UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Luka AUSEC

BAKTERIJSKE LAKAZE OD GENA DO ENCIMA

DOKTORSKA DISERTACIJA

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BACTERIAL LACCASES FROM GENE TO ENZYME

DOCTORAL DISSERTATION

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Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa 20. seje Komisije za doktorski študij UL z dne 21. 9. 2011 je bilo potrjeno, da Luka Ausec izpolnjuje pogoje za neposreden prehod na doktorski Podiplomski študij bioloških in biotehniških znanosti ter opravljanje doktorata znanosti s področja biotehnologije. Doktorsko delo je bilo opravljeno v laboratorijih Katedre za mikrobiologijo Oddelka za živilstvo, na Biotehniški fakulteti Univerze v Ljubljani ter v laboratorijih Univerze v Groningenu na Nizozemskem (Centre for Ecological and Evolutionary Studies) ter Univerze v Bielefeldu v Nemčiji (CeBiTec). Za mentorico je bila imenovana prof. dr. Ines Mandić-Mulec.

Mentorica: prof. dr. Ines Mandić-Mulec

Komisija za oceno in zagovor:

Predsednik: prof. dr. Franc POHLEVEN
Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za lesarstvo

Članica: prof. dr. Ines MANDIĆ-MULEC
Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo

Član: prof. dr. Gregor ANDERLUH
Kemijski institute, Laboratorij za molekularno biologijo in nanobiotehnologijo

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Delo je rezultat lastnega raziskovalnega dela. Podpisani se strinjam z objavo svojega dela na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je delo, ki sem ga oddal v elektronski obliki, identično tiskani verziji.

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OP X, 76 str., 9 sl., 80 vir, 3 pril.
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AI Bakterijske lakaze so oksidoreduktaze, ki sklapljajo oksidacijo fenolnih in drugih snovi z redukcijo kisika do vode. Zanimive so za uporabo v različnih biotehnoloških aplikacijah, najbolj pa so preučene pri glivah, čeprav jih najdemo v vseh živih bitjih. Pričujoče delo se ukvarja z bakterijskimi lakazami, njih pojavnostjo in raznolikostjo pri bakterijah ter s preučevanjem genov za te encime neposredno v naravi. Izbrano okolje so bila izsuuševana šotna tla Ljubljanskega barja, ki zaradi pestre mikrobne združbe in naravne prisotnosti fenolnih snovi predstavljajo potencialno zanimiv vir novih genov za lakaze. V njih smo z metodo kloniranja in določanja nukleotidnega zaporedja prepoznali več skupin povsem novih genov za lakazam podobne encime. Z bioinformatskimi analizami sekvenciranih genomov smo ugotovili prisotnost takih genov v vseh bakterijskih deblih ter zbrali več dokazov o njihovi mobilnosti znotraj bakterijske domene. V zadnjem delu smo gen za lakazo iz bakterije Thioalkalivibrio sp. heterologno izrazili, očistili in osnovno biokemijsko opredelili. Novi encim je alkalifilna lakaza, ki lahko oksidira fenolne snovi v bazičnem pH, kar je zanimiva lastnost za potencialno uporabo v biotehnologiji.

Key words documentation (KWD)

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AA MANDIĆ-MULEC, Ines (supervisor)
PP SI-1000 Ljubljana, Jamnikarjeva 101
PB University of Ljubljana, Biotechnical Faculty, Postgraduate Study of Biological and Biotechnical Sciences, Field: Biotechnology
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AB Bacterial laccases are oxidoreductases that couple the oxidation of phenolic and other substrates with reduction of oxygen to water. They have been used in a variety of biotechnological applications and have been mostly studied in fungi in spite of having been found in all domains of life. The present work addresses bacterial laccases, their occurrence and diversity within bacteria and aims to uncover these genes in the natural environment. The drained peat soils of the Ljubljana marsh were chosen for this purpose, since they harbor a diverse bacterial community and are naturally enriched in phenolic compounds. Multiple novel groups of genes were identified in this soil using a cloning and sequencing approach. Moreover, bioinformatics analyses of available bacterial sequenced genomes showed that genes for laccase-like enzymes were present in all bacterial phyla. Evidence evidence of the mobility of these genes within bacterial domain is also presented. In the final part of the thesis, heterologous expression, purification and partial biochemical characterization of one selected laccase gene from *Thioalkalivibrio* sp. is described. The novel enzyme is an alkaliphilic laccase that could oxidize phenolic compounds in alkaline pH and could thus potentially be useful in future biotechnological applications.

Kazalo vsebine

Kazalo znanstvenih del

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Kazalo slik

Kazalo prilog

Okrajšave in simboli

ABTS 2,2'-azino-bis(3-etilbenztiazolin-6-sulfonska kislina)

BR bazalna respiracija

DGGE gelska elektroforeza v gradientu denaturanta (ang. denaturing gradient gel electrophoresis)

DMP 2,6-dimetoksi-fenol

DTT ditiotreitol

OTU operacijska taksonomska enota (ang. operational taxonomic unit)

PCR verižna reakcija s polimerazo (ang. polymerase chain reaction)

pHMM skriti model Markova (ang. profile Hidden Markov Model)

SDS-PAGE poliakrilamidna gelska elektroforeza z natrijevim dodecil-sulfatom (ang. sodium dodecylsulfate polyacrylamide gel electrophoresis)

SIR s substratom inducirana respiracija

T-RFLP polimorfizem dolžin končnih restrikcijskih fragmentov (ang. terminal restriction fragment length polymorphism)

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 PREGLED OBJAV

1.1.1 Ljubljansko barje kot vir mikrobne raznolikosti

Ljubljansko barje je pokrajina med Ljubljano, Vrhniko, Krimom in Škofljico. Je najjužnajše evropsko mokrišče in predstavlja zatočišče za številne ogrožene rastlinske in živalske vrste. Od leta 2008 je zaščiteno kot krajinski park ter kot eno izmed varstvenih območij Natura 2000. Barja so izredno pomembna pri globalnem kroženju ogljika. Kljub temu, da v celoti predstavljajo le 3 % kopnega na Zemlji, po ocenah skladiščijo do tretjine v tleh vezanega ogljiko (Gorham, 1995). Šota se je na Ljubljanskem barju nabirala približno 6000 let, danes pa je zaradi intenzivnega rezanja šote in izsuševanja v zadnjih 200 letih od prvotnih 20 tisoč hektarjev ostalo le še 135 hektarjev fragmentiranih barjanskih površin (Martinčič, 2003).

Današnji ostanki prvotnih mokrišč na Ljubljanskem barju so dveh tipov: visoko barje (ang. bog) in nizko barje (ang. fen). Visoka barja so prehransko povsem odvisna od padavin, saj niso povezana s podtalnico ali hudourniško vodo, ki bi hranila prinašala od drugod. Zato visoka barja tipično poseljujejo šotni mahovi, ki močno zakisajo tla. Zaradi specifičnih lastnosti se oblikuje ne le posebna rastlinska, ampak tudi značilna mikrobna združba takih ekosistemov (Morales in sod., 2006). V sibirskih visokih barjih so tako odkrili samosvojo in pestro bakterijsko združbo, ki so jo močno zaznamovale bakterije iz debla Acidobacteria (Dedysh in sod., 2006). Nasprotno pa so nizka barja bogatejša s hranili in običajno gostijo bolj raznoliko rastlinsko združbo.

Nizko barje na Ljubljanskem barju je z vidika lastnosti tal in raznolikosti mikrobne združbe dokaj dobro preučeno. Kraigher in sod. (2006) so ugotovili, da sta mikrobni združbi v dveh tipih tal z različno vsebnostjo organske snovi podobni ter stabilni preko leta. pH tal je blizu 7, vsebnost organske snovi v tleh pa je med 20 in 30 %, saj je šota na tem območju zaradi izsuševanja in kmetijske rabe tal močno razgrajena. Danes so tam travišča, šota pa je nastala iz ostankov rogoza, ločja in šašev. Ostanki visokega barja na Ljubljanskem barju pa so precej drugačni, saj tam še danes najdemo šotne mahove ter gozdne sestoje jelše, bora in breze. pH tal je kisel. Talna voda se pogosto približa površini

ali celo poplavlja, zato so v tleh pogosti anoksični pogoji. Šota iz šotnih mahov je zato le slabo razgrajena, vsebnost organske snovi pa je tu dosti višja (do 90 %) kot v tleh nizkega barja.

Kisla tla visokega barja bi bila zaradi oligotrofnosti, a sicer visoke vsebnosti težko razgradljivih organskih snovi (lignini, huminske kisline in druge fenolne spojine), lahko zanimiv vir robustnih ligninolitičnih encimov, na primer lakaz, vendar je o dejanski mikrobni in funkcionalni raznolikosti v teh tleh malo znanega. S primerjavo različnih tipov tal Severne in Južne Amerike so ugotovili, da je pH tisti okoljski dejavnik, ki najbolj vpliva na strukturo mikrobne združbe, ter da je raznolikost v kislih tleh manjša kot v neutralnih tleh (Fierer in Jackson, 2006). V prvi fazi bi bilo zato treba podrobneje spoznati tla visokega barja in mikrobno združbo, ki je v njih prisotna, da bi lahko utemeljili smotrnost iskanja bakterijskih encimov v tem okolju. To je bil eden od ciljev doktorske naloge.

1.1.2 Lakaze: pojavljanje in aktivnost

Lakaze so skupina raznolikih encimov, ki jih najdemo v vseh živih bitjih. Uvrščamo jih med modre bakrove oksidaze skupaj z encimi, kot so rastlinske askorbatne oksidaze in sesalčji ceruloplazmin. Lakaze so bile med prvimi odkritimi encimi že v poznem 19. stoletju, ko so jih našli v mlečku octovca (*Toxicodendron vernicifluum*, prej *Rhus vernicifera*, ang. lacquer tree, od tod ime lakaza). Kasneje so jih našli še v mnogih drugih rastlinah, kjer lakaze igrajo pomembno vlogo pri sintezi komponent celične stene (Gavnholt in Larsen, 2002).

Nasprotno pa so lakaze pri glivah vedno povezovali z razgradnjo rastlinskega materiala in so jih našli v skoraj vsaki glivi bele trohnobe, kjer so jih iskali (Hoeger in sod., 2006). Danes vemo, da imajo lakaze v glivah še druge funkcije poleg izrabe substrata (globalno pomembna vloga pri kroženju ogljika), saj sodelujejo pri številnih odnosih z drugimi organizmi. Lakaza je npr. virulenčni dejavnik pri človeškem patogenu *Cryptococcus neoformans* (Zhu in Williamson, 2004). Pri glivi *Coprinopsis cinerea* so odkrili kar sedemnajst genov za lakaze, ki so regulirani z različnimi promotorji in tako sodelujejo v različnih fazah ontogenetskega razvoja glive (Kilaru in sod., 2006).

V zadnjih letih so se pojavile študije o pomenu lakaz pri drugih organizmih. Pri komarjih tako lakaze sodelujejo pri sintezi barvila v kutikuli ter pri detoksifikaciji živalske krvi

(Lang in sod., 2012). *In silico* analize so pokazale prisotnost genov za lakazam podobne encime pri arhejah (Sharma in Kuhad, 2009). Eno arhenjsko lakazo iz *Haloferax volcanii* so že očistili iz originalnega seva in hetorolognega producenta *E. coli*, encim pa je zelo stabilen tudi pri povišanih slanosti in visoki temperaturi (Uthandi in sod., 2010; Uthandi in sod., 2012). Vedno več pa je znanega tudi o bakterijskih lakazah, ki so tema pričujočega dela.

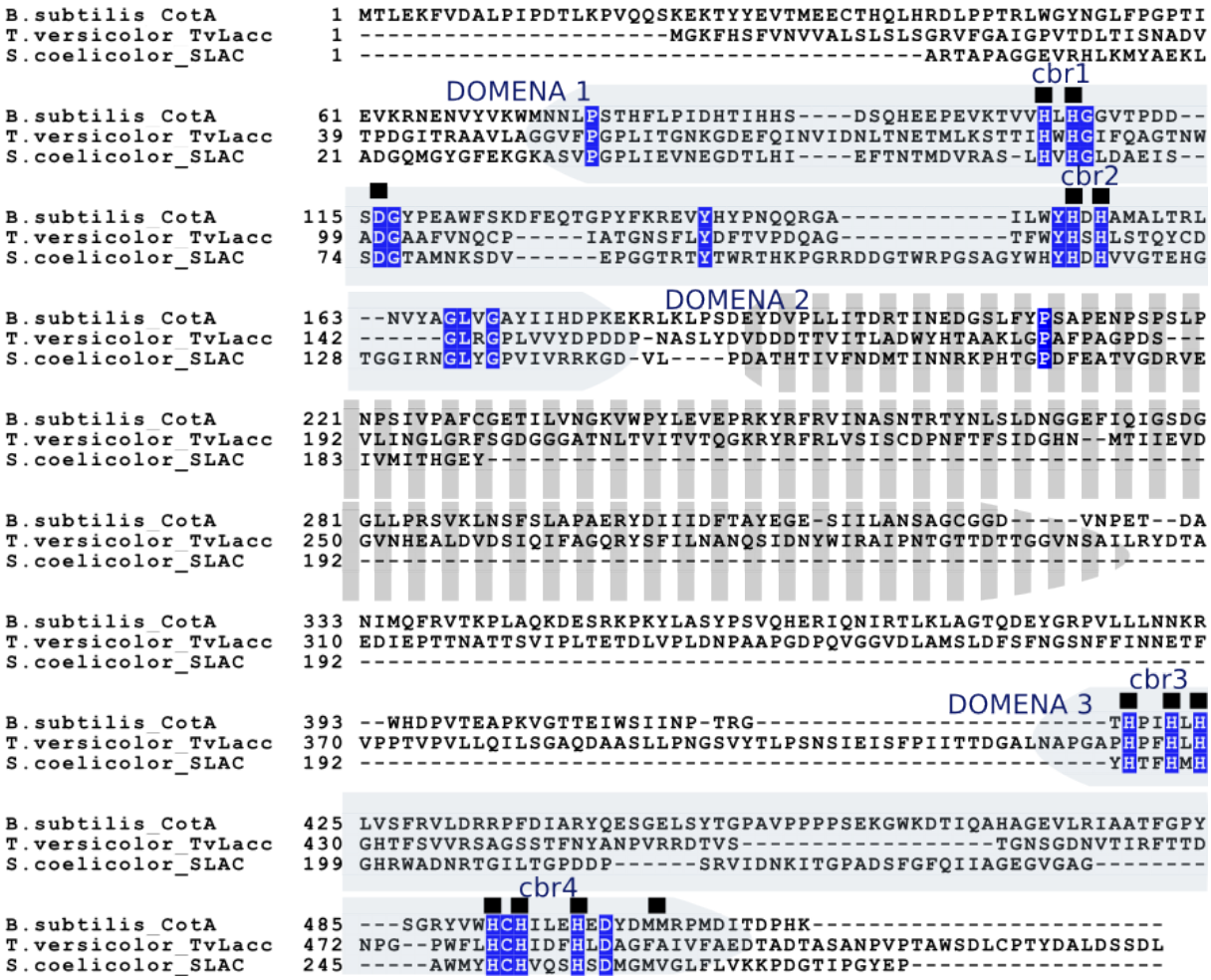
Tradicionalno lakaze razvrščamo med benzendiol:kisikove oksidoreduktaze (EC 1.10.3.2), vendar je za te encime značilno, da lahko oksidirajo širok spekter različnih mono- in polifenolnih spojin ter nekatere nefenolne ali celo anorganske spojine. Njihova aktivnost se pogosto prekriva z ostalimi oksidoreduktazami, na primer s peroksidazami, vendar lakaze od ostalih loči sprejemnik elektronov, ki je vedno molekularni kisik. Lakaze tako sklopijo oksidacijo enega substrata z redukcijo kisika do vode (Slika 2A). Zaradi tako neškodljivih stranskih produktov so lakaze še posebej primerne za okoljske aplikacije, saj za njihovo delovanje ni potrebno dodajati škodljivih (in dragih) snovi, kot je vodikov peroksid za peroksidaze.

Lakaze običajno opisujejo kot monomerne encime s tremi kupredoksinskimi domenami (Slika 1). V prvi in tretji domeni se nahajajo štiri vezavna mesta za baker, ki imajo tipično aminokislinko zaporedje HXH (H pomeni histidin, X predstavlja različne aminokisline). Druga domena pa je precej variabilna in se med lakazami iz različnih skupin organizmov zelo razlikuje (Claus, 2004). Domene v aktivnem mestu držijo štiri bakrove atome, zaradi katerih imajo lakaze v raztopini tipično modro barvo. Na bakrovih atomih potekajo oksidoredukcijske reakcije in mehanizem elektronskega prenosa je danes precej dobro poznan (Bento in sod., 2010).

Ta enostavna slika strukture lakaz se je precej zakomplicirala z odkritjem dvodomenskih lakaz pri bakterijah in arhejah. Sprva so njihov obstoj predpostavili na podlagi redkih DNA zaporedij ter teoretičnega razumevanja evolucijskega razvoja bakrovih oksidoreduktaz (Nakamura in sod., 2003), kasneje pa so encime tudi eksperimentalno potrdili z biokemijskimi in kristalografskimi metodami. Dvodomenske lakaze imajo ohranjene vse štiri regije, ki vežejo baker, a ker manjka srednja variabilna domena, so nekoliko krajše od trodomenskih lakaz (Slika 1). Ker encim ni dovolj dolg, da bi samostojno tvoril obe reakcijski mesti, je potrebna oligomerizacija, zato dvodomenske lakaze v aktivni obliki nastopajo kot homotrimeri (ali redkeje kot homodimeri), katalitični domeni pa se nahajata v eni izmed domen posamezne monomere ter na stiku med dvema monomerama. Glede na orientacijo domen ločimo dva tipa 2-domenskih lakaz: tip B - SLAC iz *Streptomyces*

coelicolor (Machzynski in sod., 2004) in EpoA iz *Streptomyces griseus* (Endo in sod., 2003) ter tip C - lakaza iz metagenoma (Komori in sod., 2009) ter lakaza iz po Gramu negativne bakterije *Nitrosomonas europea* (Lawton in sod., 2009).

Dvodomenske lakaze lahko oksidirajo podoben razpon substratov, vendar sta njihova struktura in evolucijski razvoj povsem drugačna od trodomenskih lakaz, ki jih najdemo

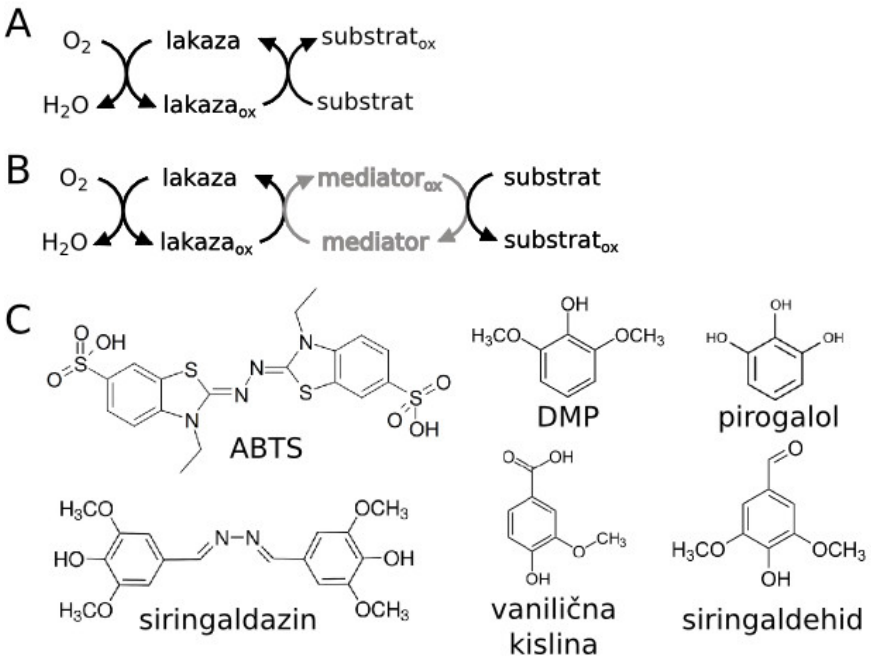


Slika 1: Primarna struktura bakterijskih lakaz. Prikazana so poravnana zaporedja aminokislin izbranih lakaz: trodomenska bakterijska lakaza (CotA iz *Bacillus subtilis*), trodomenska glivna lakaza (TvLacc iz *Trametes versicolor*) in dvodomenska bakterijska lakaza (SLAC iz *Streptomyces coelicolor*). Označene so tri domene in lepo se vidi, da dvodomenska lakaza nima druge variabilne domene. Označene so štiri vezavne regije za baker (cbr1-4). Z modro bavo so označene amino kisline, ki so skupne vsem trem beljakovinam, s kvadratki nad poravnavo pa tiste ključne aminokisline, ki sodelujejo v aktivnih centrih vseh lakazam podobnih encimov.

Figure 1: Primary structure of bacterial laccases. Amino acid sequence alignment of representative laccases is shown: a three-domain bacterial lacccase CotA from the bacterium *Bacillus subtilis*, a three-domain laccases TvLacc from the fungus *Trametes versicolor* and a two-domain bacterial laccase SLAC from *Streptomyces coelicolor*. The three domains are clearly marked and the two-domain laccase is clearly missing the middle domain. Four copper-binding sites are marked with black squares, and the amino acid residues that are common in all three proteins are highlighted in blue.

tudi pri evkariontskih organizmih. Poleg tega danes prepoznavamo zelo veliko raznolikost tudi znotraj teh dveh velikih skupin encimov, zato se za konglomerat teh različnih encimov danes (Reiss in sod., 2013) pogosto uporablja izraz “lakazam podobne bakrove oksidaze” (ang. *laccase-like multi-copper oxidases* – LMCO).

Aktivnost lakaze v laboratoriju preprosto merimo s spektrofotometrom, saj oksidacijo mnogih fenolnih snovi vidimo kot pojav ali spremembo barve. Nekaj tipičnih lakaznih substratov, ki smo jih tudi mi uporabili pri naših raziskavah, je prikazanih na Sliki 2C.



Slika 2: Shematičen prikaz oksidacije substrata z lakazo in nekaj tipičnih substratov. A – Lakaza sklaplja oksidacijo substrata z redukcijo molekularnega kisika do vode. B - Oksidacija je lahko posredna, tako da lakaza najprej oksidira majhno molekulo (mediator), ki nato oksidira nek končni substrat. Tudi v tem primeru po oksidaciji štirih molekul mediatorja poteče redukcija molekularnega kisika do vode (povzeto po Morozova in sod., 2007). C- Tipične spojine, ki jih oksidirajo lakaze.
Figure 2: Oxidation of a substrate with laccase and several representative laccase substrates. A – Laccase couples the oxidation of the substrate with the reduction of molecular oxygen to water. B – Oxidation can be mediated by a small molecule called the mediator, which is oxidized by the laccase and can then in turn oxidize another substrate. Again, the oxidation of 4 molecules of mediator is coupled with the reduction of molecular oxygen to water. C – Typical laccase substrates.

Spekter substratov, ki jih lahko oksidira posamezna lakaza, lahko razširimo z uporabo tako imenovanih mediatorjev. To so lahko sintetične spojine kot na primer 1-hidroksibenzotriazol (HBT), acetosiringon in ABTS, lahko pa so naravne fenolne spojine, ki sicer sestavljajo lignocelulozo (Morozova in sod., 2007; Cañas in Camarero, 2010). Slednje predstavljajo poceni in naravi prijazno rešitev za okoljske aplikacije z lakazami,

poleg tega pa kažejo na pomembnost tega mehanizma oksidacije v naravi. Lakaze mediatorje oksidirajo do radikalov, ti pa nato oksidirajo druge substrate, na primer lignin (Slika 2B). S pomočjo mediatorjev tako lakaze rešujejo dva glavna problema, ki sta pomembna tudi v biotehnologiji, in sicer dokaj nizek redoks potencial lakaznih encimov ter njihova velikost, ki jim onemogoča oksidacijo kompleksnih in trdnih struktur naravnih polimerov (npr. lignina).

Uporabo lakaz z mediatorji omogoča širok razpon biotehnoloških aplikacij, od postopkov v industriji papirja, hrane in tekstila do okoljskih projektov čiščenja odpadnih voda in bioremediacije tal (Couto in Herrera, 2006; Alcalde, 2007; Kunamneni in sod., 2008). Več proizvajalcev ponuja različne glivne lakaze za eksperimente na laboratorijskem nivoju (Sigma, Novozymes). Nekateri produkti se že leta uporabljajo v industrijskem merilu, denimo DeniLite (Novo Nordisk) za razbarvanje tekstila, katerega glavna komponenta je lakaza.

1.1.3 Glivne lakaze

Glivne lakaze so danes pomembne v biotehnologiji zaradi več lastnosti. Najpomembnejša sta relativno visok redoks potencial (v primerjavi z bakterijskimi lakazami) in povečan razpon substratov, ki jih glivne lakaze lahko oksidirajo z mediatorji. Poleg tega pa je glivne lakaze relativno enostavno proizvajati z izvornimi glivnimi sevi v tekočih kulturah, saj so glivne lakaze ekstracelulari encimi. Njihovo izražanje lahko induciramo z dodatkom enostavnih monofenolnih spojin. Izrabljeno gojišče je nato možno neposredno uporabiti kot grobo encimsko frakcijo skoraj brez nadaljnjih korakov čiščenja. Taka proizvodnja encimov je dovolj enostavna in poceni, da omogoča uporabo v industrijskem merilu.

Optimalni pogoji za aktivnost glivnih lakaz so definirani z naravnimi pogoji, v katerih glive najbolje uspevajo. To pomeni, da glivne lakaze najbolje delujejo pri temperaturah pod 50 stopinj celzija ter pri kislih pH vrednostih. Te značilnosti se večkrat izkažejo za omejujoče pri dejanskih industrijskih ali okoljskih procesih, zato se je v zadnjih letih iskanje novih lakaz razširilo na bakterijske encime.

1.1.4 Primeri dobro opisanih bakterijskih lakaz

Bakterije iz rodu *Azospirillum* so bile med prvimi bakterijskimi sevi z lakazno aktivnostjo,

ki so jih našli že pred več kot dvajsetimi leti (Givaudan in sod., 1993). Kljub temu so se resne raziskave bakterijskih lakaz začele s študijem beljakovine CotA iz bakterije *Bacillus subtilis* (Hullo in sod., 2001; Martins in sod., 2002), ki je pomemben gradnik plašča spore te bakterije. Poleg strukturne vloge ima tudi encimsko vlogo, saj sodeluje pri izgradnji melaninu podobnega barvila, ki sporo varuje pred UV sevanjem. Aktivnost tega encima, pa tudi njegova tridimenzionalna zgradba (Enguita in sod., 2003), so pokazale podobnosti s tridomenskimi lakazami iz gliv. Homologne encime proizvajajo tudi drugi predstavniki rodu *Bacillus*, kot so *B. licheniformis* (Koschorreck in sod., 2008) in *B. pumilus* (Reiss in sod., 2011) in *B. amyloliquefaciens* (Lončar in sod., 2013). To so stabilni encimi, ki ohranjajo aktivnost pri neobičajno visokih koncentracijah kloridnih ionov, ki so sicer za lakaze inhibitorni, poleg tega lahko razgrajujejo fenolne spojine pri nevtralnih ali rahlo bazičnih pH vrednostih. Lakaza CotA je tudi značilno termostabilna, saj ima največjo hitrost oksidacije pri 75 ºC in ima pri 80 ºC razpolovno dobo dve uri (Martins in sod., 2002).

Različne bakterije iz rodu *Streptomyces* proizvajajo encime z lakazno aktivnostjo. *S. lavendulae* izraža termostabilno lakazo (Suzuki in sod., 2003), *S. coelicolor* pa majhno lakazo SLAC, ki je bila prva izolirana dvodomenska lakaza (Machczynski in sod., 2004). Je zelo stabilna ob prisotnosti detergenta, visokih temperatur in pH vrednosti nad 9.

