDL
MANVAGLELVERHADFERRRFDASSRYHVSVYRAAASA
9151..10464
Ccb1 - neznámá funkce
MHLDPTTHSGGLTAADLALSAQDYDFLLIERPDFPGVIGVVHTY
RGQADASAVRKTLSRLAPYLGRLGAVLRRAPQDAAEPYFEPVPFDAGAHVDEWTTDEE
WPDAVLAEITRRQLPTDRPLMRVHVIQGPQRWWLVFSFHHVTIDGATMGRLLMTDPGR
DPAPAEPTALTADTALRVRRDAIGTAPPPARTDDSAAADDGFPAILPAHWKHRSLATA
TLGAQWRRLREETGSTKGALAAWLSGAAIAGAVTTARGRCTTLGLNITVDLRRAAPPP
VGGNWTSTILCPWAPGMEPGENLAVIGARLSGTDRSALVDQALAYEHECRDVPYAARR
EAAASWGENSFIVSPDGQRADWHAMVTVLPRLPAWHEPDDCTGHFVLPGAWLGAPWSL
TWAVGEDSASVCALSSSAEEPAQALIEALLRAADTTPVLPRDDHR
10493..11050
CcbK - předpokládaná fosfatáza histidiolu, podobnost s LmbK z lmb shluku
MSGRAAVLFDRDGVLVQALVRDGRPYPPRSLRELRLDEDAQDVV
RRLTEAGWGVAVVTNQPDVPRGLMTEETVHRINRAVAAGLGLSESCFFTCFHDDDSGC
ECRKPGPALLVEAARSLGADLARCTVVGDRWRDIGAAHRAGCGAVWIDRGYREQAPQP
PYDRVTSTAAAVDLVLGGRLRAESG
11066..12376
CcbL - předpokládaná dehydrogenáza dehydrogenase,podobnost s LmbL z lmb shluku
MTSDDQGGAAALPAEISVCGLWHLGTTVAAGLLSRGHQVVGYDP
DDGLRRGAGRAQAPTGEPGVGDALAAGLREQRLRITGDIADWARSTLCLLTYDSAVDA
EGGVADDRLDAAVAAFAEYGPRGAVLVVLSQVPAGTHTRWRGGLLAGRPDLRIVHVPE

NLRLGEALADFLEPPRLVIGAEDDTAARLAAALFPAVESANVSLTEAELVKHATNAYL
GLCVAFANELAWIGFRLGADPTVVARLLKTDPRISPRAPLLPGAAFSGSTLQRDLVAL
RRLGTEHGRAELFDSVLSANQRHAYFAVDLLRAHYGGLAGRRVAVAGLTYKPTTRTLR
DSLPLRLVHQLLAEGAVVSAYDPLAEELPGDLAVERAGSLAACVKGADALLIGSALPE
LADTDWSRLAPAERLVADGCVALSPEVLRAAGWQYLGMRQVSGS"
12373..13350
CcbM - předpokládaná dehydrogenáza, podobnost s LmbM z lmb shluku
MTRYFVTGGAGFIGSHFVERLLSDAHTTSVIAYDDLSNSTTEWI
EPLLKDPRLTFVEADILDTPELTAAMSEAGMTDRDVVVHLASSVDMRKGLSDSSFDLR
QCANGTLSVLEAMREVGPRKVLFSSSSTVYGEPTVLPTPEHAGPYAPISLYGAGKLSA
EALLSAFCHLYGFTAHAFRFGNVVGGRMNHGVIYDFIVKLTEDSSRLHILGDGKQRKN
YFLVEECVEGILTTSEKLGEGFHVLNLGNPGTVTVDEIASVIIDEMGLQDVQITYEGG
SRGWPGDVPVVEYDLSKLGGLGWTAEHGCAAAVRECARRLLAERGWTQP
13347..13955
CcbN - předpokládaná izomeráza, podobnost s LmbN z lmb shluku
MNSVKNLLVDAIEHTTALDSGDIESAARLIFRTWQQGGTVISCG
NGGSASTASHFAADLAKLTIVPGCPRLRTLCLNDNASAFSAWTNDEGFDTVYTEQAEP
WLDDKATLISFSVHGGSRATDASAVSSNLGKVAELAKSRGSSVIAVTGFDGGAAGDVA
DAHINVPHFTEPVATPLIESLHVLIHHALCVGVRQLIQEDAK
13980..15263
CcbZ - předpokládaná oxidoreduktáza, podobnost s LmbZ z lmb shluku, C
koncová část kóduje předpokládaný přenášeč
MTSPTRHDAPPRVGIIGTGLQARRRIAVAGANVVAVAGHDAAAG
EAFAAEHGIRAEKDWHALVGAADIDIVLVCTPPHLHAEMSIAALEAHKHVLCEKPFAR