Tudi proteobakterije izražajo lakaze, med drugimi *Escherichia coli*. Njen encim CueO omogoča toleranco za baker (Grass in Rensing, 2001), homologe tega encima pa najdemo tudi pri drugih po Gramu negativnih in pozitivnih bakterijah in pri aktinobakterijah (Classen in sod., 2013). Tudi CopA pri bakteriji *Pseudomonas syringae* ima zaščitno vlogo (Cha in Cooksey, 1991), homologe tega encima pa pogosto najdejo v okoljih s povečanimi koncentracijami bakra, kot so na primer morski sedimenti (Besaury in sod., 2013).

Lakaze so našli tudi v ekstremofilnih organizmih. Ni presenetljivo, da so ti encimi zelo robustni v pogojih, ki so primerljivi s tistimi v industrijskih procesih. Lakaza iz *Thermus aquaticus* je bila tako najbolj aktivna pri temperaturi 92 ºC in je imela razpolovno dobo aktivnosti kar 14 ur pri 80 ºC (Miyazaki, 2005). Taki bakterijski encimi imajo očitno lastnosti, ki bi lahko obšle pomankljivosti obstoječih glivnih lakaz, zato je vztrajno iskanje bakterijskih encimov upravičeno.

1.1.5 Raznolikost in razširjenost lakaz med bakterijami

Ob predlaganju teme za doktorsko nalogo je bilo o raznolikosti lakazam podobnih encimov pri bakterijah zelo malo znanega. Redki pregledni članki (Claus, 2003; Sharma in sod., 2007) so navajali peščico poznanih bakterijskih encimov z lakazno aktivnostjo, pri katerih včasih encim niti ni bil izoliran do čiste oblike, v več primerih pa je bilo molekularno zaporedje encima ali njegovega gena popolnoma neznano. Čeprav so bile že znane nekatere bakterijski lakaze, ki so bistveno bolj odporne na visoke temperature in pH vrednosti, se je malo raziskovalnih skupin ukvarjalo z njimi, saj so imele glivne lakaze zaradi enostavnosti pridobivanja in izolacije očitne prednosti. Čeprav so bile prve dvodomenske lakaze znane že leta 2004, ti encimi niso resneje prišli v razprave o bakterijskih lakazah vse do konca leta 2011, ko sta bili objavljeni dve bioinformatski študiji raznolikosti bakterijskih lakaz (Ausec in sod., 2011b; Sirim in sod., 2011).

Ključna študija, ki nas je motivirala v smer preučevanja raznolikosti bakterijskih lakaz, je bila raziskava nemške skupine, ki je po večletnem delu z glivnimi lakazami objavila okoljsko raziskavo raznolikosti bakterijskih genov za lakazam podobne encime v transektu gozdnih in travniških tal (Kellner in sod., 2008). Razvili so močno degenerirane začetne oligonukleotide, s katerimi so lahko pomnoževali kratke odseke genov (<150 bp) med prvo in drugo vezavno regijo za baker v prvi kupredoksinski domeni teh encimov (Slika 1). Odkrili so nekatere značilnosti, ki so te fragmente nedvoumno ločili od glivnih genov, in zaključili, da je raznolikost teh encimov v humusnem sloju gozdnih tal zelo velika. Več skupin DNA zaporedij so povezali s proteobakterijami, nekaj z debli *Actinomycetes* in *Firmicutes*. Mnogim sekvencam niso mogli najti ničesar podobnega v javnih podatkovnih zbirkah. Primerjave s podobnimi študijami genov pri glivah, nekatere so naredili v isti raziskovalni skupini (Luis in sod., 2004), so pokazale, da je raznolikost bakterijskih genov vsaj tolikšna kot pri glivah.

Kako torej iskati nove bakterijske lakaze? Najočitnejši pristop je iskanje novih lakaz v organizmih, ki so sorodni že poznanim producentom lakaz. Tako so ob pregledu več sevov rodu *Bacillus* s pomočjo testov lakazne aktivnosti ali pa z genetskim presejanjem našli lakaze, ki so jih nato bolj ali manj podrobno preučili v nativni obliki ali kot heterologne beljakovine, izražene v *E. coli* (Ruijssenaars in Hartmans, 2004; Koschorreck in sod., 2008; Reiss in sod., 2011; Lončar in sod., 2013). Ti encimi so homologi prej poznani lakazi CotA iz *B. subtilis* ter so v organizmu pomembni kot plaščni proteini spore. Tudi

raznovrstnost substratov ter pH in temperaturna območja za njihovo delovanje so zelo primerljiva s tistimi za CotA. Morda lahko izpostavimo le izrazito halotolerantno lakazo iz bolj ekstremofilnega seva *B. halodurans* (Ruijssenaars and Hartmans, 2004). Tak pristop izrablja raznolikost, ki je naravno prisotna v sorodnih bakterijskih sevih, zato lahko hitro pripravimo različice znanih encimov, vendar so ti encimi le delno drugačni od že poznanih. V primerjavi z glivnimi encimi je bilo do sedaj bolj malo poskusov, da bi obstoječe encime načrtno spreminjali z naključno ali usmerjeno mutagenezo (Gupta and Farinas, 2010; Liu in sod., 2011).

Drugi pristop izrablja zmogljive *in silico* bioinformatske metode, s katerimi prepoznavamo gene za lakazam podobne encime v obstoječih podatkih DNA zaporedij sekvenciranih genomov. Začetni pristopi za iskanje homologov so temeljili na algoritmu BLAST (Alexandre and Zhulin, 2000). Nekaj zaporedij bakterijskih lakaz so v svojo študijo raznolikosti lakaz (predvsem glivnih) vključili Hoegger in sod. (2006), vendar njihova študija nikakor ne odraža realne raznolikosti lakaz pri bakterijah in zanemarja celotne skupine encimov, denimo 2-domenske lakaze. Trenutni uspešni pristopi pa vključujejo zmogljive verjetnostne modele genov (ang. p*rofile Hidden Markov Models, pHMM*), s katerimi lahko zaznamo tudi bolj oddaljene homologe. Tak poskus katalogizacije lakazam podobnih encimov je baza *The Laccase Engineering Database* (Sirim in sod., 2011), ki encime združuje v skupine na podlagi modelov, s katerimi so jih našli. Tak pristop smo izbrali tudi mi pri bioinformatskem presejanju bakterijskih genomov (Ausec in sod., 2011b). Tovrstne zbirke potencialnih novih biotehnoloških učinkovin je v naslednjih stopnjah treba izrabiti s poglobljenim preučevanjem posameznih genov oziroma encimov. Ta pristop že prinaša prve sadove, kot je na primer opis nove rekombinantne lakaze iz *Rhodococcus erythropolis* (Classen in sod., 2013).

Tretji način odkrivanja novih lakaz pa so različne metagenomske študije, ki raziskujejo celokupno (genetsko) raznolikost posameznega okolja, ki nam je v veliki meri neznana. Do celokupne okoljske DNA dostopamo neposredno s sekvenciranjem ali pa preko kloniranja v omejeno število gostiteljskih sevov. V slednjem primeru lahko klone presejamo s testi aktivnosti ali pa iščemo znane vzorce v zaporedjih DNA. Neposredna vzpodbuda za iskanje bakterijskih lakaz na tak način prihaja iz dveh raziskav, ki sta odkrili nesluteno raznolikost lakaznih genov v gozdnih in šotnih tleh (Kellner in sod., 2008; Ausec in sod., 2011a). V primerjavi s prvima dvema se metagenomski pristop najmanj opira na že obstoječe znanje, zato obstaja možnost odkritja nečesa povsem novega. Vendar pa je treba povedati, da je iskanje določenega encima z metagenomiko precej podobno iskanju šivanke v kupu sena, saj je zelo težko presejati zajeto diverziteto, ker potrebujemo ali

veliko sekvenciranja ali pa avtomatizirano presejanje klonov za želeno aktivnost ter zmožnost, da hitro in zanesljivo ravnamo s stotisoči klonov. Zato je bilo do danes relativno malo poročil o novih encimih z lakazno aktivnostjo, ki bi jih našli z metagenomiko (Beloqui in sod., 2006; Ye in sod., 2010; Fang in sod., 2011; Fang in sod., 2012).

1.1.6 Namen dela

Raziskovalno delo ima tri glavne dele.

Prvi del je mikrobno-ekološki. Raziskovali smo mikrobno združbo Ljubljanskega barja in njeno raznolikost lakaznih genov. Izbrana tla vsebujejo veliko organskih snovi s potencialno visokimi vsebnostmi lakaznih substratov. Bakterijsko združbo visokega barja, ki živi v izrazito kislem okolju, smo primerjali z združbo iz nizkega barja. Nadalje smo želeli uporabiti metodo Kellnerja in sodelavcev (2008), ki so raziskovali pestrost bakterijskih genov za lakaze v gozdnih in travniških tleh, in ugotavljati raznolikost teh genov v barjanskih tleh. Nazadnje smo raziskavo razširili z razvojem dodatne molekularne metode, s katero smo lahko pomnoževali daljše odseke genov ter tako prišli do natančnejšega uvida v raznolikost bakterijskih genov za lakaze v preučevanem okolju Ljubljanskega barja.

Drugi del je bioinformatski. Poleg okoljske raznolikosti smo želeli preučiti biotsko raznovrstnost, ki jo že vsebujejo javne podatkovne baze. Namen dela je bilo raziskati razširjenost genov za lakazam podobne encime v razpoložljivih bakterijskih genomih z uporabo naprednejših metod, kot je iskanje podobnosti z algoritmi tipa BLAST. Zanimalo nas je, ali lahko poleg katalogizacije genov in taksonomskih kategorij s preučevanjem lastnosti genov in njihovih proteinskih produktova dobimo uvid v širšo biologijo teh encimov.

Tretji del je najbolj biotehnološki. Oslanja se na v drugem delu prepoznano raznolikost bakterijskih genov za lakazam podobne encime. Namen raziskave je ugotoviti, ali gen, ki smo ga izbrali iz nabora domnevnih lakaznih genov, resnično kodira funkcionalno lakazo in ali lahko na podlagi lastnosti izvornega organizma sklepamo o lastnostih encima. Gen za lakazo iz obligatnega alkalifila *Thioalkalivibrio* sp. smo tako izrazili v gostitelju *Escherichia coli*, ga očistili in biokemijsko okarakterizirali.

1.2 RAZISKOVALNE HIPOTEZE

Javne zbirke DNA zaporedij bakterijskih genomov ter metagenomskih študij zelo hitro rastejo. Z modernimi bioinformatskimi pristopi lahko raziskujemo razširjenost in raznolikost genov za lakaze pri bakterijah *in silico*. Predpostavljamo (hipoteza 1), da so lakaze raznolike in splošno razširjene v svetu bakterij.

Lakaze imajo štiri regije, ki vežejo baker in ki so ohranjene pri različnih organizmih. Pripraviti želimo nove začetne oligonukleotide, ki bodo komplementarni tem ohranjenim regijam, tako da nam bodo omogočili pomnoževanje fragmentov bakterijskih genov za lakaze neposredno iz okoljskih vzorcev. S takim pristopom bomo lahko preučevali raznolikost bakterijskih genov za lakaze *in situ*. Predvidevamo (hipoteza 2), da bomo z uporabo kloniranja in sekvenciranja našli povsem nove gene za lakaze, ki se bodo razlikovali od tistih v javnih bazah podatkov.

S študijem raznolikosti lakaz v okoljskih vzorcih ter v genomih bakterij bomo identificirali potencialno zanimive nove gene, ki kodirajo lakazam podobne proteine. Eno takih lakaz bomo izrazili v *E. coli*. Predvidevamo (hipoteza 3), da bo nova lakaza imela drugačno toplotno in/ali pH stabilnost od do sedaj opisanih lakaz ter da bo zato zanimiva za prihodnjo biotehnološko uporabo.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Razlike v aktivnosti in strukturi bakterijske združbe v osušenih travnatih in gozdnih šotnih tleh

Ausec L., Kraigher B., Mandic-Mulec I. 2009. Differences in the activity and bacterial community structure of drained grassland and forest peat soils. Soil Biology and Biochemistry, 41: 1874–1881.

Delo predstavlja eno temeljnih študij šotnih tal Ljubljanskega barja - okolja, s katerim se na Katedri za mikrobiologijo ukvarjamo že vrsto let. Preučevali smo dva tipa tal, in sicer pretežno osušena travniška tla nizkega barja (ang. *grassland fen soil*) ter gozdnata tla visokega barja (ang. *forest bog soil*). Na nizkem barju je šota, izvorno sestavljna iz rastlinskih ostankov šašja in ločja, v glavnem odstranjena. Na visokem barju vsaj deloma ohranjena plast šote, sestavljena pretežno iz šotnih mahov, dviguje področje nad okolico in onemogoča stik s podlago. Področje visokega barja je zato revnejše s hranili in glede vode odvisno od padavin. Ključna razlika med obema tipoma tal je v deležu vsebnosti organske snovi (16 % v nizkem in 45 % v visokem barju) ter v pH vrednosti, ki je v nizkem barju blizu nevtralnega (7,5), v visokem barju pa je pH kisel (4,6). V pričujoči raziskavi smo preučevali strukturo in aktivnost bakterijske združbe v obeh tipih tal. Bakterijsko aktivnost smo merili z metodo s substratom inducirane respiracije (SIR), pri kateri s plinskim kromatografom spremljamo količino ogljikovega dioksida, ki nastane v določenem času v eksperimentalnem vzorcu tal po dodatku glukoze. V nasprotju s pričakovanji smo ugotovili, da je SIR kljub fiziološko manj ugodnemu pH večji v tleh visokega barja, če rezultate podamo klasično na enoto mase suhih tal. Ravno nasproten pa je rezultat, če preračunamo rezultate na volumen tal, kar je v primeru šotnih tal z zelo visoko vsebnostjo organskih snovi (in posledično majhno gostoto) gotovo smiselno. V zaključku članka zato priporočamo tak izračun za tla z mejnimi vrednostmi gostote. Strukturo bakterijske združbe smo pregledovali z dvema molekularnima metodama. Najprej smo z analizo polimorfizmov dolžin terminalnih restrikcijskih fragmentov (T-RFLP) ugotovili, da se profila združb močno razlikujeta, in sicer ne glede na sezonska nihanja. Nato smo iz vzorca tal visokega barja pripravili gensko knjižnico s fragmenti genov za ribosomalno RNA (16S

rRNA) ter jo primerjali s knjižnico tal nizkega barja (Kraigher in sod., 2006). Nukleotidna zaporedja so pokazala veliko raznolikost bakterij v obeh tipih tal z neobičajno visokim deležem bakterij iz debla *Acidobacteria* (24 in 42 % v nizkem in visokem barju). Zanimivo pa je, da so bile v obeh tipih tal prisotne druge skupine teh bakterij. V skladu z našimi pričakovanji smo opazili, da se bakterijski združbi v obeh tipih tal močno razlikujeta, nismo pa opazili manjše biotske raznovrstnosti v tleh visokega barja, kjer je pH nižji.

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Differences in the activity and bacterial community structure of drained grassland and forest peat soils

Luka Ausec, Barbara Kraigher, Ines Mandic-Mulec*

Biotechnical Faculty, Department of Food Science and Technology, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

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ABSTRACT

The microbial activity and bacterial community structure were investigated in two types of peat soil in a temperate marsh. The first, a drained grassland fen soil, has a neutral pH with partially degraded peat in the upper oxic soil horizons (16% soil organic carbon). The second, a bog soil, was sampled in a swampy forest and has a very high soil organic carbon content (45%), a low pH (4.5), and has occasional anoxic conditions in the upper soil horizons due to the high water table level. The microbial activity in the two soils was measured as the basal and substrate-induced respiration (SIR). Unexpectedly, the SIR ($\mu l\ CO_2\ g^{-1}$ dry soil) was higher in the bog than in the fen soil, but lower when CO_2 production was expressed per volume of soil. This may be explained by the notable difference in the bulk densities of the two soils. The bacterial communities were assessed by terminal restriction fragment length polymorphism (T-RFLP) profiling of 16S rRNA genes and indicated differences between the two soils. The differences were determined by the soil characteristics rather than the season in which the soil was sampled. The 16S rRNA gene libraries, constructed from the two soils, revealed high proportions of sequences assigned to the *Acidobacteria* phylum. Each library contained a distinct set of phylogenetic subgroups of this important group of bacteria.

1. Introduction

Marshes and other types of wetlands are globally important as reservoirs of soil organic carbon (SOC), and are locally important as hydro-regulators and biodiversity hotspots. These wetlands cover approximately 3% of the land-surface, store up to 30% of the Earth's terrestrial carbon and play a vital role in carbon cycling (Gorham, 1995). Their biomass production exceeds decomposition due to low pH and the anoxic conditions often found in the soil, caused by high ground water or flooding (Augustin et al., 1996). Despite the fact that bacteria play a major role in the turnover of energy and matter in the soil, there have been only a few attempts to study the bacterial diversity in the peat soils (Dedysh et al., 2006; Kraigher et al., 2006; Morales et al., 2006). Terminal restriction fragment length polymorphism (T-RFLP) analysis of 24 bogs in the USA revealed a high bacterial diversity with a marked similarity among the sites (Morales et al., 2006). The composition of the bacterial community was also studied in a Siberian peat bog (Dedysh et al., 2006). Data from that study showed that the largest number of sequences (24 out of 84) in the 16S rRNA gene library was from the recently described phylum, *Acidobacteria*. When counted by fluorescent *in situ* hybridization (FISH), *Acidobacteria* comprised 0.1–4.1% of the total bacterial cell number in nine different *Sphagnum*-dominated bogs in Northern Russia (Pankratov et al., 2008). Other studies on the bacterial diversity and activity in peat soil have focused on specific taxonomic (e.g., *Actinobacteria*, Rheims et al., 1996) or functional groups, e.g., methanogenic archaea (Basiliko et al., 2003; Horn et al., 2003) and methanotrophic bacteria (Dedysh et al., 2001). In addition to the northern peat soils, *Acidobacteria* were also dominant in the Alaskan acidic soils, representing approximately 40% and 30% of clones in the tussock and intertussock soil clone libraries, respectively. However, *Acidobacteria* were poorly represented in the shrub organic and mineral soils from the same area, which were dominated by *Proteobacteria* (Wallenstein et al., 2007).

Little is known about the microbial communities in the temperate bogs and fens. Usually these marshes have been subject to drainage and altered land use and therefore the microbial communities might not follow the patterns observed in the relatively undisturbed marshes of the high latitudes. The aim of this study was to investigate the microbial activity and bacterial community structure in a temperate marsh. These soils have been

* Correspondence to: Ines Mandic-Mulec, Biotechnical Faculty, Department of Food Science and Technology, University of Ljubljana, Chair of Microbiology, Vecna pot 111, 1000 Ljubljana, Slovenia. Tel.: +386 1 4233388; fax: +386 1 257 3390.
E-mail address: ines.mandic@bf.uni-lj.si (I. Mandic-Mulec).

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severely degraded in the past two centuries due to drainage, peat collection and agriculture. The area with neutral pH drained sedge peat soil (fen area) has primarily been converted into farmland (25%) and grasslands (65%), but a few fragments of the lowland bog with low pH *Sphagnum*-derived peat soil are still present (Hacin et al., 2001). While both of the soils are exposed to the same climate conditions, they differ most notably in SOC and pH. SOC was shown to be an important factor for microbial activity but not for the bacterial community structure of the two drained grassland fen soils of the European temperate region (Kraigher et al., 2006). Microbial activity was measured as the substrate-induced respiration (SIR) or dehydrogenase activity, and changed with the season and the soil organic matter content; in contrast, the bacterial community structure did not respond to these environmental factors.

Soil pH has been shown to have significant effects on microbial communities. It was found to be the best predictor of bacterial diversity and richness in 98 soil samples from a range of ecosystems in North and South America (Fierer and Jackson, 2006) and in temperate freshwater wetlands from two North American sites (Hartman et al., 2008). Here, the hypothesis that pH may influence the bacterial communities in drained marsh soils was tested by comparing the acidic pH peat soil (bog) and the neutral pH peat soil (fen). The bacterial community structure was evaluated by T-RFLP profiling. A comparative analysis of bacterial diversity and phylogenetic composition was performed on the bog 16S rRNA gene clone library obtained in this study and the fen soil clone library data from Kraigher et al. (2006). In addition, a comparative study of both of the soil types involved an assessment of microbial activity as represented by the basal and substrate-induced respiration. Based on the pH difference between the two soils, we hypothesized that both the soil activity and diversity would be lower in the acidic bog soil.

2. Materials and methods

2.1. Soil sampling

A sampling of the fen and bog soils for the basal and substrate-induced respiration measurements was performed in September 2008 at two nearby locations (less than 3 km apart) in the Ljubljana marsh, Slovenia (45°58′N, 14°28′E). The first site was a grassland sedge-derived peat fen soil with a neutral pH (Rheic Fibric Histosol). The peat had been degraded in the upper 30–50 cm due to drainage, peat collection and agriculture. The second site was an acidic (pH 4.5) bog soil (Dystric Rheic Fibric Histosol) in a swampy forest where the *Sphagnum*-derived peat had been better preserved. Three field replicates of both of the soil types were taken. For each field replicate, 5 individual soil cores (2.5 cm wide and 30 cm deep) were obtained, pooled and homogenized through a 4 mm sieve. The soil samples were refrigerated overnight prior to the experiments. T-RFLP analysis was performed on the soil sampled at the same sites in August 2003 and in March 2004, as described by Kraigher et al. (2006). For T-RFLP analysis, an additional fen soil sample was obtained in 2003 and 2004, approximately 50 m from the fen soil sampling site, and this sample had a markedly lower SOC (therefore the designation fenLC). Two field replicates (20 soil cores each) were obtained and pooled, passed through a 4 mm sieve, frozen and stored at −20 °C in several aliquots of 0.5 g. The fen soil sampling sites were located on a meadow that is mown twice a year but that has not been amended by fertilizer; both of the fen soils were previously described by Kraigher et al. (2006). The bog soil sample, obtained in August 2003, was used for the 16S rRNA gene library construction.

2.2. Soil properties

Measurements were performed on the soil samples obtained from the upper 30 cm of soil. SOC was measured with a LECO CNS-2000 analyzer (LECO, USA) and $CaCO_3$ was subtracted. The organic soil nitrogen was determined by a standard Kjeldahl analysis (Bundy and Meisinger, 1994), using a Tecator 2012 digestion apparatus (Tecator AB, Sweden) and a micro Kjeldahl distillation apparatus. The soil pH was measured in a water slurry (1:5 soil:liquid ratio). The soil water holding capacity (WHC) was measured by packing the soil into soil sample rings (standard diameter 53 mm, volume 100 cm^3; Eijkelkamp, Giesbeek, NL) fitted with cloth at the bottom, and immersing the rings into water for 24 h. The soil moisture content (100% WHC) was measured after allowing the rings to drain freely on a funnel until a constant weight was reached (7 h). The soil water content was determined after drying at 105 °C for 24 h, and is expressed on an oven-dry (105 °C) weight basis and the characteristics of both the bog and fen soil types are presented in Table 1.

2.3. Basal and substrate-induced respiration (SIR)

The substrate-induced respiration was measured in 3 field replicates from the fen and bog soils, each with 3 methodological replicates, as described by Sparling (1995). 0.5 g glucose in the form of a 40% solution was added to 25 g of sieved fresh soil. CO_2 production at different soil moisture contents was measured prior to the experiment, and a water holding capacity (WHC) of 70% was determined to give a maximum response in all of the tested soil samples. This WHC was adopted for future analyses of the basal respiration and the SIR measurements. Sealed bottles with soil samples were incubated at room temperature for 3 h. The gas samples were taken at the beginning and end of incubation, and analyzed for CO_2 by gas chromatography (Network GC System 6890 N; Agilent Technologies, Palo Alto, CA, USA). The SIR is expressed as μl CO_2 g^{-1} dried soil h^{-1}, and μl CO_2 cm^{-3} dried soil h^{-1}, taking into account the soil bulk density. The basal respiration was measured in the same way, except that glucose was not added. The amount of added glucose proved to be sufficient to trigger a maximum SIR response in both of the soil samples since a higher amount of glucose did not result in a higher SIR response (data not shown).

2.4. Analysis of microbial community structure

Terminal restriction fragment length polymorphism (T-RFLP) was used to characterize the soil microbial community structure. The DNA was extracted from 0.5 g soil subsamples of a field replicate (two independent isolations for each type of soil, i.e. bog soil, fen soil

Table 1
Properties of the fen, fenLC and bog soils (values represent the mean ± standard deviation for the upper 30 cm of soil).

	Fen	fenLC	Bog
C_{org} content (%)	16.3 ± 0.21	9.73 ± 0.1 4	45.4 ± 0.21
N_{org} content (%)	1.40 ± 0.01	0.88 ± 0.01	2.75 ± 0.01
C:N ratio	11.7 ± 0.2	11.1 ± 0.25	16.5 ± 0.01
pH	7.55 ± 0.02	7.63 ± 0.01	4.58 ± 0.1 6
WHC[a] (g_{H_2O}/g_{soil})	1.65 ± 0.21	6.99 ± 0.01	8.14 ± 0.29
Bulk density (g cm^{-3})	0.59 ± 0.05	1.41 ± 0.20	0.16 ± 0.05
Mean annual T(°C)	17.8 ± 13.3	0.74 ± 0.04	12.6 ± 10.2
Mean annual water table (cm)	−53.2 ± 22.0	−17.2 ± 13.5	−24.4 ± 13.8
Soil classification[b]	Rheic Fibric Histosol	Mollic Gleysol (Thaptohistic)	Rheic Fibric Histosol (Dystric)

[a] WHC – water holding capacity.
[b] Soils have been classified under the World Reference Base for Soil Resources (2006).

and fenLC soil) using the Ultra Clean soil DNA isolation kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. The 16S rRNA genes were amplified with primer 27f [5′ – AGAGTTTGATCCTGGCTCAG – 3′] (Weisburg et al., 1991) labeled with 6-FAM (6-carboxyfluorescein) at the 5′ end, and primer 927r [5′ – CCGTCAATTCCTTTRAGTTT – 3′) (Lane, 1991). The 25-μl reaction mixtures contained a 200 μM dNTP mixture, 0.2 μM of each primer, 1% of deionized formamide, 0.2–0.4 mg ml^{-1} of bovine serum albumin (BSA), 2 mM $MgCl_2$, 1× Mg-free reaction buffer, 2 U of Taq polymerase (Promega, Madison, WI, USA), and 1 μl of template DNA solution. Thermocycling was performed in a Biometra UNO Thermoblock (Biotron, Göttingen) with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C, 1.5 min at 72 °C, ending with 10 min at 72 °C. Two amplification products were prepared from each DNA sample and were pooled to reduce PCR variability. The pooled PCR amplification products (approx. 200 ng) were digested with *Hae*III (MBI Fermentas, Litva) or *Alu*I (New England Biolabs, UK) in 30 μl reactions and purified by ethanol precipitation. The digests were mixed with 0.5 μl of the Genescan 500 ROX size standard (Applied Biosystems Inc.) and 10 μl of deionized formamide. Prior to fragment analysis, the samples were denatured at 95 °C for 2 min and chilled on ice. Analysis of the terminal restriction fragment (TRF) sizes and quantities was performed on an ABI PRISM 310 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). The profiles were analyzed using the Genescan analysis software (ABI). The TRFs that were less than 50 bp long were excluded from the analysis.

2.5. Construction of the 16S rRNA gene clone library and sequencing

DNA was isolated from four 0.5 g bog soil samples, as described above, and the 16S rRNA genes were amplified by PCR with primers 27f and 1495r [5′ – CTACGGCTACCTTGTTACGA – 3′] (Weisburg et al., 1991), with the following touchdown PCR protocol: an initial denaturation at 94 °C for 5 min, for the first 10 cycles a 1 min denaturation at 94 °C, 1 min annealing at 58–53 °C (at each cycle, the temperature was decreased by 0.5 °C), 1.5 min at 72 °C, for the last 25 cycles 1 min at 94 °C, 1 min at 53 °C, 1.5 min at 72 °C, ending with 10 min at 72 °C. The resulting PCR amplification products were pooled. Cloning was performed using plasmid vectors (pGEM-T and pGEM-T Easy Vector Systems, Promega) and competent *Escherichia coli* cells; selection of the clones was based on blue–white appearance and resistance to ampicillin. The size of the inserted fragments was checked by PCR prior to sequencing with the primer 1495R (Macrogen, Seoul, Korea). The sequences were manually proofread and corrected, if necessary, with the Chromas Version 2.3 (Technelysium Pty Ltd). Any putative chimeras were detected by the Chimera Check program version 2.7 of the RDP and by partial treeing analysis (Hugenholtz and Huber, 2003); identified chimeras were removed from further analysis. A gene library of 154 partial (approximately 800 bp) 16S rRNA genes was thus obtained.

2.6. Sequence analysis

The sequences of the bog soil gene library were compared to the 16S rRNA gene library that was obtained from the fen soil sample by Kraigher et al. (2006). A phylogenetic analysis of the gene libraries was performed using the ARB software (Ludwig et al., 2004). A maximum parsimony phylogenetic tree was inferred and sequences were assigned to bacterial phyla. A database of 2222 aligned acidobacterial sequences was downloaded from the Ribosomal Database Project (Cole et al., 2007) in February 2008 and used to align and analyze acidobacterial sequences in the ARB. A maximum parsimony phylogenetic tree was made and sequences were assigned to the 26 subgroups proposed by Barns et al., 2007. An additional alignment of the sequences of both the bog and fen libraries was made in the ARB, and distance matrices were exported for the purposes of calculating rarefaction curves and diversity indices with the DOTUR software (Schloss and Handelsman, 2005). The sequences were grouped into operational taxonomic units (OTU) using the farthest-neighbor approach, implemented by the DOTUR software, for the genetic distance < 0.03. The classic Shannon–Weaver diversity index (Shannon and Weaver, 1963) was used as a measure of the general diversity. The Chao1 estimator (Chao, 1984) was calculated as an alternative to the Shannon diversity. The sequences of the bog soil library were submitted to GenBank, accession numbers FJ405427–FJ405580.

2.7. Statistical analysis

For the basal and substrate-induced respiration, the arithmetic means and standard deviations of nine replicates for each type of soil (three field replicates, each with three methodological replicates) were calculated, and *t*-tests were performed to check the significance of the differences between them. For analysis of the microbial community structure, the T-RFLP profiles of duplicate DNA isolations per sample were analyzed using the Bionumerics program version 3.0 (Applied Maths). The normalized intensity values and position of the detected bands were used for cluster analysis. The peaks representing less than 1% of the total fluorescence of all peaks in a sample were excluded from the analysis (less than 5% gave similar results). The Pearson correlation coefficient, which takes into account both the fragment length and the peak height, was used to calculate the similarity coefficients. A dendrogram was constructed using the unweighted pair-group method with the arithmetic means (UPGMA) algorithm.

3. Results

3.1. Microbial activity

The basal respiration and substrate-induced respiration were significantly higher ($P < 0.05$) in the bog soil than in the fen soil (Fig. 1a). CO_2 production was additionally expressed as μl CO_2 cm^{-3} dried soil h^{-1}. The basal respiration calculated per unit volume was slightly ($P < 0.05$) higher in the bog soil. However, the SIR was 100% greater in the fen soil than in the bog soil if the activity was expressed per volume of soil (Fig. 1b).

3.2. Bacterial community structure analysis

T-RFLP analysis was performed on the bog soil and on two drained grassland peat soils that differed in SOC (fen and fenLC, with a higher and lower organic C content, respectively), as described and studied previously by Kraigher et al. (2006). The T-RFLP profiles of fenLC were added to the present analysis in order to emphasize the differences in the bacterial community structure in the bog and fen soils.

The samples were taken in March and August, which represent two contrasting seasons, i.e. damp and cold or warm and dry, respectively (Stres et al., 2008). The T-RFLP profiles of the bog soil samples could be clearly distinguished from both of the fen soil profiles (Fig. 2). The differences between the soil chemical properties (carbon content and pH) appear to be more important than the seasonal variations since the profiles from the same site were similar (less than 10% difference) at the two contrasting sampling times. Therefore, the soil type but not the seasonal change determined the community structure in the peat soils.

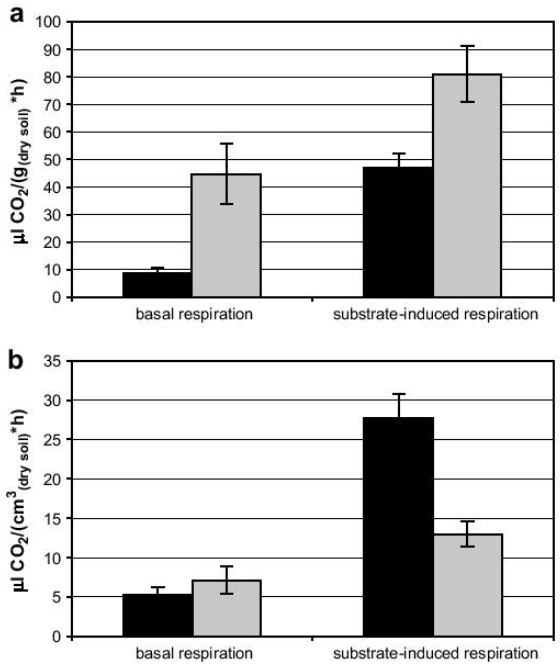

Fig. 1. Basal and substrate-induced respiration in the fen (black) and bog (grey) soils expressed as CO_2 production per mass (a) and volume (b) of the dried soil. The bars indicate a standard deviation of arithmetic means of the 3 subsamples, each with 3 replicates.

3.3. *Phylogenetic analysis and diversity indices*

To examine the phylogenetic composition of the bog soil, 154 partial 16S rRNA sequences were obtained from the bog soil library and were placed within the major bacterial taxa with a maximum parsimony phylogenetic tree. The *Proteobacteria* and *Acidobacteria* phyla were the predominant groups found in the library (62 and 64 sequences, respectively), constituting a total of 81% of the library. Other bacterial phyla identified were *Actinobacteria* (11), *Planctomycetes* (8), *Verrucomicrobia* (8) and *Spirochaetes* (1). The phylogenetic composition of the bog soil library was compared to the fen soil library (Fig. 3), as described previously by Kraigher et al. (2006), and the data were reanalyzed in this study.

Since both of the libraries contained a high proportion of *Acidobacteria*, this group was subjected to a more in-depth analysis. The phylum *Acidobacteria* was established based on the molecular data of Ludwig et al. (1997). It has been phylogenetically reanalyzed several times with the ever increasing number of 16S rRNA sequences in the public databases. For example, six (Barns et al., 1999), eight (Hugenholtz et al., 1998), eleven (Zimmermann et al., 2005) or more major sequence clusters have been proposed. In this study, the acidobacterial sequences were assigned to 26 subgroups within the *Acidobacteria* phylum that were recently proposed by Barns et al. (2007). The majority of these sequences were assigned to the first six subgroups, which are well represented in the public databases, with 92% in the bog soil library belonging to groups 1, 2 and 3, and 82% in the fen soil library belonging to groups 1, 4 and 6. Only one group, GP1, was found in both libraries. Therefore, the composition of the *Acidobacteria* clones differed significantly between the two soils (Table 2).

Operational taxonomic units (OTU) were formed at a 3% genetic distance and the rarefaction curves were inferred with the DOTUR software (Schloss and Handelsman, 2005). The rarefaction curves (Fig. 4) of the total libraries (as well as those that took only *Acidobacteria*-associated sequences into account) failed to plateau, indicating that many more sequences would be necessary to completely cover the bacterial diversity in the investigated soils. In addition, the Chao1 estimators were much higher than the number of OTUs that were detected (Table 3). Both diversity indices were calculated for the genetic distance < 0.035 to avoid PCR amplification and sequencing artifacts (Upchurch et al., 2008).

The *Acidobacteria* sequences within the bog library showed less variation in the number and size of OTUs than those in the fen library. In the fen library, 60% of the acidobacterial clones were found in OTUs represented by a single sequence. The bog library

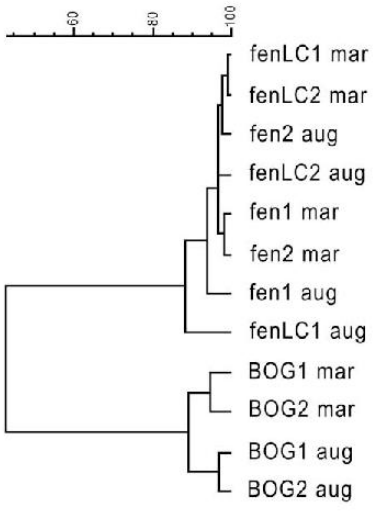

Fig. 2. Comparison of the T-RFLP profiles of the three types of peat soils from the Ljubljana marsh, as analyzed with the Bionumerics 3.0 software. The dendrogram represents similarities between the profiles of the samples based on the Pearson correlation with the UPGMA clustering algorithm. fen, fenLC: two types of drained grassland peat soil; BOG: bog soil; mar, aug: sampled in March and August, respectively; 1, 2: represent DNA isolations from the duplicate samples.

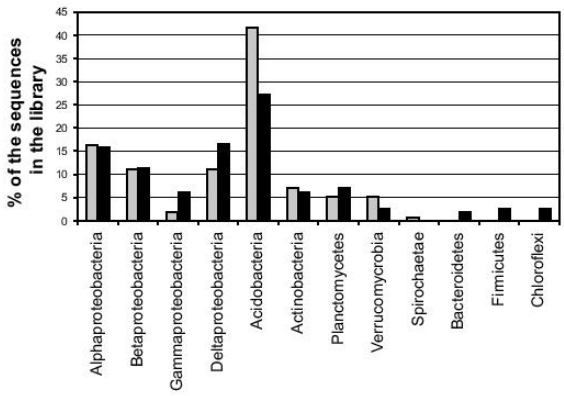

Fig. 3. Distribution of the bacterial 16S rRNA gene sequences of the bog soil (this study) and fen soil (Kraigher et al., 2006) libraries among the various phylogenetic groups. The bog and fen soil libraries have 154 (grey) and 114 (black) clones, respectively.

Table 2
Distribution of the *Acidobacteria* found in the fen soil and bog soil libraries within the subgroups proposed by Barns et al. (2007). The numbers in brackets represent the percentage of acidobacterial sequences in the library belonging to the respective subgroup.

	Fen library	Bog library
GP1	5 (18.5)	26 (40.6)
GP2		22 (34.4)
GP3		11 (17.2)
GP4	5 (18.5)	
GP5		1 (1.6)
GP6	12 (44.4)	
GP7		2 (3.1)
GP10	1 (3.7)	
GP11	1 (3.7)	
GP13		2 (3.1)
GP25	2 (7.4)	
Unclassified	1 (3.7)	
Total[a]	27 (23.7%)	64 (41.6%)

[a] Total number (and proportion) of the acidobacterial sequences in the library.

contained 33 OTUs with only one sequence, while the remaining 31 sequences were clustered into 10 OTUs, two of which contained 6 sequences (Table 4). This resulted in a less steep rarefaction curve (Fig. 4) for the bog library, despite having a greater Shannon–Weaver diversity index (4.68 as compared to 4.52). The rarefaction curves for all of the bacteria and *Proteobacteria* were almost identical; however, the curve for the acidobacterial sequences was less steep (Fig. 4).

4. Discussion

4.1. Basal and substrate-induced respiration in fen and bog soils

The microbial activity was evaluated by measuring the basal respiration and SIR in the fen and bog soils. Based on the neutral pH in the fen soil the microbial activity was expected to be higher. Contrary to our expectations, the basal respiration was higher in the bog soil than in the fen soil. This is in agreement with Fisk et al. (2003), who found a moderately strong negative relationship between the basal respiration and the peat pH. However, the proportion of microbial biomass capable of metabolizing glucose is likely to be lower in the peat than in other soils (Fisk et al., 2003). Enwall et al. (2007) found that the metabolic quotient, calculated as

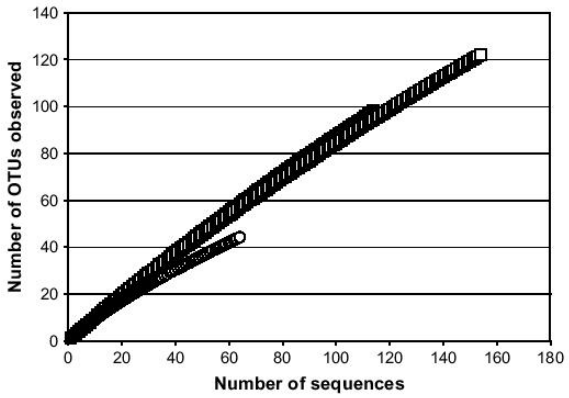

Fig. 4. Rarefaction curves for the bacterial 16S rRNA gene libraries from the bog (□) and fen (■) soils and for the acidobacterial sequences in the two libraries (○ for bog library and ● for fen library). The OTUs were formed at genetic distance of <0.035.

Table 3
Shannon–Weaver and Chao1 diversity indices. The OTUs were formed and calculated for the genetic distance of <0.035. The numbers in brackets represent 95% confidence intervals for the Shannon–Weaver diversity index and the Chao1 estimator of the number of OTUs in the original sample.

	Shannon–Weaver	Chao1	Chao1 acido[a]	No. of observed OTU
Bog library	4.68 (4.55, 4.82)	526 (334, 888)	163 (89, 362)	122
Fen library	4.52 (4.38, 4.66)	463 (277, 840)	84 (45, 205)	98

[a] Chao1 acido – estimation of acidobacterial OTUs in the original sample.

the ratio of basal respiration and SIR, is negatively correlated with pH, indicating a decreased efficiency of organic carbon conversion at low pH. The metabolic quotient also decreases with depth (Brake et al., 1999) and occasional anoxic conditions in the bog soil will reduce CO_2 production compared to the oxic horizons of the fen soil. The bog soil with a lower pH had a higher metabolic quotient (0.55 in bog soil and 0.18 in fen soil), consistent with Enwall et al. (2007). However, the SIR in the bog soil was up to twice that measured in the neutral fen soil. The same trends were observed in similar experiments conducted in March 2003, August 2004 and September 2006 (data not shown). This trend was not expected, given the findings from other studies (Kaiser et al., 1992; Enwall et al., 2007; Lin and Brookes, 1999), and several explanations can be proposed.

Firstly, the microbial biomass (which can be estimated from SIR measurements) increases with soil organic matter (Dornbush, 2007 and references therein), but this is not always the case (Lin and Brookes, 1999), and microbial biomass may thus be higher in the bog soil. However, Kaiser et al. (1992) suggested that the peat soil might be an exception to the trend that is generally observed. Biomass has been positively correlated to soil C and N (Kaiser et al., 1992; Wardle and Ghani, 1995; Kraigher et al., 2006), but also to pH (Enwall et al., 2007), which is again contrary to our observations. Secondly, more CO_2 will dissolve in the soil water at pH values > 6.5 (Lin and Brookes, 1999), potentially leading to an underestimation of the CO_2 in the fen soil. Thirdly, the distribution of added glucose may have been more efficient in the bog soil because of its higher WHC. For this reason, Lin and Brookes (1999) preferred 120% WHC and incubation with shaking. However, this method drastically changes the conditions for aerobic microbes, the activity of which is mainly measured with SIR. Therefore, it can be concluded from this short review of the literature that it is difficult to establish general trends when relating microbial activity to soil organic carbon (maybe even more so when the peat soils are in question). However, when comparing different types of organic soils the marked difference in the bulk densities of the investigated soils is important. The soil bulk density is much lower in the bog soil (0.16) than in the fen soil (0.59). This results in a large volumetric difference when the soils are gravimetrically compared. For example, 25 g of soil with WHC set at 70% would contain 3.7 and 11.6 g of dried bog and fen soils, but the volumes were 23.11 and 19.7 cm^3, respectively.

Table 4
The number of OTUs of the acidobacterial sequences with the indicated size (n) in the fen soil and bog soil libraries. OTUs were formed at genetic distance of <0.035.

Size of OTU (n)	Fen	Bog
1	16	33
2	2	5
3	1	3
4	1	0
6	0	2
Total no. of OTUs	20	43
No. of sequences	27	64

Therefore, SIR was calculated with respect to soil volume rather than the mass. Using this approach, CO_2 emissions in the fen soil were almost twice than those from the bog soil. Brake et al. (1999) confirmed the reliability of the basal respiration and SIR methods for analyzing peat soils; however, it may be advisable to calculate the activities per volume, rather than per weight, when comparing two different soils with significantly different bulk densities. To our knowledge, this is the first time this approach has been adopted.

4.2. Bacterial community structure and diversity in two soils

The 16S rRNA bacterial gene libraries from the bog soil (obtained in this study) and from the fen soil (obtained by Kraigher et al., 2006) were dominated by bacterial species of the *Acidobacteria* and *Proteobacteria* phyla. The bacterial phyla *Actinobacteria*, *Planctomycetes* and *Verrucomicrobia* each comprised less than 10% of the sequences. In the bog soil library there was one sequence belonging to *Spirochaetes*, while *Bacteroidetes*, *Firmicutes* and *Chloroflexi* were represented by a few sequences and only in the fen library.

A higher resolution analysis by T-RFLP indicated differences between the soils regardless of the season in which the soil was sampled. Stres et al. (2008) studied the same fen soil that was used in this study and also found that the bacterial and denitrifier communities were stable and did not respond significantly to seasonal changes in the soil conditions. These conclusions were confirmed by DGGE (denaturing gradient gel electrophoresis) analysis, which also indicated a significant difference between the bacterial communities in the bog and fen soils (data not shown).

The Shannon–Weaver diversity indices, calculated for the two clone libraries at a genetic distance of 3%, did not correspond to the general trend that was observed by Fierer and Jackson (2006) who found that the pH was by far the best predictor of the soil diversity and richness, with the lowest values in acidic soils. Our data suggest that the diversity in the two soils that differ greatly in pH were actually rather similar, and may even be higher in the acidic bog soil. This was contrary to our hypothesis and indicated that the peat soil with high soil organic carbon may be an exception to the general trend.

4.3. Diversity of Acidobacteria in the two clone libraries

The widespread use of rRNA sequence-based analysis for the molecular characterization of complex environments led to the recognition of the phylum *Acidobacteria* (Ludwig et al., 1997), a phylogenetically coherent yet highly diverse group (Quaiser et al., 2003). Initially, six subgroups were proposed by Barns et al. (1999) based on the 16S rRNA gene sequences; however, *Acidobacteria* are now grouped into 26 separate lineages (Barns et al., 2007). The *Acidobacteria* are found in many soil and freshwater habitats, wastewater treatment bioreactors, microbial mats of hot springs and habitats that are contaminated with heavy metals, acids or radioactive elements (Quaiser et al., 2003 and references therein, Barns et al., 2007). Only a few representatives of *Acidobacteria* have been cultivated, which seriously impedes the ecophysiological characterization of this group. However, their wide distribution implies that they might be as metabolically diverse and as ecologically important as the *Proteobacteria* (Barns et al., 1999). The *Proteobacteria* represent the majority of known gram-negative bacteria of medical (*Salmonella*, *Legionella*, *Campylobacter*, *Yersinia* etc.), industrial (*Gluconobacter*, *Acetobacter*, *E. coli*) and agricultural (*Rhizobium*, *Erwinia*, *Agrobacterium* etc.) significance.

A substantial portion of the sequences in the bog (41.6%) and fen (23.7%) soil 16S rRNA gene libraries was assigned to *Acidobacteria*, and they were the most abundant group in the bog library. Members of the phylum *Acidobacteria* make up between 5 and 46% of the soil bacterial communities, and in combination with the *Proteobacteria*, are the most abundant soil bacteria as determined by analysis of 16S rRNA genes in the 32 clone libraries prepared from a variety of soils (Janssen, 2006). Urich et al. (2008), using a RNA-centered meta-transcriptomic approach to asses microbial community structure and function simultaneously, found *Acidobacteria* to be among the dominant bacterial phyla in the nutrient-poor sandy lawn, comprising 11.9% of the 99,061 bacterial small subunit rRNA-tags. Smit et al. (2001) suggested that the ratio between the *Proteobacteria* and the *Acidobacteria* in the clone library could indicate the nutrient status of the soil ecosystem, with a low ratio indicating oligotrophic conditions and vice versa. The ratio in the examined bog soil was lower (1.0) than in the fen soil (2.3), indicating that the bog soil is more oligotrophic. This is in accordance with the general notion that bogs are nutrient-poor. In addition, the higher C:N ratio in the bog soil, as compared to the fen soil, indicates a lower availability of organic carbon to the microorganisms. Similarly, the *Acidobacteria* dominated the Artic tussock soils, which are rich in organic C of low-quality while the *Proteobacteria* dominated the shrub soils with a small, highly labile pool of bioavailable C (Wallenstein et al., 2007). A recent study (Hartman et al., 2008), investigating several sites of the North Carolina pocosin bogs and Florida Everglades, showed that the bacterial community composition and diversity responded strongly to soil pH, with an increase in the abundance of *Acidobacteria* with lower pH. This strongly supported our findings, which extended their work with an in-depth comparison of the acidobacterial sequences linking soil pH with the noted community change.

Phylogenetic analysis of the acidobacterial sequences showed that each library contained a distinct set of phylogenetic subgroups, apart from subgroup 1 which was present in both libraries. Already at this high taxonomic level, the differences between the soil bacterial communities were obvious. The name of this phylum implies that these bacteria preferentially inhabit slightly to moderately acidic environments. Although some subgroups appear to be neutrophilic (Barns et al., 1999), the occurrence of the subgroup 1 *Acidobacteria* has been correlated with a low habitat pH (<6) (Sait et al., 2006). Therefore, it is not surprising that the majority of the bog library sequences fell into this subgroup. Subgroups 1, 3, 4, and 6 are the most abundant subgroups in soil surveys worldwide (Barns et al., 2007), but little is known of their metabolic and ecological role.

Although the *Acidobacteria* sequences were abundant in the bog soil clone library, they were rather similar to each other. Defining OTUs, at a genetic distance of 3%, resulted in 64 sequences grouped into 43 OTUs, two of which contained as many as 6 sequences. However, it is impossible to conclude from our data that the *Acidobacteria* were very numerous in the bog soil, and further research is needed to confirm this hypothesis, possibly by FISH counts (Dedysh et al., 2006) or by quantitative PCR. Despite this fact, the Chao1 estimator predicted there to be almost twice as many acidobacterial OTUs in the bog soil as compared to the fen soil. The molecular data therefore indicated a high diversity and a possible abundance of *Acidobacteria* in the peat soils of the Ljubljana marsh.

4.4. Conclusions

This study showed that high organic soils might be an important exception to the general trend when relating SOC to microbial activity or the soil pH to bacterial diversity. A common method of calculating the CO_2 production in microbial activity measurements on a gravimetric basis may not be appropriate when comparing soils with markedly different bulk densities. For this reason, expressing CO_2 emissions per soil volume rather than mass is recommended. Using this approach, the SIR in the fen soil was almost

twice that from the bog soil. T-RFLP profiling showed clear differences between communities in the two soils. This was confirmed by a phylogenetic analysis of the sequences assigned to *Acidobacteria* that comprised a substantial portion of both libraries. Apart from subgroup 1, each library contained a distinct set of subgroups of these bacteria. The results suggested that the soil pH affected the bacterial community structure, consistent with our hypothesis. However, the investigated soils did not follow the trend of a negative relationship between the soil pH and bacterial diversity.

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2.1.2 Geni za dvo- in trodomenske bakterijske lakaze so prisotni v izsuševanih šotnih tleh

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V tem delu smo ugotavljali prisotnost in raznolikost bakterijskih genov za lakazam podobne encime v dveh tipih šotnih tal z Ljubljanskega barja. Ta tla smo izbrali zaradi dveh razlogov: ker dobro poznamo njihove fizikalne in biotske značilnosti in ker smo v njih izmerili visoko aktivnost lakaz. Slednjo smo merili spektrofotometrično s spremljanjem oksidacije reagenta ABTS in ugotovili, da je v šotnih barjanskih tleh lahko nekajkrat višja kot v gozdnih, travniških in kmetijskih tleh, ki smo jih vključili v raziskavo. Raznolikost bakterijskih genov za lakaze smo nato preučevali z molekularno tehniko kloniranja in določanja nukleotidnega zaporedja kratkih odsekov DNA (150 baznih parov). Rezultati so pokazali nepričakovano visoko raznolikost preučevanih genov, s filogenetskimi metodami smo prepoznali več skupin zaporedij DNA, za katere smo v javnih bazah podatkov lahko našli le redke ali sploh nobenih podobnih zaporedij. Podobno kot pri analizi genov za 16S rRNA (Ausec in sod., 2009) smo tudi tu tretjino sekvenc povezali z deblom *Acidobacteria*. Da bi lahko pridobili vpogled v daljše dele genov (600-1500 bp), smo zasnovali nove začetne oligonukelotide za pomnoževanje z verižno reakcijo s polimerazo (PCR). S njimi smo lahko pomnoževali odseke iz genov za trodomenske in dvodomenske iz raznolikih organizmov. Mnoge trodomenske (26 %) smo ponovno povezali z acidobakterijami. Za večino zaporedij pa je bila značilna majhna podobnost z že poznanimi sekvencami iz dostopnih podatkovnih baz (tipično <55 % identičnost na nivoju proteina). Študija predstavlja enega prvih poskusov preučevanja raznolikosti bakterijskih genov za lakazam podobne encime neposredno v okolju. Pokazala je na veliko raznolikost teh genov v šotnih tleh Ljubljanskega barja, poleg tega pa novo zasnovani začetni oligonukleoti predstavljajo novo orodje za preučevanje bakterijskih lakaz.

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Two- and three-domain bacterial laccase-like genes are present in drained peat soils

Luka Ausec [a], Jan Dirk van Elsas [b], Ines Mandic-Mulec [a,*]

[a] Biotechnical Faculty, Department of Food Science and Technology, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia
[b] University of Groningen, Centre for Ecological and Evolutionary Science, Department of Microbial Ecology, NL9751 NN Haren, Netherlands

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ABSTRACT

Laccases of fungal origin have been intensively studied due to their importance in various biotechnological applications. There is a constant demand for new laccases with improved properties such as stability at higher temperatures or at an alkaline pH. Growing molecular evidence suggests that laccases may also be widespread in bacteria. While only a handful of bacterial laccases have been purified and characterized, several novel traits have already been discovered (e.g. pH-stability and 2-domain organization of the enzyme as opposed to the usual 3-domain structure of fungal laccases). The aim of this study was to examine the diversity of bacterial laccase-like genes in two types of high-organic peat soil using a cloning and sequencing approach. Gene libraries prepared of small fragments (150 base pairs) revealed an amazing diversity of bacterial laccases. The fragments clustered in 11 major lineages, and one third of the 241 sequences resembled laccase-like genes of *Acidobacteria*. Additionally, a new primer was used to retrieve several larger fragments of the putative bacterial laccase genes that spanned all four copper-binding sites. Both "conventional" 3-domain laccases and the recently described 2-domain small laccases have been obtained using this approach, demonstrating the potential of the primer. The present study thus contributes to the understanding of the diversity of bacterial laccases and provides a new tool for finding laccase-like sequences in bacterial strains and soil samples.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to a group of multicopper enzymes found in all domains of life (Hoegger et al., 2006). They have low specificity and can oxidize a variety of phenolic compounds and some non-phenolic substrates, usually via mediators (Widstein and Kandelbauer, 2008), by coupling the oxidation of a substrate to the reduction of molecular oxygen to water. The reaction is catalyzed by four copper atoms that are held in place in the reaction center of the enzyme by four histidine-rich copper-binding regions (Claus, 2004). The amino acid sequences of these regions are conserved in different organisms and the respective gene regions have been utilized as primer-binding sites for PCR amplification (Luis et al., 2004; Kellner et al., 2008). Fungal laccases are most extensively studied (Sharma, et al. 2007; Baldrian, 2006) and significantly contribute to degradation of lignin, but are also associated with other processes in morphogenesis and pathogenesis of fungi (Arora and Sharma, 2010). They have already been applied in a range of biotechnological processes in the paper, textile, pharmaceutical and petrochemical industries (Rodriguez Couto and Herrera, 2006).