TGAEAERMLQAARTHGKALACGFNHRHHPALAELRRLVTQGRMGRVLWARFAYGIGGR
DGYENEWRADPAQVAGGQLMEQGIHAVDLLRTLLGDVESTTAVRNTAVWPAMAPLEDD
AMVLLRHHGGAMSTIHSTLTQWVNLFRLELGGERATAEVQGLAGSYGTQTLTVWDRTD
GPFSAARTEFRGGDRSWQQEWEYFLRLVDNPGDLSSAEDGAAAVRIVEAAYQGADGLS
WARPATGADGSAAPAPSLLVDVLELLRPLLPSADTELTPDTELFSSQLLDSLALEEIQ
AAIESRWVPLPPEELTLANFNTPAAIAETIARTST
15290..16540
CcbF - předpokládaná aminotransferáza, podobnost s LmbF z lmb
MSDLAAVDDWSTLRRIAIDAVSTGRNPELKWLADHPEGTPAYAL
HLADPLEGAPEGLRQCLREAWDEPLDSYVLSHHGLPELRQAMERWFADDENWPRRRRL
LTTATMTGTGPAMYDLLRTIKAREPEGPMAALVPRPGWDYRLFAHDVGYEPIGYHVPF
TSPTGPEPGDLDRAVEQTRAKGLRPTVLVLNPQHYATGGNWTPEFVRYALSLADTLGM
WVLVDNAYHGMTAAGTQPTSTVRLALDGGFEERLIHVRTLGKQFACNGWAVGSVTAMP
DVIDEFAHRWRGFREYPGHAREQAAFAGWLNNPESRKWADERREAIRSNGDALLDALA
EVSNTTRHCHGGSPFVLFEVPGGWSQEDFRQRLFADTGVLLASAQIPYAPDWVKVFLG
RRPDRFLPAVEALRTRPSRAWQPR
16660..17484
CcbE -předpokládaná GlcNAc acetyláza, podobnost s LmbE z lmb
MQRKILTVHAHPDDEVSRGGATIAHYTAQGVQAVLVTCTDGAAG
EILNPDFGALPQGRTLAAQRAEELAASARALGYSTVHSLGFADSGFDGTAGGTDAFVK
ASTDAAAERLAALIEKEKPQVIVGYGTNHARDPHPDHIRANQVLTRCVELLGERDGSR
VPDVFHVAFSRRRHRVLHEACGTAGVPSPYAPGLREPDTSFDDAEINTVISLSETAIE
RRLSALRCHKTQIAANSGWFALSASELYQAFPYDEYIRVAAPPGGRPCDDLFVASS
17493..18560
CcbD -neznámá funkce, podobnost s LmbD z lmb
MAQSKGSVDQTLHAPHCEVGCAANVARRVGVDLARQVICAHWAS
RMLVREVGTFPQPLLDRTQVTFSAQGEGWPALLARMTGGEVTSRHVPREELLSTLHAD
RAEGGTLLFMEDRACPWLDSAHSPGMLPHVVVPDGVAPDGSWQLIEGHSWWRGRYAMS
FQDLLAASYPDPDPHHVAGRVLSLRIRPSAERAAQLDTLARQELAAGLRTYLAAECGE
TETPAGRIVWANGPQSVPLLVERLRGWDYLCPLAARNDLSTEHARDVALGRYLFLALT
DELAFAAYARAGTLRLVEGLGLAGAVGGLRPDEAWRLAWRSGQKLYRRLDRQNLSALF
SALEKAAEVDVEYARRLLKEL
18559..20076
CcbC -adenylace prolinu, podobnost s LmbC z lmb shluku
MNTSTVRNSLSTLLADTAAATPGATALREPGGDTTYRDLAALAG
GIQTALAEAGVRPGDRVAVLAPQTAAAVAAVHAVLSACAAYVPLDPASPPARWAAVCG
MSTPRAVVGTRDALAKLTAELPHLATVDLEAPWPEAPLAPVEREAADLGAVLPTSGST

GVPKGVAISREALLAFASWMVDTFEVGADDVIAGFSPLHFDVSVFSLFGAALTGAALA
PAPPQLAGFPHELASWADDAGITVWYSVPYPLARLGTLDAVVLEQRLGRLRCVCFAGE
VFPHRQLATLMSKMPWVRYTNLYGPTETNVCTFEQVLAPPDEAIAIGSPACGAECWVE
RDGERVDTEGAVGELIVAGPTVADGYWGNEDETARRFRFGREYRSGRAYATGDQVRIL
PDGRYAYLGRQDHMVKIRGQRLELEEVESVLGTADGVTGCCVVAVTPPGRPALLCAAV
VGDQLDHAALRSHCRGRLPSWAIPQRFLEVPAIPLTSTGKTDRQEVRRRVMAELV
20084..21055
CcbP -předpokládaná kináza, podobnost s LmbP z lmb shluku
MITTTAPLRLSLAGGGTDLPEHYEEFGCRLLAVTLDLTVTVTVN