On the basis of molecular data, it was proposed that diverse laccase genes may also be present in bacteria (Alexandre and Zhulin, 2000; Claus, 2003). The ecological roles of bacterial laccases are not understood to the full extent, but they may also play an important role in the degradation of recalcitrant (poly)phenolic compounds (Claus, 2003; Bugg et al., 2010). Bacterial laccase are also involved in other physiological processes, such as pigment formation in spores of *Bacillus subtilis* (Martins et al., 2002). A novel lineage of two-domain bacterial laccases has been recently described (Nakamura et al., 2003) in addition to the "regular" three-domain laccases similar to those found in fungi. Representative enzymes have been found in *Streptomycetes* (Machczynski et al., 2004; Endo et al., 2003) and *Nitrosomonas europea* (Lawton et al., 2009) These enzymes are smaller than "conventional" three-domain (fungal) laccases, and in the active form assume a homotrimeric structure. Metagenomic data suggests that two-domain laccases may also be present in other bacterial groups, such as the *Bacteroidetes* (Komori et al., 2009). However, only a few bacterial laccase-like genes and/or protein products have been

* Corresponding author. Tel.: +386 1 4233388; fax: +386 1 257 3390.
E-mail address: ines.mandic@bf.uni-lj.si (I. Mandic-Mulec).

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characterized (reviewed by Sharma et al., 2007). This seriously impedes our understanding of the physiological and environmental role of these enzymes as well as their introduction into biotechnological processes, which are now exclusively operated with fungal laccases.

Studies addressing the diversity and activity of bacterial laccases in soil are extremely scarce (Theuerl and Buscot, 2010; Luis et al., 2004). To our knowledge, there has been only one study describing the diversity and distribution of bacterial laccases in environmental samples. Kellner et al. (2008) devised a primer set to amplify short fragments (approximately 150 bp) of bacterial laccase-like genes to study their diversity and distribution in vertical profile of forest soil and in grassland soil. They found an unusually high diversity of putative bacterial laccases with the sequences clustering into 16 distinct groups, several of which had no closely related sequence in public databases. The highest diversity was found in the humic horizon of the forest soil, as found previously for fungal laccases (Luis et al., 2004).

A colorimetric assay for measuring phenol oxidase activity was recently developed by Floch et al. (2007) which was based on the oxidation of 2,2′-azinobis-(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). ABTS, which is one of the substrates used for testing the enzymatic activity of laccases is readily oxidized by the bacterial laccases CotA of *B. subtilis* (Martins et al., 2002) and SLAC of *Streptomyces coelicolor* (Dube et al., 2008). However, measuring laccase enzymatic activity in complex environments such as soils is not an easy task due to the lack of specific substrates or inhibitors for laccases, and due to their overlapping activities with other groups of enzymes. A modification of this assay was used in the present study to assess phenol oxidase enzymatic activities in a number of different soils as the first step to evaluate the potential of these soils to produce laccase-like enzymes.

On the basis of soil phenol oxidase activity, two soils (high-organic peat soils) with the highest relative activity were selected to investigate the diversity and composition of bacterial laccase-like genes using molecular approaches. The soils differed in pH and soil organic matter content but had been exposed to the same climatic conditions. Laccase gene libraries were constructed from both types of soil using existing and new primers, developed in this work that allow the examination of almost-complete genes of the target enzymes. Laccase diversity in both peat soils was assessed using phylogenetic methods and diversity indices. This study thus provides a novel view of laccase diversity within soil bacterial communities in two divergent peat soils.

2. Materials and methods

2.1. Sampling of soils from sites known for different land use and screening for phenol oxidase activity

Five soil samples were selected for phenol oxidase activity measurements. The top 30 cm were sampled (5 samples were obtained by inserting an auger) and homogenized through a sieve. The samples included: (I) forest soil sampled in Slovenia near Škofja Loka (46°9′, 14°17′), with organic carbon content of 12.7% and pH 4.1, (II) grassland soil sampled in Jesenice, Slovenia (46°26′, 14°0′), with organic carbon content of 7.5%, C:N ratio of 0.26, and pH 6.7, (III) agricultural soil sampled in the Netherlands near Vredepeel (51°32′, 5°52′). It is a sandy soil with the organic carbon content of 3.5% and pH 5.5, and (IV, V) two types of peat soils that were chosen for further studies are described in more detail below and in Table 1.

Phenol oxidase activity was measured in five soil samples using the modified method of Floch et al. (2007) to fit the reactions into a microtiter plate setup. 10-g samples were shaken (150 rpm) in flasks with 90 ml of sterile 0.9% NaCl solution for 1 h 10 ml were transferred to 15-ml centrifuge tubes and shaken vigorously on a horizontal shaker with glass beads (diameter 2 mm) for 15 min. This soil suspension was used for measuring the enzymatic activity. Each reaction in a microtiter plate well contained 130 µl of citrate-phosphate buffer (pH 4.0, 5.8 or 7.6), 20 µl of soil suspension and 50 µl of ABTS (stock concentration 50 mM). The microtiter plates were incubated with shaking (100 rpm) for 1 h then centrifuged at 3500 rpm for 4 min and the supernatant was transferred to a new plate. The absorbance was measured using a Multiscan Spectrum spectrophotometer (Thermo, Vantaa, Finland). The controls were prepared in the same way, with the difference that soil samples were autoclaved beforehand (20 min at 121 °C). All measurements were made in triplicates. Absorbances of the controls that were due to nonbiological oxidation were subtracted from the absorbances in the samples. Mean values and standard errors of absorbances were calculated, normalized to the dry weight of the added samples, and multiplied by a factor of 1000 – these are the arbitrary units reported in Fig. 1.

Table 1
Properties of the fen and bog soils. Values represent the mean ± standard error [$N = 3$] for the upper 30 cm of soil. *WHC – water holding capacity. **Soils have been classified under the World reference base for soil resources (2006). The table was adopted from Ausec et al. (2009).

	Fen soil	Bog soil
C_{org} content (%)	16.3 ± 0.12	45.4 ± 0.12
N_{org} content (%)	1.40 ± 0.006	2.75 ± 0.006
CN ratio	11.7 ± 0.12	16.5 ± 0.006
pH	7.55 ± 0.12	4.58 ± 0.09
WHC* (g_{H20}/g_{soil})	1.65 ± 0.12	8.14 ± 0.17
Bulk density (g cm^{-3})	0.59 ± 0.03	0.16 ± 0.03
Mean annual T (°C)	17.8 ± 7.7	12.6 ± 5.9
Mean annual water table (cm)	−53.2 ± 12.7	−24.4 ± 8.0
Vegetation	Grassland	Forest
Soil classification**	Rheic Fibric Histosol	Rheic Fibric Histosol (Dystric)

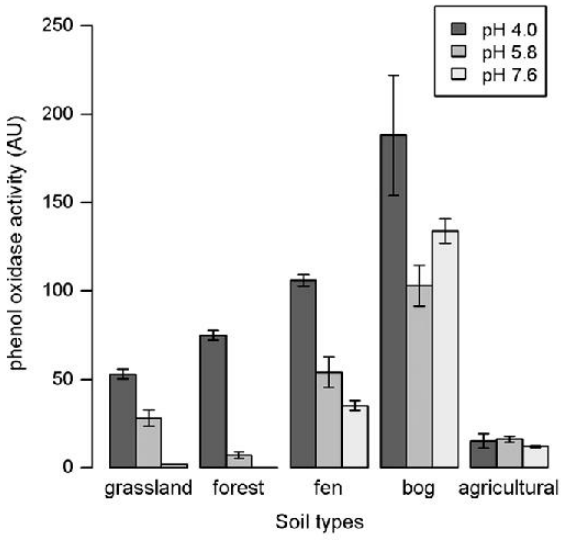

Fig. 1. Phenol oxidase activity in five different soil samples measured in a colorimetric assay using ABTS as a substrate. Measurements were made at three different pH values: 4.0 (black), 5.8 (dark gray) and 7.6 (light gray). Error bars represent standard errors of arithmetic means ($N = 3$). Activity is expressed in arbitrary units that are proportional to the measured change in absorbance.

2.2. Sampling site and characteristics of peat soils

Drained peat soil was sampled at the bog and fen experimental field locations that are positioned less than 3 km apart in the temperate wetland region of the Ljubljana Marsh, Slovenia (45°58′ N, 14°28′ E). Bog soil is an acidic *Sphagnum*-peat soil with high water table level and occasional anoxic conditions. Fen soil is neutral-pH sedge-peat soil with partially degraded peat in the upper horizons. The soils differ most notably in pH, soil carbon content and vegetation, and their properties are summarized in Table 1.

2.3. Soil sampling and DNA isolation

Fen and bog soils were sampled in December 2008. At each location, three field replicates were taken (F1, F2, F3, and B1, B2, B3 for fen and bog soils, respectively) by pooling 4 cores of the upper 30 cm of soil for each replicate. Soil was homogenized through a 4-mm mesh-size sieve. Three DNA isolations from each field replicate were obtained from 0.5-g subsamples using the PowerSoil DNA Isolation Kit (MoBio, Ca, USA). Between 50 and 100 ng DNA per μl were retrieved in each isolation.

2.4. PCR amplification of short fragments (cbr1–cbr2)

Target laccase gene fragments were amplified by PCR with primers Cu1AF (5′-ACM WCB GTY CAY TGG CAY GG-3′) and Cu2R (5′-G RCT GTG GTA CCA GAA NGT NCC-3′) (Kellner et al., 2008) with the following protocol: an initial denaturation at 94 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 48 °C, 1 min at 72 °C, ending with 5 min at 72 °C. 25-μl reactions contained 2.5 μl of each primer (20 μM), 0.5 μl of dNTP (10 μM), 3 μl of BSA (10 mg/ml), 3 μl of MgCl2 (25 mM), approximately 500 ng of DNA template, 2.5 μl of PCR buffer and 1 U of Taq DNA polymerase (Roche, Switzerland). For each type of soil, nine PCR reactions were made (3 field replicates, each with three DNA isolations).

2.5. Construction and evaluation of PCR-primer Cu4R

A new degenerate primer Cu4R (5′-TGC TCV AGB AKR TGG CAG TG-3′) was constructed for the copper-binding region 4 (cbr4) to amplify a larger portion of the putative laccase genes. Template for primer construction consisted only of sequences in public databases (from the genera *Bacillus*, *Escherichia*, *Streptomyces*, *Thermus*, *Marinomonas*, a total of 8 sequences), for which the "usual" three-domain laccase is known at the protein level. The primer was evaluated on selected pure cultures by PCR amplification as described below. For each strain, one or at most 2 bands could be visualized on an agarose gel, signifying a relatively low amount of unspecific products. The product was of expected size, as estimated from the laccase-like sequences in the genomes of these strains in public databases. *Acidobacterium capsulatum* ATCC 51196 and *Paracoccus denitrificans* PD1222 served as negative controls for primer testing, as no laccase-like genes could be found in their genome sequences using BLAST.

2.6. PCR amplification of long fragments (cbr1–cbr4)

PCR amplification was performed with the primers Cu1AF (Kellner et al., 2008) and Cu4R (this study) using the same conditions described for the short fragments, except that approximately 80 ng of template DNA was added to the 25-μl reaction and the polymerization step of the cycle lasted for 90 s at 72 °C.

2.7. Fragment purification and clone library construction

Three different templates were used for the clone library construction. Firstly, gene libraries of short fragments (cbr1–cbr4, 150 bp long) were prepared from each soil in triplicates. Secondly, a small library from each soil was prepared using the amplicons ranging in size between 600 and 1200 bp; they were excised together from the gel and used for cloning. In the third approach, two strong bands of long laccase fragments of around 600 (2-domain laccases) and 1200 bp (3-domain laccases) were purified separately from the gel, avoiding potential unspecific products. The fragments were then cloned separately, so two small gene libraries were made from each type of soil. Fragments of the expected sizes were excised from the 1% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. The fragments were cloned into the plasmid vector pGEM-T Easy Vector System (Promega, USA) and competent *Escherichia. coli* JM109 cells were transformed as described by the manufacturer. Selection of the clones was based on the blue-white colony appearance and resistance to ampicillin. The size of the inserted fragment was checked by PCR prior to sequencing (Macrogen, Seoul, Korea) with the primers SP6 and T7. The sequences were stored in GenBank under the accession numbers HM045518–HM045758 (short fragments) and HM045759–HM045777 (long fragments). For the third set of libraries, the sequences were stored with the accessions HQ286694–HQ286735 (2-domain laccases) and HQ286736–HQ286789 (3-domain laccases).

2.8. Sequence analysis and statistics

Sequences were manually proofread and similar sequences were obtained from GenBank using BLAST. Nucleotide sequences were translated and aligned as amino acid sequences using ClustalW, implemented in the Mega4 software package (Tamura et al., 2007). Mega4 software was used for sequence manipulation and phylogenetic reconstruction and a neighbor-joining tree was inferred from the nucleic acid sequence alignment. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. CD-search tool was used to find conserved multicopper oxidase domains in the protein sequences (Marchler-Bauer and Bryant, 2004).

For the gene libraries of short fragments, operational taxonomic units (OTUs) were established at the nucleotide genetic distance of 1%, 3% and 10%. Then, the OTU-based analyses (rarefaction and collector's curves, Shannon diversity indices, Chao1 richness estimators) were performed using the Mothur software (Schloss et al., 2009). The 95% confidence intervals for the indices were computed in Mothur and the significance of the differences between the fen and bog library was estimated as recommended by Cumming et al. (2007). The rarefaction curves and phenol oxidase activity measurements were visualized using the R package (R Development Core Team, 2008). Sequences from Kellner et al. (2008) were obtained from GenBank and clustered and analyzed in the same way, to compare the diversity found by us to that of the gene libraries produced in their study. As the Shannon diversity index depends on the total number of sequences, the index for the data of Kellner et al. (2008) was calculated for 120 sequences that were randomly sampled from the total dataset to compare gene libraries of similar size.

3. Results

3.1. Activity of soil phenol oxidases

Phenol oxidase activity was measured in five soil samples using a colorimetric assay at three different pH values and with ABTS as

the substrate. Results (Fig. 1) are expressed in arbitrary units (AU) that were normalized to dry weight of soil and are proportional to the observed changes in absorbance (with autoclaved controls subtracted). Peat soils exhibited the highest enzymatic activities at all three pH in the range 4.0–7.6, and were thus chosen for further study of bacterial laccases.

3.2. Diversity of short laccase-like sequences (cbr1–cbr2) in two peat soils

Three small gene libraries were constructed from both fen and bog soils, and 64 clones of each were sequenced. The sequencing yield was on average 63%, the loss being due to non-target fragments that had been amplified with the degenerate primers. A total of 124 and 117 sequences were thus obtained from fen and bog soil (Table 2), respectively. These were distributed in the libraries denoted as fenA (42 sequences), fenB (27 sequences), fenC (55 sequences), and bogA (40 sequences), bogB (45 sequences) and bogC (32 sequences). An unrooted neighbor-joining tree was constructed (Fig. 2) and the sequences were assigned to 11 major clusters.

The phylogenetic tree contained two *Acidobacteria*-associated clusters with long branches, indicating that these sequences were notably different from their neighboring groups. These two clusters each had only one reference sequence in the public databases, i.e. type 3 multicopper oxidase from *Solibacter usitatus* Ellin6076 (*Acidobacteria* I) and type 3 multicopper oxidase from *Candidatus Koribacter versatilis* Ellin345 (*Acidobacteria* II), which BLASTx search indicated were present in two of the three hitherto sequenced acidobacterial genomes (Ward et al., 2009). Together, these sequences account for 33% of all sequences, and were present in all six libraries. The *Solibacter*-associated sequences were especially numerous in the bog soil libraries bogA (23) and bogC (15), where they accounted for 57% and 47% of the sequences, respectively (Table 2).

Four distinct proteobacterial clusters were identified (Fig. 2), each with reference sequences from different subphyla of *Proteobacteria*. Protein sequences within these groups were >70% identical to the reference sequences from the public databases, which belonged to genera such as *Nitrobacter*, *Roseobacter*, *Caulobacter* (*Alphaproteobacteria*), *Polaromonas*, *Burkholderia* (*Betaproteobacteria*), *Pseudomonas*, *Stenotrophomonas* (*Gammaproteobacteria*) and *Sorangium* (*Deltaproteobacteria*).

The phylogenetic association of the Group I sequences (six sequences only from fen soil libraries) in the tree was not clear, since no reference sequence clustered in this group. A BLASTx search indicated a multicopper oxidase from *Campylobacter* (*Epsilonproteobacteria*) to be most similar to one of the sequences (fenA-G10), but the resemblance was low (52% identity, *e* value only 0.22). Group I might therefore be associated with laccases from the *Delta- or Epsilonproteobacteria*, but its position in the phylogenetic tree indicated that it could also be an acidobacterial lineage. More reference sequences from acidobacterial genomes are required to support this hypothesis.

Group II sequences (Fig. 2) could not be unambiguously placed into any major lineage and no reference sequences clustered in this group. The BogB-E12 sequence, for example, was similar to that of an enzyme from *Streptomyces* (*Actinobacteria*, $e = 10^{-4}$, identity 65%), fenC-D4 is similar to a *Sorangium* (*Deltaproteobacteria*, $e = 10^{-6}$, 63% identity) enzyme, but also to sequence EF 394768 from the library of Kellner et al. (2008), which had been assigned to an *Aplhaproteobacterial* lineage.

Sequence groups III and IV appeared to be affiliated with sequences from Gram-positive bacteria. As with the *Firmicutes*/*Actinobacteria* cluster, these sequences were assigned to either of these two phyla, suggesting that laccase genes of *Firmicutes* and *Actinobacteria* are not well separated. Among others, the genera *Bacillus*, *Streptomyces* and *Alicyclobacillus* occurred most frequently in BLASTx searches.

Rarefaction curves (Fig. 3) of the fen soil composite library were markedly steeper than those of the bog soil library, which was dominated by highly similar (>97%) *Acidobacteria*-associated sequences. While this indicated considerably higher coverage in the bog soil library, many more sequences would need to be sampled in both soil libraries for the rarefaction curves to reach a plateau. As expected, the bog soil library curves were significantly lower ($p < 0.01$) than those of the fen soil library, indicating a lower diversity of the laccase genes in the bog soil. The collector's curves for the Shannon diversity index for both libraries were smooth and leveled off toward the *x* axis (data not shown), which indicated that this index was a suitable diversity estimator for this data. The diversity of laccase gene fragments in the composite libraries of this study was thus compared to that of the composite library of Kellner et al. (2008) using the Shannon diversity index (Table 3). It was lower for the bog soil composite library than for the other two libraries, which were not statistically different ($p < 0.05$). The same was true for the Chao1 estimators of richness; the bog soil library had significantly lower richness while the confidence intervals for this estimator overlapped between the composite libraries of the fen soil and that of Kellner et al. (2008) and thus no statistical difference in richness could be detected (Table 3) between these two libraries.

Table 2
Phylogenetic affiliation of the clones in the gene libraries. The groups were named according to the reference sequences that clustered in those groups in the phylogenetic tree. No reference sequences clustered within the groups I–IV. A, B, C designate replicate libraries for each soil, % refers to the percentage of clones in that replicate library.

	Total		Fen soil						Bog soil					
			A		B		C		A		B		C	
	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
Acidobacteria I	59	0.24	4	0.10	10	0.37	4	0.07	20	0.50	7	0.16	14	0.44
Acidobacteria II	20	0.08	5	0.12		0	5	0.09	3	0.08	6	0.13	1	0.03
Proteobacteria I	45	0.19	16	0.38	5	0.19	14	0.25	3	0.08	4	0.09	3	0.09
Proteobacteria II	9	0.04	2	0.05	1	0.04	0	0	1	0.03	2	0.04	3	0.09
Proteobacteria III	28	0.12	6	0.14	1	0.04	10	0.18	1	0.03	7	0.16	3	0.09
Proteobacteria IV	18	0.07	0	0	2	0.07	0	0	10	0.25	5	0.11	1	0.03
Actinobacteria/Firmicutes	5	0.02	2	0.05	1	0.04	0	0	0	0	2	0.04	0	0
Group I	6	0.02	2	0.05	1	0.04	3	0.05	0	0	0	0	0	0
Group II	17	0.07	1	0.02	3	0.11	10	0.18	0	0	3	0.07	0	0
Group III	13	0.05	0	0		0	3	0.05	2	0.05	2	0.04	6	0.19
Group IV	21	0.09	4	0.10	3	0.11	6	0.11	0	0	7	0.16	1	0.03
Total	241		42	1	27	1	55	1	40	1	45	1	32	1

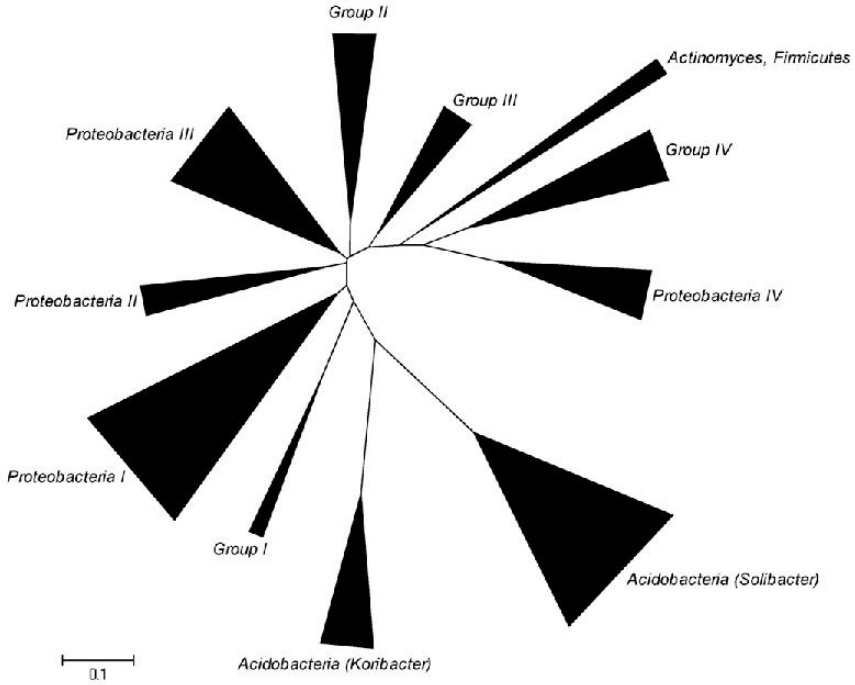

Fig. 2. Unrooted neighbor-joining tree of the 241 short laccase fragments (cbr1–cbr2) obtained in this study. There were 93 positions in the nucleotide sequence alignment after elimination of gaps.

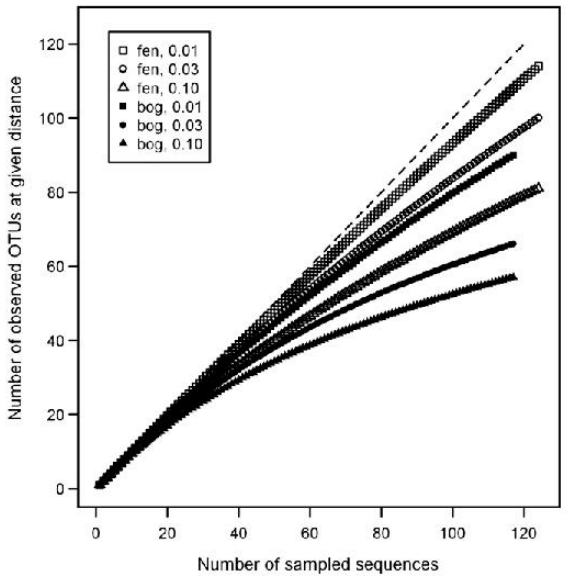

Fig. 3. Rarefaction curves for the fen and bog composite libraries of short laccase fragments (cbr1–cbr2). Operational taxonomic units (OTU) were formed at genetic distances of 1%, 3% and 10%.

3.3. Two- and three-domain laccases in the peat soils

Longer sections of the putative soil laccases were investigated using the new degenerate primer Cu4R that was designed to amplify the region between the first and the fourth copper-binding region of the bacterial laccase genes. A survey of bacterial laccase-like genes in available genomes indicated that amplified fragments would vary in length in the range between 600 and 1500 bp. The Cu4R primer was first tested on several bacterial isolates, including some that were not used for primer construction. Fragments of expected lengths (based on the multicopper oxidase sequences within sequenced genomes, available from public databases) were successfully amplified using the primer pair CuA1F–Cu4R: CotA of *B. subtilis* (1185 bp, NP_388511), PcoA of *E. coli* (1479 bp, YP_001481473), a putative

Table 3
Shannon diversity indices and Chao1 richness estimates for OTUs defined at genetic distances of 1, 3 and 10%. The fragments of the bog and fen composite libraries of short laccase gene fragments (cbr1–cbr2) are shown in comparison with the random 120 sequences of the total diversity of the data of Kellner et al. (2008). The numbers in brackets represent 95% confidence intervals of the indices.

	Distance	Shannon	Chao1
Bog ($N = 117$)	0.01	4.42 (4.28–4.56)	237 (165–380)
	0.03	4.04 (3.90–4.18)	105 (83–157)
	0.11	3.82 (3.67–3.97)	96 (73–156)
Fen ($N = 124$)	0.01	4.70 (4.58–4.83)	721 (413–1344)
	0.03	4.53 (4.39–4.66)	302 (207–485)
	0.10	4.20 (4.05–4.36)	191 (135–306)
Kellner ($N = 120$)	0.01	4.73 (4.60 4.86)	1114 (568–2317)
	0.03	4.67 (4.54–4.80)	676 (378–1301)
	0.10	4.28 (4.12–4.45)	282 (183–484)

multicopper oxidase of *Staphylococcus epidermidis* (984 bp, gi|27316888: 122271–123704) and CopA of *Pseudomonas fluorescens* (1365 bp, YP_259999). A fragment of approximately 1200 bp was amplified from *Rhizobium rhizogenes*. Kellner et al. (2008) showed that this strain had laccase activity and they were able to amplify a short fragment of the laccase gene from chromosomal DNA. However, the absence of relevant sequence data for this bacterium prevents estimation of the expected size of the PCR product. No laccase-like gene was found in the genomes of *A. capsulatum* ATCC 51196 and *P. denitrificans* PD1222. These two strains were then used to serve as negative controls, generating no amplification product with the Cu1AF–Cu4R primer pair.

Next, the Cu1AF–Cu4R primer pair was used to amplify long fragments of laccase genes from soil DNA. Two approaches were used for cloning of the long fragments. Firstly, DNA fragments between 600 and roughly 1500 bp were excised from the gel in one piece, together with the non-specific amplicons visible between the bands as smear, and secondly, two bands of different sizes were excised from the gel and cloned separately. Using the first approach, a small gene library (48 clones) from each type of soil was prepared and sequenced. The data revealed that 10 (bog) and 9 (fen) sequences were similar to those of laccase-like multicopper oxidases based on BLAST searches. The remainder of the sequences did not resemble any sequence available in public databases and could also not be translated into protein sequences, as they contained stop codons in all six reading frames. However, the total of 19 sequences (yield about 20%) appeared to represent true bacterial multicopper oxidases containing the copper-binding domains, which is characteristic of laccases (Claus, 2004; Hoegger et al., 2006). Furthermore, apparently two classes of bacterial laccases were obtained, "regular" three-domain enzymes that were predominant (16 sequences), and two-domain laccases (3 sequences).

Of the two-domain laccases, one was found in each library (HM045770 in the fen soil library and two identical sequences in the bog library – accession numbers HM045759 and HM045765). They were more than 70% identical and BLASTx analyses in both cases indicated the same closest hits from unrelated bacteria, such as *Nitrosococcus*, *Planctomyces* and *Poribacteria*. This illustrated the low amount of data available on the subject. These sequences were approximately 600 bp long. Seven sequences of the three-domain laccases were affiliated to a multicopper oxidase of *Sorangium cellulosum* (*Deltaproteobacteria*) with an amino acid positive hit rate of around 60% based on BLASTp search. Three sequences were most similar to a multicopper oxidase from *S. usitatus* (*Acidobacteria*). The remaining sequences were affiliated to multicopper oxidases of different genera such as *Bacillus* (HM045772) and *Bulkholderia* (HM045777). The translated amino acid sequences were less than 50% identical to the most similar sequences in the public databases.

To summarize, the putative laccase fragments amplified by primer pair Cu1AF–Cu4R were of two sizes, corresponding to two stronger bands visible on the agarose gel. In the subsequent approach, only these two bands (sizes around 600 bp and 1200 bp) were used as templates for cloning. Thus, two gene libraries were made for each type of soil, one for 2-domain laccases and one for 3-domain laccases. In this case, approximately 80% of the sequenced clones contained laccase-like DNA fragments and a total of 96 gene fragments were obtained in the four libraries: 28 and 33 in the bog soil and 14 and 21 in the fen soil libraries for 2-domain and 3-domain fragments, respectively.

Reference sequences could not be found for all clusters in the unrooted neighbor-joining phylogenetic tree of the 2-domain bacterial laccase genes (Fig. 4). This is especially evident for the uppermost cluster in the tree, the sequences of which were most similar to those from distinct bacterial phyla (*Proteobacteria*, *Planctomyces*, *Poribacteria*), as was the case with the 2-domain laccases found in the first libraries. Several other clusters were present in the tree, among which the *Chloroflexi*-associated sequences were most numerous (8 and 4 sequences from bog and fen, respectively). Two sequences were affiliated to those of laccases of *Firmicutes* (*Geobacillus*) according to BLASTx search, however, the similarity was low (identity score below 40%) and the reference is in fact a regular 3-domain enzyme.

The 3-domain laccase-like fragments, obtained in this study, grouped into several clusters (Fig. 5). No references clustered in the big cluster of 17 sequences that were affiliated to the sequences of laccases of various Proteobacteria using BLASTx. One sequence from each library was found to be similar to verrucomicrobial laccase-like genes. As with short-fragment libraries, a substantial proportion of the sequences (26%) were grouped with laccases of *Acidobacteria*, both by using BLASTx searches and the phylogenetic tree.

4. Discussion

The present study investigated the diversity of bacterial laccase-like genes in two types of high-organic drained peat soil in a temperate marsh. The two peat soils were selected from among a broader suite of soils, based on the very high relative phenol oxidase activity measured in these soils. This is in agreement with the findings of Kellner et al. (2008) that bacterial laccases are most diverse in the uppermost humic horizon of forest soil, which shares a high-organic matter content with peat soils.

The diversity of bacterial laccases was determined by constructing and analyzing gene libraries, initially constructed using the primer set designed by Kellner et al. (2008). This approach generated 241 putative laccase fragments of approximately 150 bp between copper-binding regions 1 and 2. In contrast with the theoretically limited diversity of such short fragments, the diversity of this region was high. The diversity of the sequences in the fen soil libraries alone was already comparable to the total (composite) diversity of all grassland and forest soil samples analyzed by Kellner et al. (2008), on the basis of rarefaction curves and Shannon diversity indices. As noted by Kellner et al. (2008), a cysteine residue (position 25 in their amino acid sequence alignment) is characteristic of fungal laccases. The cys residue was absent from all sequences retrieved in this study, suggesting that they were indeed genes of bacterial origin. The size of the fragments varied beyond the limits noted by Kellner et al. (2008), and the majority encoded 45–49 amino acid residues. Furthermore, the whole *Acidobacteria II* cluster (Fig. 2), together with the reference sequence from *Candidatus K. versatilis* Ellin345, had 53 residues, and this group of sequences was absent from the libraries of Kellner et al. (2008).

Acidobacteria-related sequences were shown to be well represented in the 16S rRNA gene libraries generated from the two peat soils (Ausec et al., 2009), representing 24 and 42% of sequences in the fen and bog soil libraries, respectively. This study again indicated a high diversity and, possibly, a high relative abundance of *Acidobacteria* in these soils, as 79 out of 241 (33%) short fragments and 14 out of 54 (26%) long fragments were associated with *Acidobacteria*. The small size of the libraries (especially those of the long fragments) was not sufficient to give high coverage and more sequence data are certainly required for robust quantitative comparison. Interestingly, no *Acidobacteria*- or *Verrucomicrobia*-related sequences were retrieved in the gene libraries of the 2-domain laccases. While it could be that these groups do not harbor any genes for 2-domain laccases, this finding may also be attributed to the low coverage of the genome sequences available for these groups of bacteria. Both libraries clearly showed that the recovered diversity of laccase-like genes was much higher than the diversity of those found in public databases, as no similar sequences

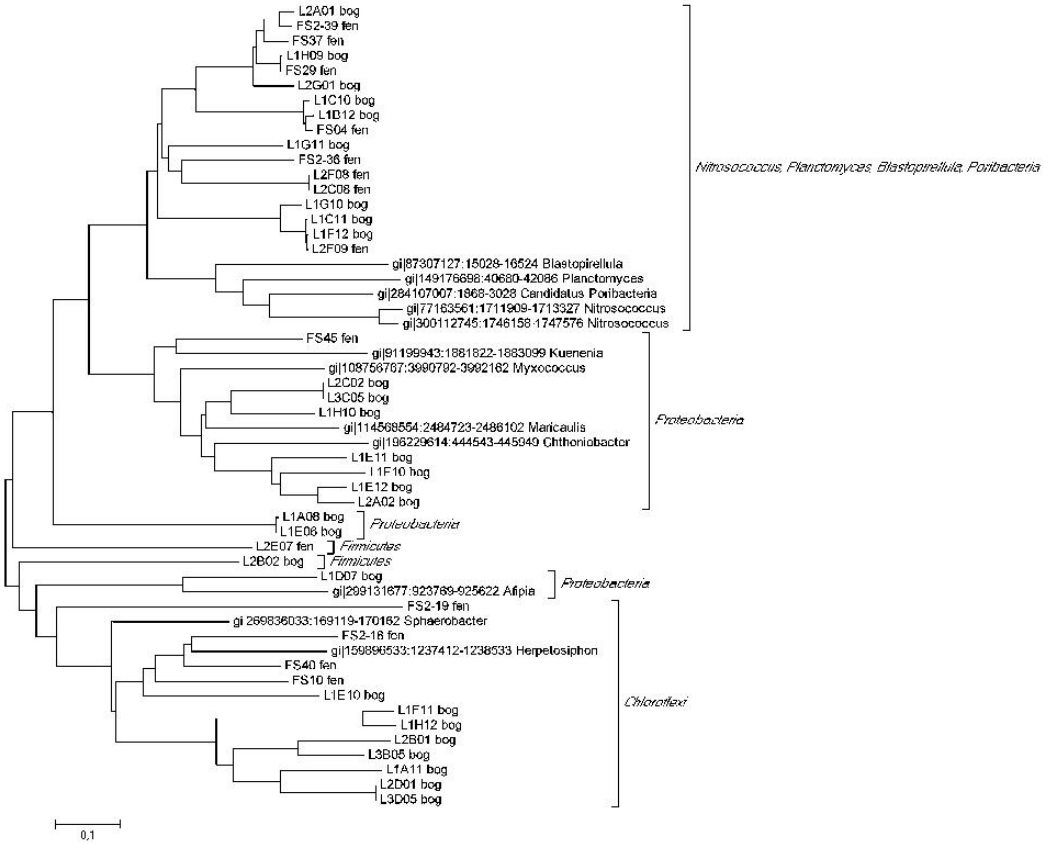

Fig. 4. Unrooted neighbor-joining tree of putative 2-domain laccase genes (cbr1–cbr4) obtained from fen and bog soil libraries. Several reference sequences were included in the phylogenetic analysis, and are designated on the tree with exact accession numbers and names of the genera. The names of the bacterial phyla, to which the first five BLASTx hits belonged, are given next to the square brackets.

could be found for several clusters of the retrieved sequences, and the similarity between those that were found and the retrieved sequences was generally low (usually identity at the protein level was below 55%).

The biological role of laccases in soil fungi is not completely understood. By studying individual fungal species, researchers have changed the stereotypical "laccases degrade lignin" with more complex points of view. Kilaru et al. (2006), for example, identified as much as 17 laccase genes in the genome of *Coprinopsis cinerea*. Although these genes probably resulted from gene duplications, the enzymes they encode vary notably in the substrate-binding regions, which presumably gives them different substrate specificities and activities (Kilaru et al., 2006). Interestingly, the promoter and terminator regions of these genes were not conserved and the genes are probably expressed under different stimuli and/or at different stages of the development of the fungus. Our understanding of bacterial laccases and their roles is even more limited. Apart from the recognized role of the CotA laccase from *B. subtilis* that is involved in pigment formation in spores (Martins et al., 2002), little is known about the biological functions of bacterial laccases. While browsing for laccase-like sequences in the sequenced genomes, we noted several genes that encode signal peptides indicating that the respective enzymes might have extracellular functions. A more thorough bioinformatics study of bacterial laccase genes may therefore give some insight into this interesting topic.

Although the design of primer Cu4R was based on the sequences of "typical" three-domain laccases, it also amplified two-domain laccases. The latter have been only recently proposed in an evolutionary model of multicopper oxidases (Nakamura et al., 2003), and only a few have been studied (Machczynski et al., 2004; Endo et al., 2003; Komori et al., 2009). The primers designed in this study were degenerated to allow the capture of as broad bacterial laccase diversity as possible since the diversity of laccases among bacteria is still virtually undescribed. This approach lowered the yield of positive clones in the gene libraries due to non-target amplification. However, when individual bands, detected after the resolution of PCR products on agarose gels via ectrophoresis, were used as templates, 80% of the clones contained laccase-like sequences and considerable diversity was discovered. Several clusters of sequences were considerably different from related sequences in public databases. This is understandable since environmental strains have not been subjected to an extensive sequencing effort. Therefore, a combined approach of studying (1) sequenced genomes and (2) environments using the primers developed in this study, should help to recognize potential subgroups of bacterial laccases and to design more specific primers for a targeted metagenomics approach. Moreover, the CuA1F–Cu4R primer pair has

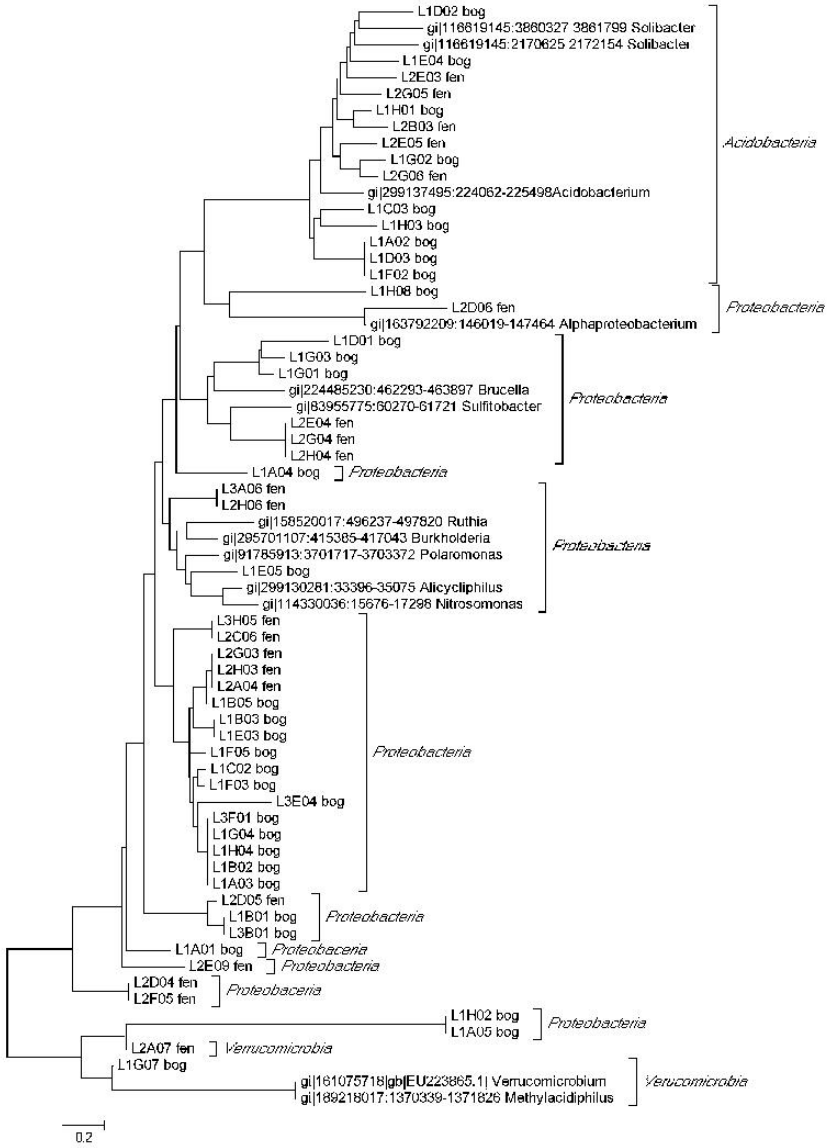

Fig. 5. Unrooted neighbor-joining tree of putative 3-domain laccase genes (cbr1–cbr4) obtained from fen and bog soil libraries. Several reference sequences were included in the phylogenetic analysis, and are designated on the tree with exact accession numbers and names of the genera. The names of the bacterial phyla, to which the first five BLASTx hits belonged, are given next to the square brackets.

proved to be useful for the amplification of putative laccase genes from several isolated strains, including *Verrucomicrobia* (Ulisses Nunes da Rocha, personal communication).

5. Conclusions

A high diversity of bacterial laccase-like genes was discovered in the drained peat soils. A new PCR-primer (Cu4R) was constructed that for the first time enabled amplification of almost full-length gene fragments directly from the soil samples. The fragments retrieved using cloning and sequencing were affiliated to both the "conventional" three-domain laccases as well as the more recently described two-domain bacterial laccases. The Cu4R primer may thus prove important for future ecological studies as well as for finding novel genes encoding laccase-like enzymatic traits for biotechnological applications.

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2.1.3 Bioinformatske analize razkrivajo visoko raznolikost bakterijskih genov za lakazam podobne encime

Ausec L., Zakrzewski M., Goesmann A., Schlüter A., Mandic-Mulec I. 2011. Bioinformatic analysis reveals high diversity of bacterial genes for laccase-like rnzymes. PLoS ONE, 6: e25724, doi:10.1371/journal.pone.0025724: 9 str.

Namen dela je bil raziskati razširjenost genov za lakazam podobne encime pri bakterijah. Z bioinformatskim pristopom smo preiskali dokončane in nedokončane bakterijske genome, ki so bili na voljo v času raziskave. Pripravili smo pet verjetnostnih modelov za bakterijske lakaze (ang. p*rofile Hidden Markov Models, pHMM*). Prednost pHMM v primerjavi z algoritmi iskanja podobnosti (npr. BLAST) je v tem, da prepozna dobro ohranjene motive, kot so aktivna mesta v encimu. Z modeli smo preiskali približno 2200 genomov in našli 1240 domnevnih genov za dvo- in trodomenske lakaze. Število lakaz, ki smo jih našli pri predstavnikih posameznih bakterijskih debel, približno posnema pristranskost javnih baz podatkov, saj so nekatere skupine bakterij (npr. *Gammaproteobacteria*) zastopane bistveno bolje kot druge (npr. *Acidobacteria*). Približno tri četrtine genov kodira signalne peptide, kar pomeni, da se ti encimi v organizmu prenesejo iz citoplaze v periplazmatski prostor ali celo iz celice, kar je dugače od splošno sprejetega mnenja, da so bakterijske lakaze intracelularni encimi. Poleg tega smo našli veliko primerov mobilnosti teh genov med bakterijami, bodisi z mobilnimi elementi ali s plazmidi. Pri nekaterih organizmih je bil gen za lakazo kodiran celo samo na plazmidu. Pokazali smo, da z verjetnostnimi profili pHMM lahko najdemo gene za lakazam podobne encime tudi v metagenomskih podatkih, četudi se geni zelo razlikujejo od vsega, kar poznamo. Odkrili smo nepričakovano veliko genov v zelo raznolikih organizmih. Ta nabor predstavlja velik potencial za biotehnološko izrabo, saj bi s pametnim izborom izvornih organizmov morda lahko našli lakaze z želenimi lastnostmi, kar prej ni bilo možno. Pomemben pa je tudi uvid v biologijo teh encimov, saj smo gene zanje našli v številnih heterotrofih, a tudi v anaerobih, avtotrofih in drugih okoljsko relavantnih bakterijah.

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Bioinformatic Analysis Reveals High Diversity of Bacterial Genes for Laccase-Like Enzymes

Luka Ausec[1], Martha Zakrzewski[2,3], Alexander Goesmann[2], Andreas Schlüter[3], Ines Mandic-Mulec[1]*

1 Department for Food Science and Technology Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia, **2** Center for Biotechnology (CeBiTec), Institute for Bioinformatics, Bielefeld University, Bielefeld Germany, **3** Center for Biotechnology (CeBiTec), Institute for Genome Research and Systems Biology, Bielefeld University, Bielefeld, Germany

Abstract

Fungal laccases have been used in various fields ranging from processes in wood and paper industries to environmental applications. Although a few bacterial laccases have been characterized in recent years, prokaryotes have largely been neglected as a source of novel enzymes, in part due to the lack of knowledge about the diversity and distribution of laccases within Bacteria. In this work genes for laccase-like enzymes were searched for in over 2,200 complete and draft bacterial genomes and four metagenomic datasets, using the custom profile Hidden Markov Models for two- and three-domain laccases. More than 1,200 putative genes for laccase-like enzymes were retrieved from chromosomes and plasmids of diverse bacteria. In 76% of the genes, signal peptides were predicted, indicating that these bacterial laccases may be exported from the cytoplasm, which contrasts with the current belief. Moreover, several examples of putatively horizontally transferred bacterial laccase genes were described. Many metagenomic sequences encoding fragments of laccase-like enzymes could not be phylogenetically assigned, indicating considerable novelty. Laccase-like genes were also found in anaerobic bacteria, autotrophs and alkaliphiles, thus opening new hypotheses regarding their ecological functions. Bacteria identified as carrying laccase genes represent potential sources for future biotechnological applications.

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* E-mail: ines.mandic@bf.uni-lj.si

Introduction

Laccases are members of the multi-copper oxidoreductases that oxidize a variety of phenolic substances including polyaromatic hydrocarbons (PAH), estrogens in wastewater [1,2] and recalcitrant biopolymers such as lignin [3,4]. Due to their broad substrate specificity laccases are of great industrial interest and have been used in paper and wood processing and in the textile industry [5,6,7].

Substrate oxidation by laccases (and subsequent reduction of molecular oxygen) creates reactive radicals which can participate in (i) polymerization (oxidative coupling of monomers), (ii) degradation of polymers or (iii) degradation of phenolics (by cleavage of aromatic rings) [8]. Substrate specificity is broadened by mediators, which are small molecular-mass compounds that are oxidized into radicals by laccases and can subsequently oxidize a variety of other (more complex) substrates such as lignin. Laccases contain four copper atoms held in place in the reaction center by conserved copper-binding regions. Nucleotide sequences specifying the copper-binding sites are suitable for molecular-ecological studies as it is possible to design PCR-primers for these sites [9-11]. Laccases have been found in all domains of life [12] but have been most intensively studied in ligninolytic fungi [13].

The first indication that laccases may be present in bacteria was based on the phenol-oxidase activity observed in *Azospirillum lipoferum* almost 20 years ago [14]. A decade ago, researchers used the BLAST algorithm to find 14 bacterial laccase genes similar to those known from fungi [15]. A few bacterial laccases have been studied since (see [16] for a more recent review). Until recently, fungal laccases have been considered extracellular enzymes while bacterial laccases were assumed to be mostly intracellular or spore-bound. It was speculated [16] that bacteria may have strategies such as rearrangement of the electron transport system to cope with the toxic molecular compounds produced by the oxidation of aromatic substrates within the cell. The simplistic view of fungal laccases as extracellular lignin-degrading enzymes has given way to a more realistic view, in which fungal laccases are involved in various intra- and extracellular developmental processes in morphogenesis and pathogenesis [7,12,17] in addition to their role in degradation of complex substrates. It was suggested that further studies are needed to verify that the diverse fungal laccases retrieved from different environmental studies are indeed extracellular ligninolytic enzymes [3].

In bacteria, the perceived role of laccases has mostly been limited to oxidation of metals and pigment formation [8,16]. The latter function is based on the well studied CotA laccase located in the spore coat of *Bacillus subtilis*, which produces a melanin-like pigment for the protection of the spore against UV-light [18]. The possibility that bacterial laccases play a role in the degradation of recalcitrant biopolymers has been suggested only recently [4,19]. However, bacterial laccases may have several properties that are not characteristic of fungal enzymes. Firstly, the laccase from *Streptomyces lavendulae* [20] shows high thermo resistance and the CotA laccase from *Bacillus subtilis* has a half-life of inactivation at 80°C of about 4 h and 2 h for the coat-associated or the purified enzyme, respectively [18]. The most termophilic laccase from

Thermus termophilus has the optimal reaction temperature of 92°C and a half life of inactivation at 80°C of over 14 hours [21]. Secondly, the laccase from *Bacillus halodurans* is stimulated rather than inhibited by chloride [22], which is a novel trait of great importance for industrial processes. Thirdly, but perhaps most importantly, several pH-tolerant bacterial laccases with pH ranges from 4 up to 9.5 have been described, e. g. from a *Gammaproteobacterium* [23], *Streptomyces* [24,25], *Bacillus halodurans* [22], and metagenomic sources [26]. The heterologous expression of bacterial laccases may be more efficient than that of fungal laccases as there are no introns or post-translational modification (fungal laccases are glycosylated). Finally, a novel evolutionary lineage of two-domain laccases has been established [27]. These laccases are different from the well-known monomeric three-domain laccases that are typical for fungi and bacteria. The two-domain laccases, which have only been identified in prokaryotes, have a homotrimeric quaternary structure and form the active site on the interface of each two monomers. Three groups of two-domain laccases were distinguished on the basis of the organization of the copper-binding regions within the protein domains, and representative enzymes of type B and type C two-domain laccases have subsequently been characterized in bacteria, while sequence data suggested the presence of type A two-domain laccases in archaea [27]. For all these reasons, studying bacterial laccases is important from the perspectives of basic science as well as for the development of novel biotechnological applications.

The aim of this study was to use the extensive sequence data of the complete and draft bacterial genomes to evaluate bacterial laccases at the level of (1) the distribution of laccase-like genes within different bacterial phyla, (2) the diversity of the genes for bacterial laccases, and (3) the structural characteristics of the putative laccases. The bioinformatic search for new genes was based on profile Hidden Markov Models (pHMMs). This approach provided the theoretical ground for new hypotheses about the roles of laccases in bacteria and may guide the future research of these interesting and biotechnologically important enzymes.

Methods

Construction of profile Hidden Markov models (pHMMs)

The construction of pHMMs [28,29] was based on a two-step approach (Figure 1). In the first step, an initial pHMM was generated using the HMMER software package [29]. For this purpose, a set of sequences was collected by applying BLAST [30] searches using known protein sequences of described bacterial laccases (Table 1) as templates. The sequences of the obtained hits were aligned using MUSCLE [39]. The alignment was manually processed to remove sequences without the four copper binding domains and duplicates to avoid bias in the models. Phylogenetic analysis was applied to identify different types of target proteins. For phylogenetic tree reconstruction, the neighbor-joining method with Jukes-Cantor genetic distances was used in MEGA4 [40]. Finally, the initial pHMM was generated for each identified group of bacterial laccase-like proteins using the HMMER3 package.

In the second step, the pHMMs for the target sequences were retrained. Firstly, the initial pHMMs were applied to search for similar sequences in the pool of protein sequences from microbial genomes stored in the NCBI protein database (described in the following section). Then the sequences were aligned using MUSCLE. Lastly, final pHMMs were constructed for the five identified types of laccases based on the modified version of the alignment. The models cover a large portion of the proteins spanning all four copper-binding domains. The five pHMMs are available as supporting information (Figure S1, S2, S3, S4, S5).

The databases – genomes and metagenomes

For the pHMM searches, several public databases were used. For the generation and testing of the pHMMs, NCBI proteins and draft proteins were used. The NCBI protein database consisting of 3,819,638 proteins was obtained from a set of 1,216 complete microbial genomes available from the NCBI (genomes – *Prokaryota* database) in September 2010. The organisms belonged to *Bacteria* (89%), *Archaea* (7%) and viruses (4%) and represented 802 different genera. 418 organisms had 1 or more plasmids (a total of 937 plasmids were included in the database). The draft proteins from NCBI is a database of 3,602,197 proteins. The proteins were obtained from 995 draft microbial genomes (apart from one viral and six archaeal genomes, all were bacterial), belonging to 517 genera. It was not distinguished between chromosomes and plasmids as the contigs were not annotated to allow this distinction.

Four different metagenome datasets were used as databases for the pHMM-based search. The metagenome obtained from a biogas plant consisted of "biogas" data contained 1,963,716

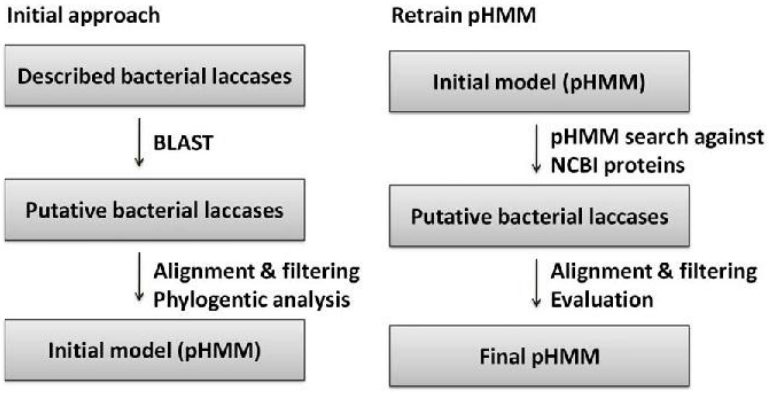

Figure 1. A two-step approach for the construction of laccase-specific profile Hidden Markov Models (pHMM). An initial set of known protein sequences was used to search for similar proteins that served for initial pHMMs building. These were refined with additional sequences and rebuilt from improved alignments. NCBI proteins – a database of all the proteins from the finished microbial genomes as described Methods.
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Table 1. List of bacterial enzymes for which laccase activity was demonstrated.

accession	source	type	reference
CAB45586	*Streptomyces coelicolor*	2-domain	[31]
BAB64332	*Streptomyces griseus*		[32]
3G5W	*Nitrosomonas europea*		[33]
2ZWN	*metagenomic library*		[34]
YP_077905	*Bacillus licheniformis* ATCC 14580	3-domain	[35]
NP_388511	*Bacillus subtilis* subsp. subtilis str. 168		[18]
NP_414665	*Escherichia coli* str. K-12 substr. MG1655		[36]
AY228142	*Bacillus halodurans*		[22]
YP_005339	*Thermus thermophilus* HB27		[21]
EF507879	*Streptomyces cyaneus* strain CECT3335		[37]
AB092576	*Streptomyces lavendulae*		[20]
AF184209	*Marinomonas mediterranea*		[38]

These sequences were used for BLAST search against the NCBI NR database to retrieve sets of sequences for initial building of profile Hidden Markov Models of bacterial laccases.
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nucleotide reads [41,42]. The sequences were obtained by sequencing on the GS FLX and Titanium platforms and assembled using the Newbler software resulting in 36,483 contigs, which were translated in six reading frames. The "termite metagenome" was a set of 82,789 proteins from the hindgut microbiome of the termite *Nasutitermes* sp. [43] obtained from the IMG/M database. Finally, the "cow rumen" consisted of 2,547,270 proteins from the cow rumen metagenome [44] obtained from the IMG/M database. The dataset of the Global Ocean Survey comprised of 12,672,518 sequences that were retrieved from the CAMERA [45] portal and translated in six reading frames.

Analyses of microbial genomes

pHMMs were applied to search for bacterial laccase-like sequences in 2,211 microbial genomes organized in two databases as described in the preceding section. Positive hits were aligned using ClustalW. The alignments were manually proof-read and filtered: the sequences without four copper-binding regions were removed. The taxonomic affiliation for the sequences in the final alignment was obtained using the NCBI taxonomy database. The web-based application Phobius [46] was used for the identification of transmembrane segments and signal peptides in the laccase sequences. Several custom-made scripts in Perl, Python and R programming languages were used for the processing and analyses of the data.