GAPSGLRFSAFGAQEHAPHADSVANPLVRAALRHFGITDGVELTVQSDVLPGSGLGGS
GAFMVALTSALAQHIGKPVTTDEAARLAFHLEREKCGFPVGQQDHWTAACGGALELRI
DRGGNATTTELPELRKAVDTLLDSELLLLRTPITRAAGAPLAAHTRALKSRHGSGMGT
IQNMVDAFHSALTSGDIDRVGALLDQHWQAKRRFNSAVTSPQIDRWYETLLAHGALGA
KVVGAGGGGHLLVAVPAAHADRVSSALASEGLRRVAVRVSEDGVRTA
21052..21750
CcbO - předpokládaná nucleotidyl transferáza, podobnost s LmbO z lmb shluku
MVALPGTPPLVVLAGGLGSRMLHHTRSTPKALLPVGGKPFLTGL
LCAFRDRGGIVEVVLCTGHKADQVRDEIGDGSQLGLRVIHSPEPEPLGPIGALRHAAD
LPDAFLLTYCDVLPTIDPRWFAEQARSSGCQAAMAVGPAPTAAEANVRIRGGKVAAYA
KKPGPAGATDCDRGLLALEKRTLDRHPGRDEQEFYGSLARDGELAAIPVQDPVVDIGT
AARYEKYLRFAETI
21757..22917
CcbS -předpokládaná aminotransferáza, podobnost s LmbS z lmb shluku
MSDYVPFAEPTLDEAEIDAVTRVLRSGWVSTGVEADSFEKEFAN
YVGTRHAVALNSCTAALHLALLALGIGPGDEVIVPTITFTATAAAVVHAGATPVLADV
EPVHLTLDPEQVEKLIGKRTKAVVPVHFTGRMASPGPLRELCDQHGIALLEDAAHTLP
ASDGPYRAGQVGDVSAFSFFATKPITTAEGGMLCTDDSRIAEEARVWSLHGLSRGAVN
RYRPGARAAYDVDRPGFKYNMSDVQAALGRVQLAKADRLHAARTTIAETYLRELDGLP
NLRLPAPDTADNLSSWYLFPVQLRLENMQADRDAFRDELHELGVGTSVHFVPLHHFSW
YAENVVRDGQRFPVADAAAGRLLSLPIFPAMDDGAVERVVTAVRKAHEGTRS
22914..23582
CcbR -předpokládaná transaldoláza, podobnost s LmbR z lmb shluku
MKIFLDTTDMEAVAQWLPHGIVDGVTTNPTLLRKAGVRNAREIV
RELAQRVAPGDVSVQVAGETHEEMVQQALGYRTLGENIVIKVPVIRPDGRPNLDVMTT
LTRQGVAVNATACLSLAQVLAAAKAGARYASLLWGRVADEGGSPEAVVADAARLLRQY
GLPTQLLIGSVRAAGDITKALSAGADVVTVPPALLERWLDHHYSRMTVQQFNSDAGMK
MGTK
23648..24805
CcbQ - neznámá funkce, podobnost s LmbQ z lmb shluku
MNAASADVRTLRHVRSATLRFADSALTSHVEHDVVEYAERSAGS
ERTRYRVVGSQECLKEAGQLAEECADVVAAASRIGTRERMGLLRELASLGPGIYGRVE
DVYGATATVDDRERTGMGTMARRNVIVTTVRDGVRRTRAVSGSPDELDVRGLALAGKP
LPQHEGPLPTQVVLEPLALAELLAEYASVGLAVQDPHTAREFHRRCMGQQVAAAGVDI
TDTPAALGGWTLDIEGTPAAPLSLLRDGRITGYATDHRGAAALGQISNGRAARSVDGL
RAYPAALTLECPPTTAPIGPYLRIERVQYISVVSAETGTVTGSTRDYCSVVENGAPRA
NVGTVRFTISVPDLLRRITGGLGPARTVPVTWGGCVVRCPAVHCADLRLVP
24835..26142
CcbT - předpokládaná glykosyltransferáza, podobnost s LmbT z lmb shluku
MAEAASKQRRIAVVWLMLECGQVSQGGVARYVENLLRAQDSIRA
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    36361 tgggcgggcg tgctgccgcc gcgcggcggc tggcggcccg aaccggggct gccggctccc
    36421 tacgcgttgc ggtccctcgt ggccgcagcc gtaaccgagt tccggtcccg tactcaggag
    36481 ttgccgtcca aactgagcat gagagccgag ctggatcgta tcgcgttaca catctggacc
    36541 cgc
//
```