Analyses of metagenomes

The pHMMs were applied to the four metagenomic datasets described in section 2.2. The translated sequences were matched against each pHMM and those matching at least one copper-binding domain were aligned to the corresponding model using the HMMER software package. The alignments were manually processed and non-matching sequences deleted. Finally, the sequences were grouped according to their origin and taxonomically assigned using the lowest common ancestor approach: (1) Each aligned sequence read was compared against the NCBI genomes database using BLAST and an e-value cutoff of e-30. (2) Hits with a bit score above 90% of the best bit score were collected. (3) The lowest common ancestor was calculated for the taxonomies of the selected hits and assigned to the read. The reads for which the lowest common ancestor was identified using this 3-step approach, were aligned to the corresponding model using the HMMER package and manually verified.

Identification of putative horizontal gene transfer (HGT) events

A parametric method [47] was implemented for a rapid detection of putative HGT events. The algorithm consisted of three steps. (i) For each laccase sequence in the input file, the parent genome was downloaded and the genomic signature was calculated using a 5 kb sliding window with a step of 500 bp as described in [47]. (ii) The distance of each local signature from the average signature was calculated and plotted for a region of $\pm$200 kbp around the locus of the putative laccase sequence. (iii) The figures were then examined by eye to select those where the position of the laccase and a stretch of unusual genomic signature overlapped. These were the putative HGT events; they were additionally examined using BLAST for the presence of other HGT-indications (such as phage integrases or insertion sequences) and to list the genes that had putatively been transferred along with the laccase gene.

Results

Identification, diversity and distribution of bacterial laccase-like genes in the genome database entries

A thorough bioinformatics survey of draft and completed bacterial genomes was performed to extensively search for bacterial laccase-like genes. A two-step approach (Figure 1) using pHMMs instead of simple BLAST searches was chosen, allowing the identification of distantly related sequences. In case of two-domain enzymes, two groups were defined corresponding to type B and type C multicopper oxidases as proposed by Nakamura et al. [27]. One profile HMM was deduced for each of these two groups, and the completed and draft bacterial genomes were searched exhaustively by using these new pHMM models. Altogether 221 sequences were obtained (Table 2). More difficulties arose when addressing the diversity of three-domain laccases since many highly diverse sequences were retrieved by searching with the initial profile HMM. Finally, three models were built for the three-domain bacterial laccases. In total, 1019 sequences were retrieved for this laccase type (Table 2).

Signal peptides and transmembrane segments in the obtained laccase amino acid sequences were identified using Phobius [46]. Three quarters of the enzymes harbored putative signal peptides (Table 2), indicating that the majority of the bacterial laccases may be exported out of the cytoplasm which is in contrast to the current knowledge [16].

In total, 1240 genes for laccase-like enzymes have been found in 807 different microorganisms (36% of 2211 organisms included in the study). The sequences are available as supplementary information (Figure S6). In 252 organisms more than one laccase gene was identified (58 organisms encoded 3 genes, 18 encoded 4 genes, 16 had 5 genes and 7 harbored more than 5 laccase-like genes). The highest number of putative laccase genes was identified in *Xanthobacter autotrophicus* Py2, where three out of the 10 laccase genes were encoded on a plasmid and both two- and three-domain enzymes were present on the chromosome and the plasmid. Both *Sulfitobacter* sp. NAS-14.1 and *Sorangium cellulosum* So ce 56 had eight genes in their chromosomes, with one two-domain laccase in each genome while the others were three-domain enzymes.

Table 2. The number of sequences retrieved with the newly defined pHMMs when searching the databases of completed and draft genomes.

		No. of genes found		No. (%) of
type	**model name**	**total[a]**	**unique[b]**	**signal peptides**
two-domain	typeC2D	63	63	40 (63.5)
	typeB2D	158	158	127 (80.4)
three-domain	small3D	822	355	303 (85.4)
	big3D	308	159	118 (74.2)
	cot3D	200	38	26 (68.4)
	more than one model[c]	/	467	329 (70.4)
		sum	1240	943 (76.0)

[a]No. of genes retrieved with the model.
[b]No. of genes not retrieved with any other model.
[c]No. of genes retrieved with more that one model.
The pHMMs and the sequences are available as supplementary information (Figure S1, S2, S3, S4, S5, S6). Signal peptides were identified using the on-line version of Phobius [46].
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Several phyla are represented with very few sequences while in other groups many laccase genes were retrieved (Figure 2). For example, as many as 368 sequences in the final dataset were affiliated to *Gammaproteobacteria*. However, only 14 of these (<4%) were two-domain laccases, which were completely absent in the groups *Deltaproteobacteria* and *Epsilonproteobacteria*. Only few two-domain laccases were identified in *Actinobacteria*, which is surprising, since two-domain laccases had predominantly been discovered in *Streptomyces* [31,32]. While *Acidobacteria* and *Bacteroidetes* seemed to lack two-domain laccases, only two-domain laccases were found in *Planctomycetes* (admittedly only two sequences in ten sequenced genomes from this phylum). Moreover, 34 genes were identified in *Cyanobacteria*. Interestingly, all 8 two-domain laccases in *Cyanobacteria* belonged to type C (Figure 2). In finished genome projects, it was possible to obtain information on the location of the genes (whether on the chromosome or a plasmid). From the 749 genes identified in the finished genomes, 76 genes (52 for three-domain and 24 for two-domain laccases) were encoded on plasmids originating from 46 different organisms (Figure 3). One third of these (34%) were associated with various *Rhizobiales* species that usually had multiple genes for laccases in their genomes. *Enterobacteria*, on the other hand, typically contained just a single gene for a three-domain laccase, except for eleven enterobacterial strains that carried two genes for laccase-like enzymes. Here, the second gene was usually encoded on a plasmid (Figure 3), e.g. *Klebsiella pneumoniae* NTUH-K20 plasmid pK2044 (NC_006625.1, protein YP_001687946.1) and *Escherichia coli* APEC O1 plasmid pAPEC-O1-R (NC_009838.1, protein YP_001481473.1). In contrast, in some organisms (e.g. species of *Mycobacterium*, *Ralstonia* and *Leuconostoc*) laccase-like genes were identified only on plasmids (Figure 3).

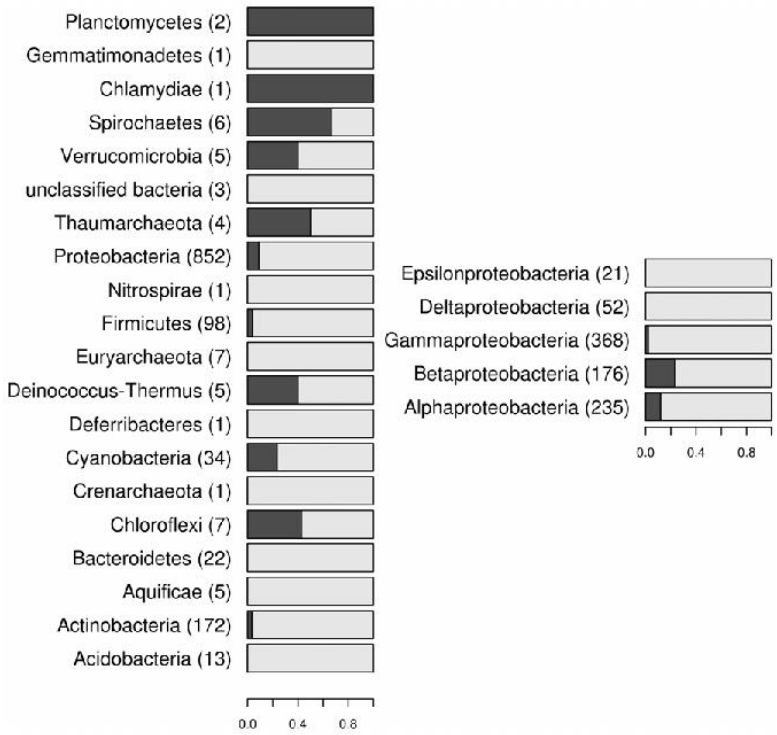

Figure 2. Proportions of two-domain (black) and three-domain (grey) laccases in different phyla (left) and classes of Proteobacteria (right). The numbers in brackets represent the total number of laccase genes found in each taxon.
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and three-domain laccase-like sequences, respectively, aligning neatly to the copper-binding regions of the models. However, only 33% of the putative three-domain laccases could be affiliated to *Bacteria* using the lowest common ancestor approach; the rest did not resemble any sequence in public databases, indicating considerable novelty. The majority (97%) of the classifiable sequences were assigned to *Proteobacteria* while the remaining were assigned to *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria* (Figure 4). At the genus level, most sequences were assigned to *Burkholderia* and *Shewanella*.

For the two-domain laccases, all four existing copper-binding regions were identified in some environmental gene tags (EGTs). The taxonomic affiliations of the sequences of the type B and type C two-domain laccases were analyzed separately (Figure 4). In total, only 53% of the type C sequences could be affiliated to *Archaea* (*Thaumarchaeota – Nitrosopumilus*), while no similar sequences could be found in the public databases for the remaining half of the dataset. For the type B two-domain laccase sequences, 87% were assigned to *Bacteria*. *Betaproteobacteria* were the dominant class (90% of all classifiable reads), mostly represented by the *Burkholderia*-associated sequences (84%) (Figure 4).

The origin of the identified laccase-like sequences was analyzed in more detail. Most of the three-domain laccase-like sequences (30%) were found in the Sargasso Sea at the station 11 where the sea temperature at the time of sampling was 20.5°C, the chlorophyll density was 0.17 µg/kg and the salinity was 36.7 ppt. 14% of the reads encoding three-domain laccase like enzymes were obtained from the sample from a Galapagos Islands sample, taken at Punta Cormorant. This location featured a high salinity of 63.4 ppt and a sea temperature of 37.6°C. The two-domain laccases were mainly obtained from the Sargasso Sea station 11 (52%) and from Sargasso Sea station 3 (7%).

Horizontal gene transfer of laccase-like sequences identified in microbial genomes

Identification of potentially horizontally transferred laccase genes was based on tetraoligonucleotide frequencies or genomic signatures [47]. Local genomic signatures covering laccase genes were compared to average signatures of respective genomes. If the local signatures were significantly different, the corresponding genes were considered to have been acquired *via* horizontal gene transfer (HGT) and the fragment was further examined for the presence of other indicators of HGT, such as remainders of insertion sequences, transposons or phages. Possible other open reading frames on the fragment were annotated to elucidate the genetic potential of the fragment for the organism. More than 40 examples representing putative HGT events were found (not all examined in further detail), and four examples are described below.

In *Nitrosococcus watsoni* C-113, a short fragment of less than 5 kbp was identified as having been horizontally transferred, carrying a putative two-domain laccase (YP_003760803) and an outer membrane efflux protein (YP_003760804) gene. There were also the remnants of genes similar to a resolvase and a transposase, again indicating past horizontal gene transfer of the fragment.

Of the 34 *Yersinia* strains included in the study, more than one gene for a laccase-like enzyme was found only in the draft genome of *Yersinia mollaretii* ATCC 43969. At the beginning of the putatively horizontally transferred fragment, remnants of a phage integrase gene were detected. In addition, several putative open reading frames could be predicted but most of them were small and did not resemble any known sequences in the database. However, four open reading frames other than the ORF for the putative three-domain laccase are homologous to database entries. These encode a protein with a beta-lactamase domain, an ABC transporter, and two cytochrome c family proteins.

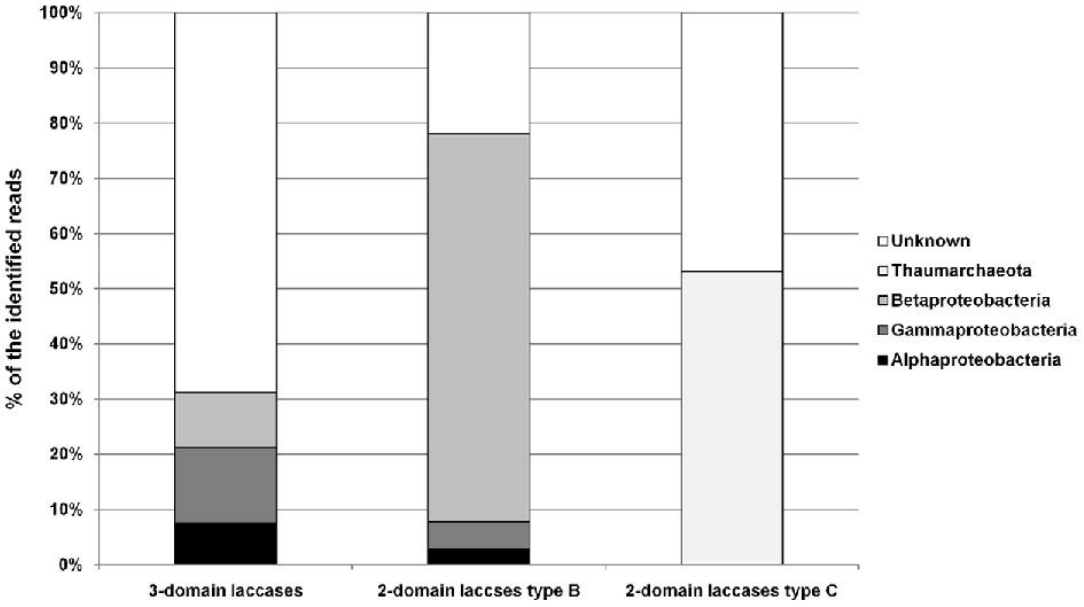

Figure 4. Taxonomic affiliation of sequences encoding laccases-like proteins in the Global Ocean Survey data. Proportions are shown for the total of 847, 245 and 32 sequences aligning well to the pHMMs of the three-domain laccases, type B two-domain laccases and type C two-domain laccases, respectively.
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Out of 40 genomes from the genus *Haemophilus* included in our study, only one strain contained two laccase-like genes. These two genes were identified on a common genomic island that has a markedly different signature than the rest of the genome. Close to the two laccase genes was a gene encoding a heavy metal translocating ATPase and a longer stretch (2500bp) of DNA with a homology to plasmids and transposons of various organisms, harboring genes for tetracycline resistance. The end of the fragment showed a similarity to putative integrase genes as revealed by BLASTn.

In *Geobacter sulfurreducens*, the putative horizontally transferred fragment encoded a ferrous ion transport protein, a set of CRISPR-associated genes (known for their mobility through HGT) and a laccase gene.

Discussion

The aim of this study was to identify laccase-like genes in published bacterial genomes and metagenome datasets. Five new profile Hidden Markov Models (pHMM) were developed for two-domain and three-domain laccases. Such probabilistic models of protein families are commonly used in the analysis of high-throughput sequencing data [48]. The main advantage of a pHMM-based approach is the high accuracy in detecting conserved domains compared to other methods such as BLAST.

Specific pHMMs were developed for type B and type C two-domain laccases that were previously identified [27]. These models are particularly important since two-domain multicopper oxidases could not be efficiently discovered with the existing models for fungal laccases. Several genes for the type A two-domain laccases have also been identified with the initial BLAST-based searches. However, these originated solely from *Archaea* such as *Halogeometricum borinquense* DSM 11551 (3 genes), *Haloterrigena turkmenica* DSM 5511 (2 genes), *Haloarcula marismortui* ATCC 43049 (2 genes), *Halobacterium sp.* NRC-1 (this gene was previously identified by [27]), and were thus excluded from further analysis.

The diversity of three-domain bacterial laccases, which are similar to those in fungi, was unexpectedly high; three models were constructed to capture most of the variability in amino acid sequences and lengths of the predicted proteins. Based on the sizes of individual domains and whole proteins, two major groups were identified: a larger group of enzymes (81% of the three-domain laccases) with the well-known representatives such as CotA from *Bacillus subtilis* (identified with the pHMM named small3D), and a smaller group (16% of the three-domain laccases) including considerably larger proteins that to our knowledge has no characterized representatives (these were retrieved with the pHMM named big3D). Bacteria of the genera *Pseudomonas*, *Geobacter*, *Xanthobacter* and *Acinetobacter* were found to possess laccases belonging to this second group. However, the diversity within these two groups, e.g. the diversity in the copper-binding regions, was also notable. Laccases from smaller taxa of closely related bacteria with few copies in each genome (e.g. *Enterobacteria*) clustered together in phylogenetic trees (data not shown) while the clustering of laccases from some phyla was not consistent with the 16S rRNA phylogeny. Notably, laccases from *Firmicutes* (low-GC) and *Actinobacteria* (high-GC) formed several mixed clusters that often included laccases from *Proteobacteria* (data not shown).

The high level of diversity within groups of related bacteria suggested two mutually non-excluding explanations. Firstly, it is evident from our data that there are several groups of bacterial laccases in terms of protein structure and, we speculate, also in terms of physiological function (substrate utilization, pigment formation, stress resistance and others yet to be discovered). Further members of these subgroups of bacterial laccases may be identified in due time on the basis of studies such as this one. Secondly, horizontal gene transfer may offer some explanations as to why laccases from the same organism can be so diverse. The present study provides some evidence that certain laccase genes were probably acquired *via* horizontal gene transfer, either alone or together with other important genes such as antibiotic resistance genes.

It is important to note that the microbial genome databases are extremely biased towards certain organisms. Although measures have been undertaken to help relieve this issue and many genomes of organisms from scarcely represented phyla are being added [49], several major bacterial groups are still represented with very few sequenced genomes. This bias is reflected in our data as several phyla were represented with less than 10 sequences (Figure 2) while some groups were large (for example, as many as 368 sequences in the final dataset were affiliated to *Gammaproteobacteria* due to the pronounced bias towards *Enterobacteria*). This fact makes it difficult to infer general conclusions about the presence or absence of genes for certain types of laccases in a particular group of organisms. Moreover, finding genes encoding laccase-like enzymes does not necessarily mean that the organism has laccase activity at its disposal.

Still, the results presented here indicate considerable diversity of laccases in bacteria and question some of the current views of bacterial laccases. Most notably, based on the presence of signal peptides around 76% of the putative proteins identified in this study appear to be secreted from the cytoplasm. Moreover, the genes for laccase-like enzymes were found in anaerobic organisms. Corresponding enzymes almost invariably had signal peptides indicating that they may be active in a more aerobic environment away from the cells. This is a possible scenario for some soil bacteria (e.g. *Geobacter*, *Clostridium*) but quite unlikely for microbes living in the anaerobic digestive systems of herbivores. This is probably the reason why very few laccases were found in metagenomes derived from these habitats – organisms living in digestive tracts probably use other enzymes for the breakdown of plant (poly)phenolics, such as diverse peroxidases. There is evidence that some bacterial laccases are indeed involved in lignin degradation [19], while others may carry out functions such as pigment formation, as shown for the CotA laccase from *Bacillus subtilis* [18]. Moreover, many autotrophs have laccases, for example *Cyanobacteria* (34 genes in 23 organisms) and nitrifying bacteria (28 genes in 9 organisms of the genera *Nitrosococcus*, *Nitrosomonas and Nitrobacter*).

As reviewed in the introduction, bacterial laccases may also be interesting for biotechnological applications. However, there have only been a few attempts to verify this in practice. Notably, the CotA laccase was able to decolorize a variety of structurally different synthetic dyes at alkaline pH and in the absence of redox mediators [50]. Azo-dyes have been degraded with an unusual two-domain laccase from *Streptomyces* that is active in a dimeric form and exhibits high thermo- and pH-stability [51]. It has been shown that xenobiotics increase the activity of the laccase from a *Gammaproteobacterium*, which may indicate the protective role of laccases against mutagens, xenobiotics and agrochemicals [52]. There is a growing body of evidence that *Bacteria* can degrade lignin [19] and that laccases are important in this process, either acting alone or together with other enzymes such as extracellular peroxidases [4]. Bacterial laccases certainly will have to be taken into account in future enzyme cocktails for lignin degradation or diverse environmental applications.

The present study can help to identify potential novel sources of laccases. These are the organisms with multiple genes for laccases;

for example, *Rhodococcus erythropolis* PR4 had one gene on a plasmid and six other genes encoded on the chromosome. Bacteria of the genus *Rhodococcus* were shown to degrade different types of lignin in the absence of hydrogen peroxide, indicating that laccases may be involved in the process [19]. *Rhodococci* are known to be potent degraders of polychlorinated biphenyls (PCBs) and since biphenyls occur naturally due to lignin degradation, it has been speculated that the enzymes responsible for PCB degradation had originally been involved in lignin degradation [19]. In this study, 26 genes for laccase-like enzymes could be identified in only five different species of the genus *Rhodococcus*.

Salt- and pH-tolerant laccases are desired for industrial applications and one laccase exhibiting such properties has been described in *Bacillus halodurans* [22]. A novel laccase, found using a metagenomic approach, was extremely halotolerant (up to 1M NaCl) and pH-stable and could degrade several synthetic dyes, some of them even in the absence of mediators [53]. Many alkaliphilic bacteria are currently being sequenced by the Joint Genome Institute (e. g. *Heliothrix oregonensis*, *Thioalkalivibrio thiocyanoxidans*, *Thioalkalimicrobium cyclicum*) and it will be interesting to search their genomes for laccase genes once they become available. However, some genes for laccase-like enzymes have been discovered in the alkaliphiles available at the time of this study, for example in *Oceanobacillus iheyensis* (*Firmicutes*) and *Thioalkalivibrio sp.* (three genes in two strains of these *Gammaproteobacteria*). These organisms may also be important sources of novel enzymes with desired properties.

Recently, the Laccase Engineering Database (LccED) was launched with an ambition to collect and manage molecular data regarding laccases and related multicopper oxidases from all domains of life [54]. Over 2200 proteins were collected in the LccED, and laccases from fungi and plants predominated. Their dataset of bacterial laccases overlapped with ours to a large extent (up to 70% of the sequences). Their collection was richer for the environmental sequences but also contained sequences which appeared not to be laccases as they contained no copper-binding domains (e.g. CAA78165.1). Conversely, our search retrieved several hundred new sequences. One of the distinctive findings of our study was to identify 220 genes for the two-domain laccase enzymes, while the LccED database listed less than 20. LccED is certainly a valuable resource that may be further enriched with sequences from studies such as the present one. By facilitating the access to taxonomic information and by enabling batch assignments to the proposed protein families, the LccED could enable the researchers to elegantly investigate topics similar to the ones addressed here.

Conclusions

In the present study, an enormous amount of sequence data was made accessible to study an increasingly important group of enzymes. Both two- and three-domain laccases were retrieved. The results supported our hypothesis that the genes for laccases were widely distributed among virtually all bacterial phyla. We showed that the localization of bacterial laccases may not be restricted to the cytoplasm and that they may be rather mobile. Moreover, these genes abounded in anaerobic organisms and autotrophs, and we pointed to some interesting organisms that could be exploited for their laccases. Admittedly, the majority of the putative enzymes discussed in this paper still need to be experimentally verified. However, elucidation of the wide distribution and enormous diversity of bacterial genes for laccase-like enzymes will undoubtedly increase scientific interest in this emerging field.

Supporting Information

Figure S1 Profile Hidden Markov Model for type C two-domain laccases. The pHMM file "typeC2D.hmm" was generated with HMMER software (28).
(HMM)

Figure S2 Profile Hidden Markov Model for type B two-domain laccases. The pHMM file "typeB2D.hmm" was generated with HMMER software (28).
(HMM)

Figure S3 Profile Hidden Markov Model for three-domain laccases. The pHMM file "small3D.hmm" was generated with HMMER software (28).
(HMM)

Figure S4 Profile Hidden Markov Model for three-domain laccases. The pHMM file "big3D.hmm" was generated with HMMER software (28).
(HMM)

Figure S5 Profile Hidden Markov Model for three-domain laccases. The pHMM file "cot3D.hmm" was generated with HMMER software (28).
(HMM)

Figure S6 Amino acid sequences of 1240 putative laccases identified in the present study. The sequences are in fasta format, each sequence has the accession number and the name of the source organism in the description.
(FAS)

Author Contributions

Conceived and designed the experiments: LA MZ AG AS IMM. Performed the experiments: LA MZ. Analyzed the data: LA MZ AS IMM. Contributed reagents/materials/analysis tools: AG AS IMM. Wrote the paper: LA MZ AS IMM.

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Nova pH-tolerantna lakaza iz *Thioalkalivibrio* sp.

Avtorji: Luka Ausec, Miha Črnigoj, Marko Šnajder, Nataša Poklar Ulrih, Ines Mandić-Mulec.
Opomba: delo je trenutno v postopku objave, zato tu navajamo ključne opise namena dela, metode in rezultate.

2.2.2.1 Uvod

Lakaze so raznovrstne oksidoreduktaze, ki se že uporabljajo v različnih biotehnoloških in industrijskih procesih. Trenutno komercialno uporabljamo le lakaze iz gliv, vendar raziskave zadnjih let kažejo, da imajo bakterijske lakaze pomembe lastnosti, s katerimi lahko kompenzirajo njihovo trenutno težavno in drago pridobivanje ter relativno nizek redoks potencial (v primerjavi z glivnimi lakazami). Bakterijske encime je na splošno enostavno pridobivati zaradi dobro razvitih metod biotehnologije, poleg tega pa so sedaj znane bakterijske lakaze precej trpežne v pogojih, ki so pomembni za industrijske procese, kot je visoka temperatura (Martins in sod., 2002; Miyazaki, 2005) in bazični pH (Reiss in sod., 2011; Singh in sod., 2007; Ye in sod., 2010; Zheng in sod., 2011). Zato ves čas iščemo nove bakterijske lakaze.

Da bi našli res nekaj pomembno novega in zanimivega za dejansko uporabo, moramo pogledati v organizme, ki niso neposredno sorodni že znanim producentom lakaz. Ker metagenomika trenutno daje relativno skromne rezultate glede na kompleksnost in ceno potrebnih metod, je logična pot bioinformatska raziskava sevov, ki so jim že določili zaporedje genoma. To so sevi, ki so lahko povsem nesorodni znanim producentom lakaz, pa vendar vsaj do neke mere poznamo njihov način življenja in tako lahko sklepamo na lastnosti njihovih encimov. Ideja te raziskave je preprosta: na podlagi obstoječega znanja iz *in silico* raziskav izbrati ekstremofilni sev, ki kodira lakazo s predvidenim signalnim peptidom (ki torej encim izloča v periplazmo ali celo v celično okolje), proizvesti tako lakazo v laboratoriju in preveriti, če ima tak encim predvidene lastnosti (denimo želeni pH in temperaturni optimum za delovanje), podobne tistim v izvornem organizmu.

Raziskava je tako neposredno nadaljevanje bioinformatske študije (Ausec in sod., 2011b) in z njo dejansko izrabljamo odkriti nabor potencialnih genov. Ker le redke glivne lakaze ostanejo aktivne pri nevtralnih ali celo bazičnih pH vrednostih, smo v pričujoči študiji iskali alkalifilno lakazo. Za vir nove lakaze, ki bi delovala v bazičnih pH vrednostih, smo izbrali bakterijo iz rodu *Thioalkalivibrio*. Vemo, da ti organizmi lahko živijo v zelo slanih (do 4M Na^+/K^+) in bazičnih okoljih s pH okoli 10 (Muyzer in sod., 2011). V genomu bakterije *Thioalkalivibrio* sp. K90mix smo predhodno našli dva gena za lakazam podobna encima, oba z predvidenim signalnim peptidom. V pričujoči raziskavi smo uporabili sev *Thioalkalivibrio* sp. ALRh, ki je ozko soroden sevu K90mix in ki je bil dostopen v javnih zbirkah sevov. Zanimalo nas je, če bo ta lakaza odporna na visoke koncentracije soli in pH vrednosti. Za potrebe osnovne biokemijske karakterizacije nove lakaze smo jo izrazili v heterolognem gostitelju *Escherichia coli* BL21.

2.2.2.2 Metode

Sev *Thioalkalivibrio* sp. ALRh smo pridobili iz nemške zbirke sevov DSMZ (DSM13533). Gojili smo ga v tekočem gojišču DSMZ št. 925 za alkalifilne sulfatne respiratorje. To je minimalno mineralno gojišče, ki ga pretežno sestavljajo Na_2CO_3 (20 g/l), $NaHCO_3$ (10 g/l), NaCl (5 g/l) in K_2HPO_4 (1 g/l). Po avtoklaviranju dodamo na liter gojišča po 1 ml raztopine magnezijevega klorida (založno raztopino pripravimo z 200 g/l $MgCl_2{\times}6H_2O$) ter 2 ml/l raztopine elementov v sledeh, ki jo predhodno pripravimo in ločeno steriliziramo. Ta raztopina je sestavljena iz EDTA (5 mg/l), $FeSO_4{\times}7H_2O$ (2 mg/l), $ZnSO_4{\times}7H_2O$ (100 mg/l), $MnCl_2{\times}4H_2O$ (30 mg/l), $CoCl_2{\times}6H2O$ (200 mg/l), $NiCl_2{\times}6H_2O$ (20 mg/l), $Na_2MoO_4{\times}2H_2O$ (30 mg/l), $CuCl_2{\times}2H_2O$ (10 mg/l), H_3BO_3 (300 mg/l). Za sev ALRh je potrebno dodati še natrijev tiosulfat (do končne koncentracije 40 mM) in KSCN (do končne koncentracije 5 mM). Tako pripravljeno gojišče ima pH 10. Sev smo gojili tri tedne pri 28 ºC. Čeprav je v tem času zrasla komaj vidna količina nepigmentiranih celic, smo s komercialnim kompletom PowerSoil DNA Isolation Kit (MoBio, CA, USA) lahko izolirali zadostne količine DNA za pomnoževanje z verižno reakcijo s polimerazo (PCR).

Kloniranje smo osnovali na genu za protein YP002361151 iz sorodnega seva *Thioalkalivibrio* sp. K90mix, saj genomsko zaporedje seva ALRh v času raziskave ni bilo znano. Pripravili smo začetne oligonukleotide za pomnoževanje želenega gena ter vključili potrebna restrikcijska mesta za kasnejši prenos gena v ekspresijski plazmid. Začetna oligonukleotida Thio5Ffull (5'-atacatatgaaacgacgtcaatttctggggctggcc-3') in Thio5R (5'-

aaactcgagggcacgcacgcggacgttcaccatc-3') uvajata restrikcijski mesti za restriktazi NdeI in XhoI (prepoznavni zaporedji sta podčrtani). Gen smo pomnožili v 50-mikrolitrski reakciji, ki je vsebovala 3 µl vsakega začetnega oligonukleotida (10 µM), 1 µl dNTP (10 mM), 1 µl BSA (10 mg/ml), 2 µl $MgCl_2$ (25 mM), približno 50 ng matrične DNA, 5 µl 5x PCR pufra in 2 enoti Taq DNA polimeraze (Promega). Uporabili smo protokol z začetno denaturacijo pri 3 min pri 94 ºC, 30 ciklov po 30 s na 94 ºC, 30 s na 63 ºC, 60 s na 72 ºC, ter končnim pomnoževanjem 5 min pri 72 ºC. Pomnožek dolg približno 1600 bp smo izrezali iz 1 % agaroznega gela in ga očistilli s komercialnim kompletom PureLink Quick Gel Extraction Kit (Invitrogen, Life Technologies). Gen smo vstavili v ekspresijski plazmid pET-21c tako, da smo na C-terminalni konec proteina dodali 6xHis označbo (6x aminokislina histidin). Celotno zaporedje tako pripravljenega konstrukta smo objavili v javni podatkovni bazi GenBank (številka za dostop: HF913729.1).

Heterologni protein smo izrazili v bakteriji *Escherichia coli* BL21(DE3) pLysE. *E. coli* smo gojili pri 37 ºC v gojišču LB z ampicilinom (10 µg/ml), kloramfenikolom (25 µg/ml) in bakrovimi ioni ($CuSO_4$ v končni koncentraciji 0,25 mM). Izražanje gena smo začeli pri optični gostoti celic OD_{650}=0,60 in nato celice celice najprej 4 ure gojili pri sobni tempraturi s stresanjem pri 200 rpm, nato pa še brez stresanja dodatnih 16 ur. Tak način inkubacije priporočajo nekateri avtorji (Durão in sod., 2008), saj se pri nižji temperaturi in v mirujočem gojišču pojavijo pogoji zmanjšane dostopnosti kisika (mikroaerofilni pogoji), kar povzroči, da celice *E. coli* privzemajo več bakra iz gojišča in se ga zato lahko več vgradi v aktivne centre nastajajočih encimov, zato je delež aktivnih lakaz večji. Heterologni encim, označen s 6x His označbo, smo nato izolirali z afinitetno kromatografijo na osnovi niklja (Ni-NTA). Izolirali smo po navodilih proizvajalca (Qiagen, Hilden, Nemčija, protokoli v QIAexpressionist protocols - Qiagen, 2003) z eno pomembno spremembo: ker smo v preliminarnih raziskavah ugotovili, da je prisotnost kloridnih ionov zniža aktivnost ThioLacc, smo vse pufre za izolacijo pripravili z natrijevim sulfatom namesto z natrijevim kloridom.

Najaktivnejše encimske frakcije smo združili in jih 15 min inkubirali pri 50 ºC ob prisotonosti 0,5 mM $CuSO_4$. Po taki *in vitro* rekonstituciji ektivnega mesta se je encimska raztopina obarvala močneje modro - barva je sicer ostala tudi po zamenjavi pufra, vendar se je aktivnost komaj zaznavno povečala (<10 %). Elucijski pufer (50 mM NaH_2PO, 300 mM NaCl, 250 mM imidazol, pH 8,0) in odvečni baker smo zamenjali z doionizirano vodo s pomočjo koncentratorja Amicon Ultracel - 30k (Merck, Millipore). Tako pripravljen encim (ThioLacc) smo uporabili pri vseh nadaljnih eksperimentih.

S poliakrilamidno gelsko elektroforezo (SDS-PAGE) smo ocenjevali čistost encimskih frakcij med procesom čiščenja. Raztopine smo segreli na 100 ºC za 10 min ob prisotnosti reducenta ditiotreitola (DTT) ter beljakovine ločili na 10 % poliakrialmidnem gelu. Za velikostno lestvico smo uporabili Page Ruler Prestained Protein Ladder (Fermentas), barvali pa smo z barvilom Page Blue Protein Staining Solution (Fermentas). Naredili smo tudi cimogram neposredno v SDS-PAGE gelu. V tem primeru vzorcev nismo kuhali, po končani elektroforezi pa smo gel izdatno spirali v destilirani vodi, da smo odstranili detergent, nato pa barvali s 100 mM raztopino ABTS (približno 0,2 ml), da bi zaznali morebitno aktivno frakcijo v gelu po nastanku zelenih lis.

Celokupno koncentracijo lakaze ThioLacc smo določili s spoektrofotometrom Nanodrop ND-1000 (ThermoScientific, Wilmington, DE, USA), uporabili smo molekularno maso 59,9 kDa in ε vrednost 67380 $M^{-1}cm^{-1}$ (to smo določili z orodjem ProtParam na spletnem mestu Expasy).

Lakazno aktivnost smo merili pri sobni temperaturi v 100 µl reakcijah. Standardna 10-µl reakcija je vsebovala 80 µl 150 mM fosfatno-citratnega pufra pH 2,5 – 8,0 ali 100 mM Tris pufra pH 7,2 – 9,5, 10 µl posameznega substrata v vodni raztopini (končna koncentracija 10 mM, substrati so opisani v nadaljevanju) in 10 µl encima v vodni raztopini. Oksidacijo smo sledili s spektrofotometrom za mikrotitrske plošče (Thermo, Ventaa, Finska) kot spremembo absorbance pri določeni valovni dolžini (glede na substrat), absorbanco smo odčitali vsaj vsako minuto prvih petnajst minut po začetku reakcije. Merili smo tudi negativne kontrole brez dodanega encima, da smo lahko odšteli morebitno spreminjanje substrata ob reakcijskih pogojih zaradi neencimskih procesov. Hitrost oksidacije smo nato izračunali iz naklona regresijske premice za prvih nekaj meritev, pri katerih se je absorbanca linearno spreminjala s časom trajanja reakcije.

Preizkusili smo aktivnost encima pri različnih pH vrednostih reakcijske mešanice. Meritve smo izvedli v treh ponovitvah za dva substrata: 2,2'-azino-bis(3-etilbenzotiazoline-6-sufonska kislina) (ABTS) in 2,6-dimetoksifenol (DMP), uporabili pa smo fosfatno-citrati (McIlvaine) puffer za pH vrednosti med 2,5 in 8,0 ter TRIS pufer za pH med 7,2 in 9,5. Reakcije smo sledili pri 420 nm (ABTS) in 468 nm (DMP).

Kinetiko smo izmerili za oba substrata v koncentracijah od 0,01 do 40 mM pri pH 5,0 (ABTS) in 8,0 (DMP). Kinetiko smo izmerili tudi v supernatantu celičnega lizata pred čiščenjem encima, da bi ocenili izkoristek čiščenja. Predpostavili smo Michaelis-Mentenovo kinetiko, modeliranje krivulje smo izvedli s statističnim paketom R.

Za preverjanje stabilnosti encima smo uporabili fluorescečno emisijsko spektrometrijo. Pri tej metodi vzbujamo aminokislino triptofan (in v manjši meri druge aromatske aminokisline) v proteinu in merimo spekter emisije, ki je odvisen od polarnosti okolja triptofana. Če protein denaturiramo in tako triptofan iz nepolarnega jedra proteina izpostavimo vodnemu okolju, bomo konformacijsko spremembo lahko zaznali kot "rdeči premik" emisijskega spektra (fluorescenca z višjo valovno dolžino). Metodo smo izvedli v sodelovanju s Katedro za biokemijo in kemijo živil Biotehniške fakultete UL, merili smo s spektrofluorimetrom Cary Eclipse (Varian) s termostatiranim držalom za kivete. Raztopino ThioLacc smo vzbujali z 280 nm in merili emisije v razponu od 300 do 380 nm. S kontrolnimi reakcijami brez proteina smo zajeli učinek topila in napako fotopomnoževalke. Spekter izsevane svetlobe smo merili v odvisnosti od:

1. pH - razpon pH od 0,5 do 12,5 s korakom po 0,2 enote pri temperaturi 20 ºC; različen pH raztopine ThioLacc smo dosegli s titracijo s HCl in NaOH, pH raztopine smo z mikroelektrodo merili v ločeni kiveti (napaka pH je bila 0,01 enote);
2. temperature – razpon od 20 do 95 ºC s korakom 2 ºC, pri pH 2, 5, 8, 10, in 12.

Valovne dolžine maksimalne intenzitete fluorescence smo prikazali v odvisnosti od pH oziroma temperature. Pri prileganju krivulje smo uporabili Savitzki-Golayev algoritem glajenja v programu OriginPro 8.1 (OriginLab Corporation, ZDA). Temperaturo denaturacije in van't Hoffovovo entalpijo denaturacije ThioLacc smo določili tako, kot je opisano v Poklar in Vesnaver (2000).

Denaturacijo lakaze smo smo spremljali tudi kalorimetrično (ang. differential scanning calorimetry) z NANO-DSC III (TA Instruments, ZDA). Liofiliziran vzorec lakaze smo raztopili v 10 mM HEPES pufru pH 8,0 v koncentraciji 0,33 mg/ml. Vzorec in kontrolo (pufer) smo odzračili v vakumu. Vzorce smo v treh korakih segrevali, hladili in zopet segrevali v temperaturnem razponu med 10 in 100 ºC s hitrostjo spreminjanja temperature 1 ºC/min. Prvi korak (segrevanje) smo uporabili za določitev temperature denaturacije in kalorimetrično entalpijo ThioLacc. Z nadaljnjima dvema koraka smo določili reverzibilnost denaturacije.

Obstojnost lakaze ThioLacc pri različnih temperaturah smo vrednotili tudi preko merjenja encimske aktivnosti na dva načina:

- začetno hitrost oksidacije encima smo merili v standardnih reakcijah z ABTS v treh ponovitvah pri temperaturah od 40 do 90 ºC,
- rezidualno aktivnost encima smo izmerili po dolgotrajni (do 70 min) inkubaciji

encima na temperaturah od 40 do 70 ºC. Alikvote encima smo jemali v želenih časovnih intervalih ter nato izmerili oksidacijo ABTS v standardnih reakcijah pri sobni temperaturi.

Da bi ocenili, kakšne substrate lahko oksidira lakaza ThioLacc, smo merili aktivnost oksidacije še ene nefenolne in devetih fenolnih spojin pri pH vrednostih 5, 6, 7 in 8. Uporabili smo naslednje spojine (valovne dolžine, pri katerih smo spremljali morebitne spremembe izvornih snovi, so navedene v oklepaju): $K_4[Fe(CN)_6]$ (405 nm), pirokatehol (410 nm), pirogalol (450 nm), siringaldehid (365 nm), siringična kislina (360 nm), siringaldazin (525 nm), gvajakol (468 nm), vanilična kislina (350 nm), ferulična kislina (320 nm) and tirozin (475 nm). Inhibitorni učinek soli smo merili v standardnih reakcijah z ABTS pri pH 5,0, uporabili pa smo tri različnih soli (NaCl, Na_2SO_4, and K_2SO_4) pri koncentracijah od 0,5 do 100 mM.

2.2.2.3 Rezultati z razpravo

Na začetku raziskave genom *Thioalkalivibrio* sp. ALRh ni bil poznan, zato smo začetne oligonukleotide načrtovali na podlagi gena iz sorodnega seva *Thioalkalivibrio* sp. K90mix. Z njimi smo brez težav pomnožili celoten gen iz našega delovnega seva. To pomeni, da seznam 1240 potencialnih genov za lakaze, najdenih v naši bioinformatski študiji (Ausec in sod., 2011), omogoča kloniranje še dosti širšega nabora genov in posredno dostop do velike naravne raznolikosti, ki je že zajeta v sekvenciranih genomih bakterij. V našem primeru se je pomnoženi gen precej razlikoval od znanega, in sicer 9,9 % na nivoju zapisa DNA, kar se prevede v 7,8 % razliko na nivoju aminokislin (41 od 527 aminokislin je bilo drugačnih, Slika 3). Aminokisline, ki vežejo baker, so bile popolnoma ohranjene, čeprav smo našli nekaj tihih mutacij na nivoju DNA v teh regijah. Zato smo zaključili, da gre za nov protein, ki je drugačen od YP_003461151 iz *Thioalkalivibrio* sp. K90mix. Celotni genski konstrukt smo shranili v javno bazo podatkov GenBank (HF913729.1). Slika 3 prikazuje primerjavo aminokislinskih zaporedij ThioLacc, proteina iz *Thioalkalivibrio* sp. K90mix ter dvema najbolje poznanima predstavnikoma bakterijskih lakaz: CueO in CotA.

V drugi polovici leta 2013 so se v bazi GenBank pojavili osnutki genomov 24 predstavnikov bakterij iz rodu *Thioalkalivibrio*, med njimi je bil tudi sev ALRh. Primerjali smo homologe ThioLacc in ugotovili, da so na nivoju aminokislin >90 % identični. Zaporedje iz seva ALRh in dejansko izraženi gen za ThioLacc se razlikujeta v enem baznem paru, ki povzroči točkasto mutacijo N424D. Ostale sekvence se najbolj razlikujejo

v variabilni regiji, bogati z glicinom in metioninom, ki je pri ThioLacc med 325. in 355. aminokislino in je na Sliki 3 označena s črtkastim okvirom.

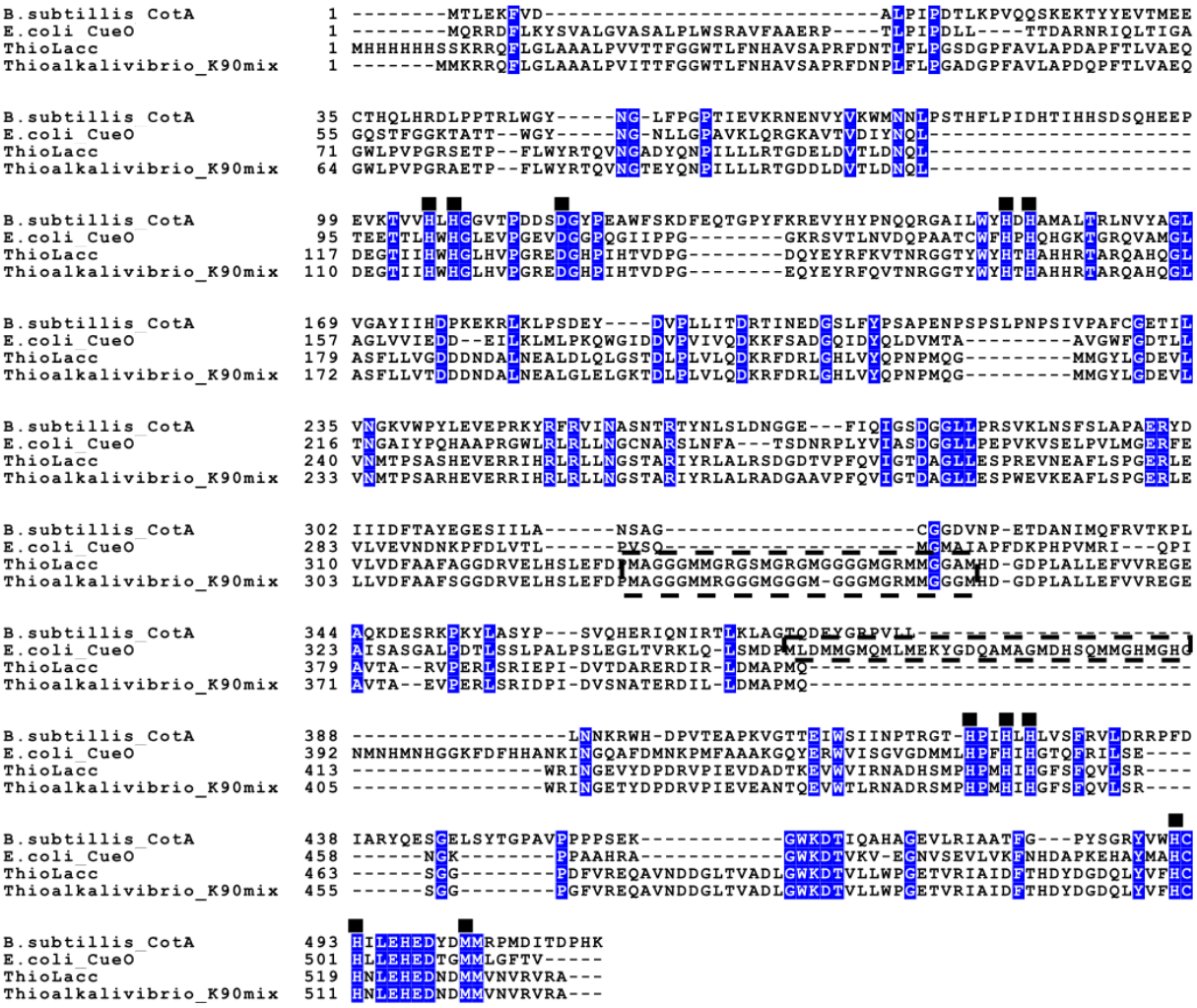
Podatkovna baza LccED (The Laccase Engeneering Database) (Sirim in sod., 2011) razvršča lakaze in lakazam podobne skupine na podlagi skritih modelov Markova (pHMM). ThioLacc uvršča v skupino encimov podobnih CueO iz *E. coli*, in sicer v podskupino z le 16 drugimi zaporedji, ki izvirajo iz bakterij rodov *Aquifex*, *Hidogenivirga*, *Pyrobaculum* in *Desulfovibrio*. Le oxsidazo McoP iz arheje *Pyrobaculum aerophilum* so doslej preučuli na nivoju encima. Pokazali so, da lahko poleg molekularnega kisika kot končni sprejemnik elektronov uporablja tudi dušikov oksid, zato bi bil lahko ta encim in vivo pomemben v denitrifikaciji (Fernandes in sod., 2010). Kljub temu, da McoP oksidira nekatere tipične lakazne substrate, *in vivo* verjetno deluje kot metalo-oxsidaza. To nakazuje, da tudi ThioLacc ni običajna lakaza in da v organizmu najverjetneje ne opravlja funkcije izrabe fenolnega substrata.

S spletnim servisom I-TASSER (Zhang, 2008) smo predvideli trodimenzionalno strukturo ThioLacc. Med najbolj podobnimi proteini sta bila CueO iz *E. coli* in McoP iz *Pyrobaculum aerophilum*. Oba proteina lahko oksidirata baker (Grass in Rensing, 2001; Fernandes in sod., 2010), kar bi lahko bila tudi potencialna *in vivo* fiziološka funkcija ThioLacc. Pri CueO so na podobnem mestu kot v ThioLacc opazili z metionini bogato regijo, ki je ovirala dostop organiskih molekul do aktivnega mesta v encimu – ko so to regijo odstranili, se je afiniteta do bakra (I) zmanjšala, do fenolnih spojin pa povečala (Kataoka in sod., 2007). Prisotnost take zanke pri ThioLacc bi lahko dokaj nizko izmerjeno aktivnost za organske spojine v primerjavi z lakazami iz predstavnikov rodu *Bacillus*, ki te strukturne posebnosti nimajo.

Čiščenje encima smo si olajšali tako, da smo protein na C-terminalnem koncu označili s 6xHis značko (šest aminokislin histidin). Tako smo protein lahko osamili iz lizata celic *E. coli* s pomočjo afinitetne kromatografije Ni-NTA. Encimska frakcija je bila rahlo modre barve, kot je to značilno za celo družino oksidoreduktaz z bakrom v aktivnem centru. Iz 600 ml tekoče kulture *E. coli* smo pridobili približno 1 mg encima oziroma 2,5 enote encima, če upoštevamo običajno definicijo lakazne encimske enote, ki pomeni oksisadicijo 1 µmol ABTS na minuto pri sobni temperaturi in optimalnem pH.

V primerjavi z objavljenimi podatki (Reiss in sod., 2011) je to precej nizka aktivnost za 1 mg encima, kar daje slutiti, da je aktiven le majhen delež encimskih molekul. Iz SDS-PAGE analize namreč vidimo, da je končna encimska frakcija precej čista. Encima torej

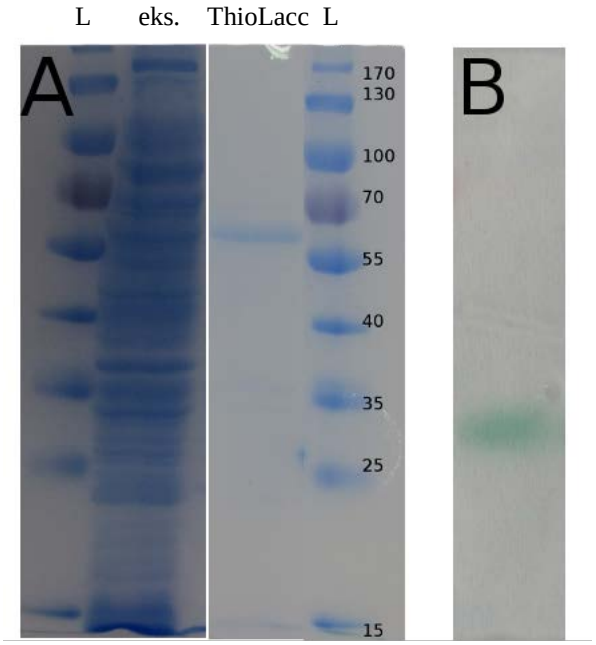
nastane dovolj, najbrž pa aktivna mesta niso zapolnjena s po štirimi bakrovimi atomi, ki so potrebni za aktivnost. Aktivnost je ostala nizka tudi po *in vitro* rekonstituciji bakra v aktivna mesta encima. Izvedli smo jo s kratkotrajno inkubacijo (15 min) pri povišani temperaturi (50 ºC) in ob prisotnosti bakrovega klorida, aktivnost pa se je v povprečju povečala za manj kot 10 % (podatki niso prikazani).



Slika 3: Primerjava zaporedij lakaze ThioLacc z znanima baktrijskima lakazama CotA iz *Bacillus subtilis* in CueO iz *Escherichia coli*. Z modro so odznačene aminokisline, ki so enake pri vseh zaporedjih, s črnimi kvadratki pa tiste, ki sodelujejo pri koordinaciji bakra v aktivnem centru. S črtkano črto so označene z metioninom in glicinom bogate regije.
Figure 3: Comparison of amino acid sequences of ThioLacc and two well-studied bacterial laccases, CotA from *Bacillus subtilis* and CueO from *Escherichia coli*. Amino acid residues that are the same in all the sequences of the alignment are shown in blue, those that are crucial for the coordination of copper in the enzyme's active center are marked with black squares. Methionin- and glycin-rich regions are marked with a dashed line.

Če encim termično denaturiramo, je njegova ocenjena velikost na SDS-PAGE gelu enaka predvideni, ki je okoli 60 kDa (Slika 4A). Izkazalo pa se je, da je encim stabilen v

prisotnosti denaturantov (detergenta natrijev dodecilsulfat in reducenta ditiotreitol - DTT), saj ohrani aktivnost v gelu po izvedeni elektroforezi SDS-PAGE, če encima ne denaturiramo s kuhanjem. Po izdatnem spiranju lahko ThioLacc oksidira ABTS v gelu (Slika 4B), kar kaže na reverzibilno kemijsko denaturacijo med SDS-PAGE.

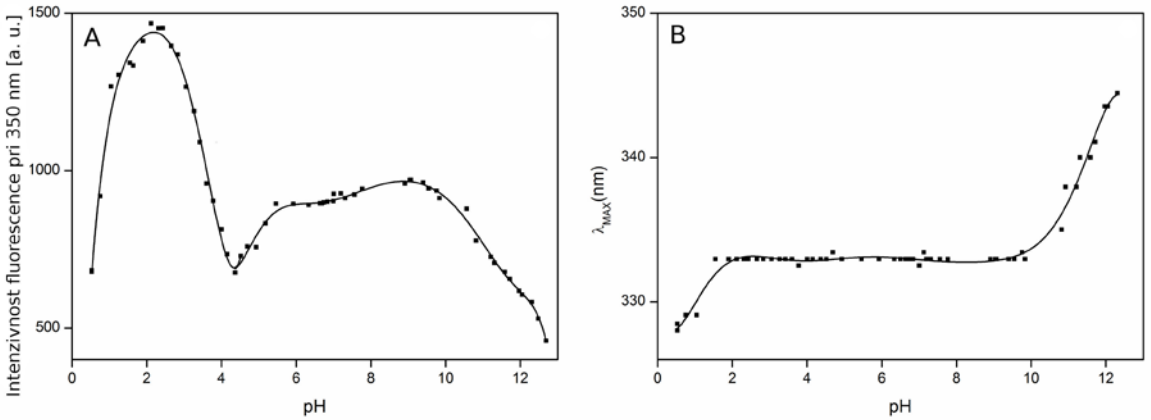


Slika 4: SDS-PAGE analiza ecimskih frakcij ThioLacc. A – Celični ekstrakt po razbitju celic (eks.), izolirana lakazna frakcija (ThioLacc), lestvica proteinskih standardov (L) z lisami znanih velikosti (v kDa). B – Nativna frakcija ThioLacc, barvana z ABTS – zelena barva je odraz encimske aktivnosti.
Figure 4: SDS-PAGE analysis of the ThioLacc enzymatic fractions. A – Cell extract after disruption of cells (eks.), isolated laccase fraction (ThioLacc), protein ladder (L) with bands of known size (in kDa). B – Native ThioLacc, coloured with ABTS – the green colour is proof of enzymatic activity.

Izvorni organizem je obligatni alkalifil. Izolirali so ga iz izjemno bazičnega jezera v Keniji v sklopu študije haloalkalifilnih sevov rodu *Thioalkalivibrio* (Foti in sod., 2006). Ker njegov gen za lakazo, ki smo ga izrazili v naši študiji, vsebuje zapis za signalni peptid (in torej ukaz za njegov prenos v periplazmo ali ven iz celice), smo sklepali, da bo tudi lakaza ThioLacc aktivna pri visokih pH vrednostih.

Srukturno stabilnost ThioLacc v odvisnosti od pH smo merili preko intrinzične fluorescence v razponu pH med 0,5 in 12,5. Intenziteta fluorescence je odvisna od lokalnega okolja 12 tirozinskih in 9 triptofanskih aminokislinskih ostankov v molekuli ThioLacc. Ko so aromatske aminokisline zaradi denaturacije proteina nenadoma izpostavljene polarnemu topilu, lahko to zaznamo kot premik intenzitete fluorescence k višjim valovnim dolžinam. Intenziteta fluorescence je bila konstantna na ozkem območju okoli pH 2,5 (± 0,5) ter med pH 5,0 do 9,0, kar pomeni, da je encim na teh območjih

konformacijsko stabilen (Slika 5A). Spremembe v intenziteti fluorescence so posledica protonacije in deprotonacije nabitih aminokislin v ThioLacc: 48 Asp (pK_a = 3,9), 31 Glu (pK_a = 4,3), 30 His (pK_a = 6,0), 1 Cys (pK_a = 8,3), 5 Lys (pK_a = 10,8), 12 Tyr (pK_a = 10,8) in 41 Arg (pK_a = 12,5). Pozicija valovnih dolžin pri maksimalni intenziteti fluorescence (Slika 5B) je bila nespremenjena v širokem razponu pH med 2,1 in 9,9, kar znova kaže na konformacijsko stabilnost ThioLacc v tem območju. ThioLacc je pH-tolerantni encim, kot smo predvidevali ob izboru tega proteina.

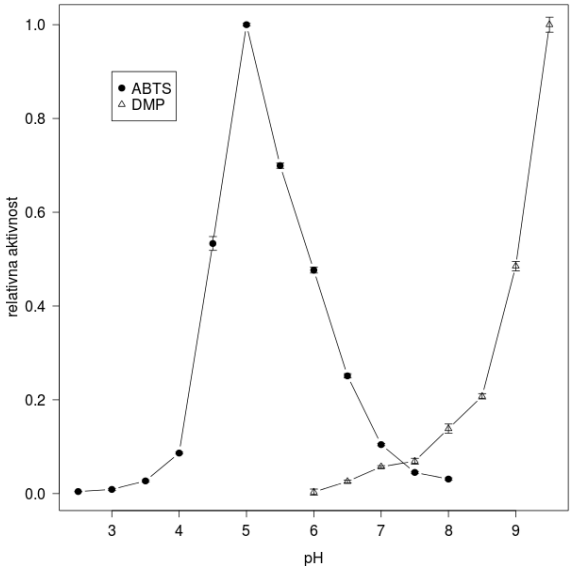


Slika 5: Intrinzična emisija fluorescence pri ThioLacc. A – Intenziteta intrinzične fluorescenčne emisije pri 350 nm v odvisnosti od pH. B –Valovna dolžina maksimalne intenzitete intrinzične fluorescence v odvisnosti od pH.
Figure 5: Intrinsic fluorescence emission of ThioLacc. A – Intensity of intrinsic fluorescence emission at 350 nm in dependence of pH. B – The wavelength of maximum intensity of the intrinsicfluorescence in dependence of pH.

Kot pri drugih lakazah je bil tudi pri ThioLacc dejanski pH optimum za oksidacijo odvisen od substrata. Za nefenolno spojino ABTS smo izmerili optimalno aktivnost pri pH 5,0 (25 % aktivnosti se ohrani celo pri pH 6,5), za fenolno spojino DMP pa nad pH 9,5 (Slika 6). Za primerjavo lahko navedemo, da dobro preučena lakaza iz glive *Trametes versicolor* najbolje razgrajuje ABTS pri pH 3,5, ohrani le polovico aktivnosti pri pH 5,0, aktivnost pa se nato s približevanjem nevtralnemu pH naglo zmanjšuje in izgine pri pH 6,0 (Han in sod., 2005). Pri večini glivnih lakaz tudi oksidacija fenolnih snovi poteka v kislem pH, navadno pri pH 4,0 - 6,0 (Baldrian, 2006).

Uporabili smo še deset drugih substratov, da bi preučili razpon spojin, ki jih ThioLacc lahko oksidira. Zaznali smo oksidacijo naslednjih spojin (v oklepaju so navedene pH vrednosti, pri katerih smo opazili spremembo začetnega UV-VIS spectra): $K_4[Fe(CN)_6]$ (pH 5,0), pirogalol (pH 7,0), pirokatehol (pH 8,0), siringaldehid (pH 8,0), siringaldazin (pH 8,0). ThioLacc je oksidiral organsko (ABTS) in anorgansko (kalijev ferocianat) nefenolno spojino in pa večino 2,6-dihidroksi in 2,6-dimetoksi substituiranih fenolnih

spojin, ne pa tudi monometeksi ali monohidroksi substituiranih spojin, kar kaže na relativno nizek redoks potencial lakaze. ThioLacc ni oksidiral tirozina, kar od prave lakaze tudi pričakujemo. Ker oksidacija fenolnih spojin najhitreje poteka pri pH 8,0 ali celo višje, lahko zaključimo, da je ThioLacc alkalifilna lakaza. Druge bakterijske lakaze, ki dobro delujejo v bazičnih pH vrednostih, večinoma izvirajo iz različnih vrst rodu *Bacillus*. Tako



Slika 6: Aktivnost ThioLacc v odvisnosti od pH reakcijske mešanice. Prikazana so povprečja in standardne napake (N=3).
Figure 6: Activity of ThioLacc in response to pH. Shown are averages and standard errors (N=3).

denimo lakaza iz *B. halodurans* (Ruijssenaars in Hartmans, 2004) lahko oksidira siringaldazin pri pH 8, lakaza iz *B. licheniformis* (Lu in sod., 2012) ohrani 60% aktivnosti oksidacije DMP pri pH 9, lakaza iz *B. pumilus* (Reiss in sod., 2011) pa optimalno oksidira ABTS pri pH 4,0 in DMP pri pH 7,0. Lakaza, ki so jo našli z metagenomiko in je podobna lakazam iz rodu *Bacillus* (Ye in sod., 2010), optimalno oksidira gvajakol pri pH 8,0. Redka izjema med alkalifilnimi lakazami je encim iz *γ-proteobacterium* JB (Singh in sod., 2007), ki je aktivna v širokem razponu pH vrednosti med 4 in 10 in lahko oksidira različne fenolne spojine pri ali rahlo pod pH 7,0. Ta lakaza izvira iz organizma iz družine *Chromatiaceae* in je tako sorodna bakteriji *Thioalkalivibrio* sp., ki sodi v družino *Ectothiorhodospiraceae* iz poddebla *Gammaproteobacteria.*

Lakaze so encimi, ki lahko oksidirajo širok spekter substratov, zato jih je težko nedvoumno klasificirati. Encim CotA iz plašča spore pri *Bacillus subtilis* je tako modelna bakterijska lakaza, vendar je hkrati med najaktivnejšimi znanimi oksidazami bilirubina (Sakasegawa in sod., 2006), lahko pa celo uporablja didušikov oksid namesto kislika kot končni sprejemnik elektronov (Fernandes in sod., 2010). Encimom CueO iz *E. coli* lahko oksidira večino klasičnih substratov za lakaze, vendar hkratu tudi odlično oksidira baker (Grass in

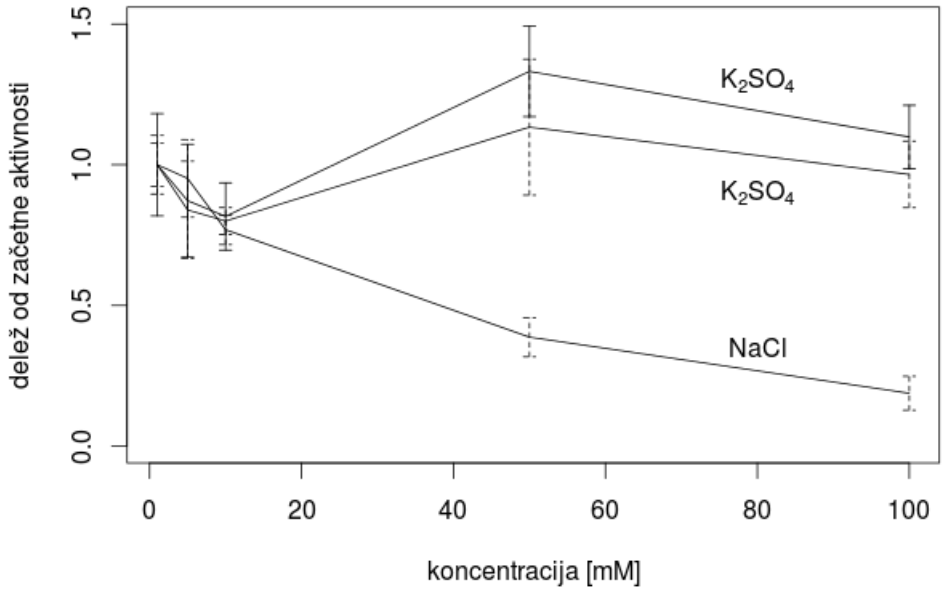
sod., 2001; Singh in sod., 2004). Podobno je tudi z encimom McoP iz arheje *Pyrobaculum aerophilum* (Fernandes in sod., 2010). Ker je ThioLacc podoben CueO in McoP, lahko sklepamo, da je tudi ta encim *in vivo* udeležen pri homeostazi bakra. Ker *Thioalkalivibrio* sp. raste v ekstremnem pH in izredno počasi, je to težko preveriti, vsekakor pa bi bilo v luči nedavnih razmišljanj o raznolikosti encimov z lakazno aktivnostjo (Reiss in sod., 2013) ThioLacc pravilneje imenovati lakazam-podobna oksidaza z bakrom (ang. laccase-like multicopper oxidase, LMCO).

Encimsko kinetiko smo študirali z dvema tipičnima substratoma za lakaze, z ABTS in DMP. Pri pH > 9 DMP počasi spontano oksidira, saj je njegova pKa vrednost 9,9 (Giardina in sod., 2010), zato smo kinetiko za ta substrat merili pri suboptimalnem pH 8,0. Izmerili smo Michaelis-Mentenovi konstanti K_M 7,6 in 3,8 mM ter maksimalne hitrosti reakcij V_{max} 1,8 in 0,1 µmol/min za ABTS in DMP. Izmerjene vrednosti so do dva velikostna reda nižje od robustnih lakaz iz bakterij rodu *Bacillus* (Reiss in sod., 2011), saj smo morali dodati zelo veliko substrata, da smo zasitili encim.

Nizka aktivnost lahko pomeni, da smo pri meritvi uporabili suboptimalen substrat, kar bi pritrjevalo hipotezi, da ThioLacc ni običajna lakaza, kot že razpravljamo zgoraj. Nam pa se zdi tudi verjetno, da nizka izmerjena aktivnost znova kaže, da je kljub dokaj visoki masi izoliranega encima le majhen delež molekul resnično aktivnih. Razlog je lahko v bodisi neučinkovitem vključevanju bakra v aktivno mesto encima med sintezo v *E. coli* bodisi v izpiranju bakra iz aktivnih mest med procesom čiščenja, vendar je razlog iz naših meritev težko ugotoviti. Kljub temu pa laho ugotovimo, da smo z afinitetno kromatografijo dobili dovolj čistega encima za njegovo karakterizacijo. Izkoristke ekspresije in izolacije bi gotovo lahko še izboljšali, vendar pa trenutni izkoristki ne vplivajo na naše zaključke, ki zadevajo meritve pH in temperaturnega optimuma za delovanje encima, njegove sposobnosti razgradnje različnih spojin ali delovanja v prisotnosti soli.

Kloridi so znani inhibitorji glivnih in bakterijskih lakaz, čeprav obstajajo tudi izjeme, znova pri lakazah iz skupine *Bacillus*, ki so ohranjale delovanje pri 1 M koncentraciji NaCl (Reiss in sod., 2011; Ye in sod., 2010) ali pa so jih kloridni ioni celo stimulirali (Ruijssenaars in Hartmans, 2004). Da bi ugotovili učinek različnih soli na delovanje lakaze ThioLacc, smo spremljali oksidacijo ABTS ob dodatku različnih koncentracij natrijevega klorida ter natrijevega in kalijevega sulfata (Slika 7). Kloridni ioni so močno inhibirali delovanje lakaze, kalijev in natrijev sulfat pa sta imela manj opazen učinek na aktivnost ThioLacc tudi pri 100 mM koncentracijah. Predstavniki rodu *Thioalkalivibrio* so ekstremofilni organizmi, ki živijo v ekstremno bazičnih in slanih okoljih, kjer je pH blizu 10 in se koncentracije Na^+ in K^+ približajo zasičenju. Zato smo pričakovali, da bo lakaza

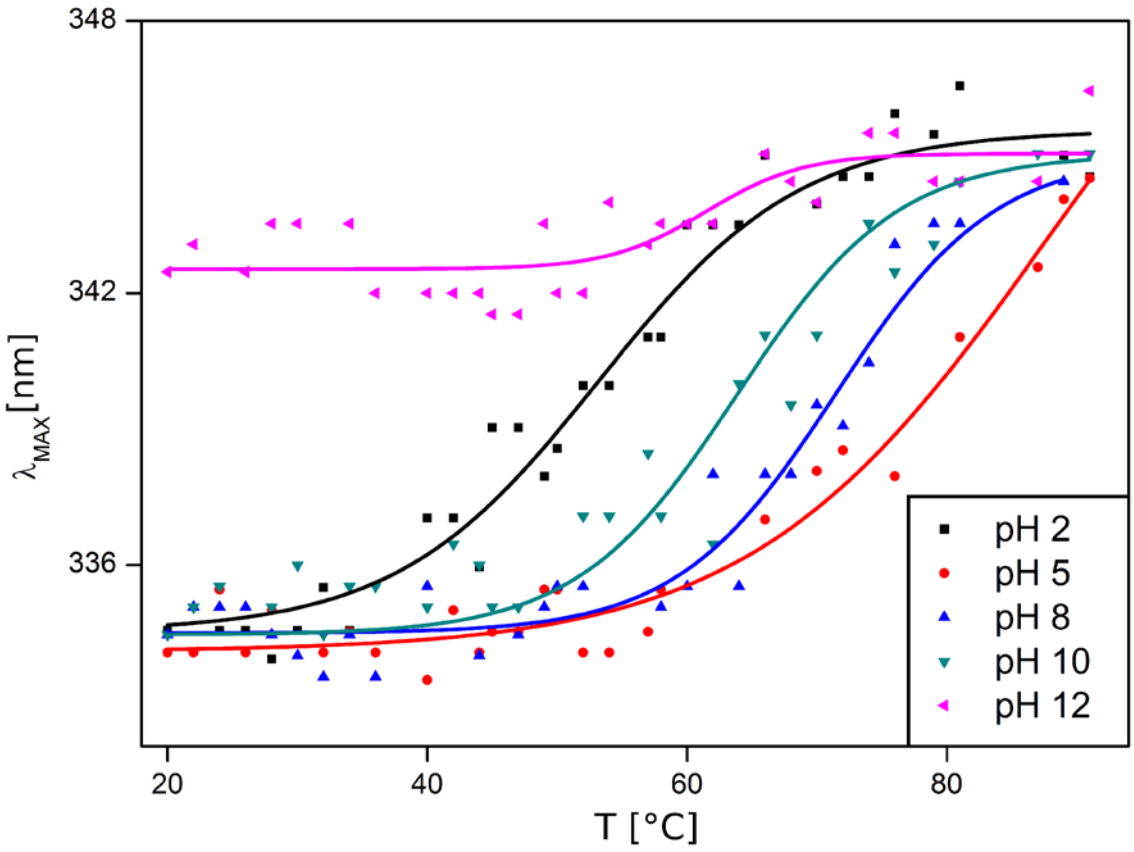
odporna na visok pH in slanost, vendar slednje ne drži. Presenetila nas je visoka občutljivost na natrijev klorid v koncentracijah pod 0,1 M. Občutljivost na kloride lahko razložimo s fiziologijo sorodnega seva *Thioalkalivibrio* sp. Mix90, ki lahko živi v izjemno visokih koncentracijah natrijevih in kalijevih ionov, vendar je občutljiv na klorid, zato so ga avtorji označili za ekstremnega natronofila in ne klasičnega halofila (Muyzer in sod., 2011). Vse to tudi nakazuje, da je ThioLacc najverjetneje periplazmatski protein v bakteriji *Thioalkalivibrio* sp. DSM13533.



Slika 7: Vpliv soli na aktivnost ThioLacc. Kalijev in natrijev sulfat ne vplivata značilno na aktivnost lakaze, natrijev klorid v koncentraciji 100 mM pa začetno aktivnost zniža na približno 20 %. Prikazana so povprečja in standardne napake (N=3).
Figure 7: Activity of ThioLacc in response to chlorides and sulfates. Potassium and sodium sulfate do not significantly influence the laccase activity. Sodium chloride, however, reduces the initial activity to 20 % at 100 mM concentration. Shown are averages and standard errors (N=3).

Termično stabilnost ThioLacc smo merili na razponu med 20 °C to 95 °C pri pH 2,0, 5,0, 8,0, 10,0 in 12,0 (Slika 8). Valovna dolžina maksimalne intenzitete fluorescence je bila 334 ± 1 nm pri pH med 2 in 10. Pri 90 °C je bil protein razvit (denaturiran), zato smo maksimalno intenziteto fluorescence zabeležili pri 344 ± 1,5 nm. Lakaza je bila tako najstabilnejša pri pH 5 in 8, temperaturo denaturacije pa smo določili grafično in sicer pri 78 °C in 72 °C. Pri pH 2 smo izmerili nižjo stabilnost in denaturacijo pri 53 °C, pri pH 12 pa je bil protein denaturiran že pri sobni temperaturi (Slika 8). S kalorimetrijo (ang. differential scanning calorimetry, DSC) lahko neposredno določimo tempreaturo denaturacije proteina in preko merjenja toplotne kapacitete tudi entalpijo denaturacije. Z DSC smo temperaturo denaturacije pri pH 8,0 določili pri 76 °C, kar se dobro ujema z

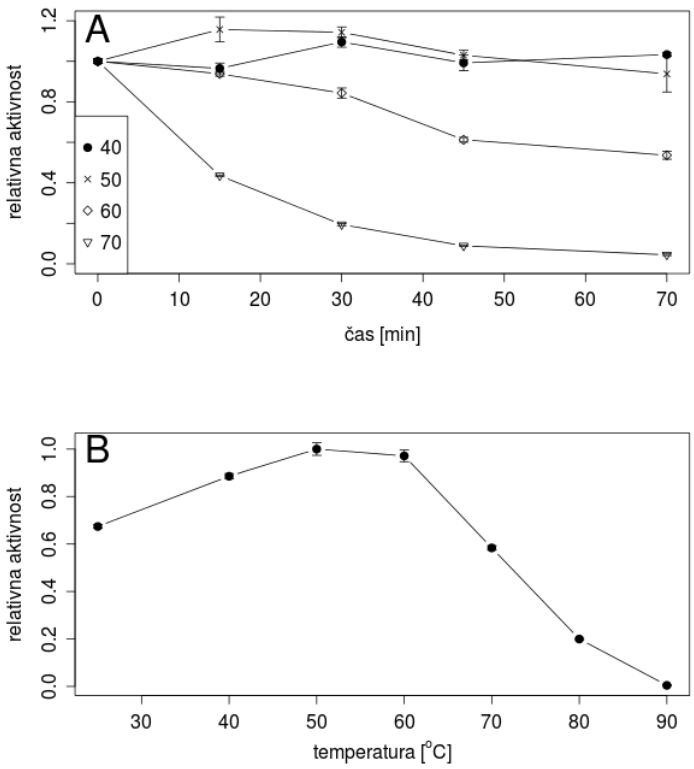
rezultati fluorescenčne spektrometrije (78 °C). Entalpija denaturacije je bila 147 kJ/mol, razlika v toplotni kapaciteti med nativno in denaturirano obliko pa 15 kJ/(mol×K).



Slika 8: Maksimalna valovna dolžina intrinzične fluorescence ThioLacc v odvisnosti od pH. Merili smo pri petih pH vrednostih (pH 2 – pH 12). Pri pH 12 je bil protein denaturiran že pri sobni temperaturi, pri ostalih pa smo opazili premik maksimalne valovne dolžine od 334 proti 344 nm.
Figure 8: Maximum wavelength of intrinsic fluorescence emission of ThioLacc measured at five pH values (pH 2 – pH 12). At pH 12 the protein is denatured already at room temperature. At other pH values a shift of maximum wavelength from 334 to 344 was observed.

Strukturno je lakaza ThioLacc torej dokaj termostabilna. Učinek dolgotrajne inkubacije (do 70 minut) pri povišanih temperaturah na encimsko aktivnost smo merili na razponu 25 – 70 ºC, nato pa smo merili preostalo (rezidualno) aktivnost encima pri sobni temperaturi. Aktivnost s časom inkubacije na 60 ali 70 ºC pada, po 15 min inkubacije na 70 ºC tako ostane manj kot polovica aktivnosti (slika 9A). Nasprotno pa se v dobri uri inkubacije na temperaturah 40 in 50 ºC aktivnost ne spremeni. Ker smo tudi najvišjo začetno hitrost oksidacije izmerili pri temperaturi 50 ºC (slika 9B), lahko zaključimo, da je lakaza ThioLacc primerna za uporabo pri daljših inkubacijskih časih na 50 ºC. Za primerjavo lahko navedemo, da se pri alkalifilni lakazi iz bakterije *γ-proteobacterium* JB aktivnost prepolovi po 120 minutah na 55 ºC oziroma po 30 minutah na 60 ºC (Singh in sod., 2007),

torej je lakaza ThioLacc obstojneša. Do sedaj poznamo le eno lakazo iz obligatnega termofila (*Thermus aquaticus*), ki je bila najbolj aktivna pri temperaturi 92 ºC in je imela razpolovno dobo aktivnosti kar 14 ur pri 80 ºC (Miyazaki, 2005).



Slika 9: Termostabilnost lakaze ThioLacc. A – Rezidualna aktivnost lakaze po inkubaciji na povišani temperaturi; inkubacija do 70 min na 40 ali 50 ºC ni zmanjšala aktivnosti lakaze ThioLacc. B – Maksimalna začetna hitrost oksidacije v odvisnosti od temperature; najvišjo aktivnost smo zabeležili pri 50 in 60 ºC. Aktivnost je podana kot delež največje začetne hitrosti oksidacije ABTS pri pH 5,0, podana so povprečja in standardne napake (N=3).
Figure 9: Thermostability of ThioLacc. A – Residual activity of laccase after the incubation of the enzyme at increased temperature. Incubation at 40 or 50 ºC for up to 70 min did not reduce the activity of ThioLacc. B – Maximum initial oxidation rate in dependence of temperature. Maximum rate was recorded at 50 and 60 ºC. Activity is given as the proportion of maximum initial rate of ABTS oxidation at pH 5.0. Shown are averages and standard errors (N=3).

2.2.2.4 Zaključki

Pričujoče delo je neposredno izrablja odkritja bioinformatske raziskave (Ausec in sod., 2011a), v kateri smo s pregledom vseh javno dostopnih genomov ustvarili seznam potencialnih genov za lakazam podobne encime. Gen iz ekstremofilne proteobakterije *Thioalkalivibrio* sp. DSM13533 smo heterologno izrazili, očistili in določili njegove osnovne lastnosti. Encim je aktiven v širokem razponu pH vrednosti in ima v primerjavi z

drugimi znanimi glivnimi in bakterijskimi lakazami visok pH optimum za različne fenolne in nefenolne spojine. Čeprav njegovo aktivnost močno inhibirajo kloridne soli, ThioLacc ohranja aktivnost ob povišani koncentraciji sulfatov. Encim ohranja stabilnost pri tempreaturah do 50 ºC, kjer je tudi najbolj aktiven, zato je encim primeren za uporabo pri daljših inkubacijah na tej tempreaturi. ThioLacc je prva lakaza, izolirana iz predstavnikov rodu *Thioalkalivibrio*. Ker so to avtotrofne bakterije, se postavlja vprašanje o funkciji lakaz pri teh organizmih.

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Premislek o metodah

Pri delu smo uporabili širok nabor metod. Temelj so bile klasične mikrobiološke tehnike gojenja bakterij v tekočih in trdnih gojiščih, običajno pri selekcijskih pogojih (visok pH ali prisotnost antibiotikov). Splošno aktivnost mikroorganizmov smo spremljali preko dihanja s sledenjem CO_2 s plinskim kromatografom. Uporabili smo številne metode molekularne biologije: verižno reakcijo s polimerazo (PCR), metode molekularnega kloniranja in elektroforezo za pregledovanje in čiščenje DNA. Sekvenciranja nismo izvajali sami (podjetje Macrogen), a smo vse rezultate analizirali z bioinformatskimi metodami. Uporabili smo nekatere filogentske metode in metode poravnav DNA zaporedij za pripravo genskih konstruktov in novih začetnih oligonukleotidov. Iskanje lakaznih genov v genomih je zahtevalo znanja uporabe javnih podatkovnih baz, priprave verjetnostnih modelov lakaz (pHMM), uporaba programiranja pri analizi rezultatov. Poleg tega pa smo pri čiščenju in preučevanju aktivnosti izbrane lakaze uporabili številne biokemijske metode (kromatografija, poliakrilamidna gelska elektroforeza, spremljanje encimske kinetike).

Ob vrednotenju tega seznama ugotavljam, da je hkrati predolg in prekratek. Predolg zato, ker je nemogoče obvladati tako širok nabor tehnik, da bi jih lahko do popolnosti izkoristili pri svojem delu, saj je znanje na nekaterih področjih nujno fragmentarno. Prekratek pa zato, ker bi z dodatnimi metodami lahko odgovorili na nekatera pomembna vprašanja. Z novimi metodami pirosekvenciranja in metagenomiko bi tako lahko podrobneje spoznali barjanske bakterijske združbe in njihove lakazne gene. Z analizo večjega števila metagenomov in njihovih metapodatkov (to je povezano z dodatnim programerskim znanjem in računsko zmogljivostjo računalnikov) bi lahko bolje sklepali na fiziološke in ekološke funkcije lakaz v naravnih okoljih. Poglobljeni algoritmi anotacije genomskih odsekov bi omogočili preučevanje gensko okolico lakaznih genov in s tem povezane encimske funkcije, pa tudi primere horizontalnih prenosov genov. Z biotehnološkimi pristopi bi lahko optimizirali gojišča za proizvodnjo lakaz, pogoje genske ekspresije in produkcije heterolognega encima. Z nekaterimi metodami fizikalne kemije (atomska

absorbcijska spektrometrija, induktivna sklopljena plazemska optična emisijska spektroskopija) bi se dalo kvantificirati dejansko vsebnost bakra v heterologni lakazi in tako sklepali o vzrokih za suboptimalno encimsko aktivnost.

Vsekakor pa lahko zaključimo, da so bili bioinformatski pristopi zelo pomembni za izvedbo pričujočega dela. Poleg uveljavljanih programov in uporabniških vmesnikov za javne baze podatkov je bilo neobhodno potrebno osnovno znanje programiranja za programatično dostopanje do baz, za obdelavo surovih podatkov, za sistematiziranje in statistično obdelavo rezultatov. Čeprav smo sodelovali z odlično skupino bioinformatikov iz Univerze v Bielefeldu (Nemčija), je bilo znanje programiranja ključno. Pri svojem delu smo uporabiljali programska jezika python, ki je izvrsten za delo s tekstovnimi datotekami in prek dodatnih paketov (bioPython) omogoča enostavno interakcijo s podatkovnimi bazami, in R, ki je zelo učinkovit za statistične analize in grafične prikaze urejenih podatkov. Pomembno je poudariti, da sta oba jezika zelo zmogljiva in brezplačna za uporabo, zato je vložek v učenje smotern, saj nadaljnja raba teh orodij ni odvisna od plačevanja licenčnin v prihodnosti. Tovrstne veščine so visoko na lestvici priporočljivih bioinformatskih znanj (Dudley in Butte, 2009). Menim, da bi njihovo učenje morali bolj spodbujati pri mladih generacijah študentov bioloških smeri, saj taki pristopi pri raziskovanju sedaj postajajo standard, cilj opolnomočenja, ki pride z računalniško avtomatizacijo nekaterih nalog, pa opravičuje časovni vložek. Bioinformatika omogoča ukvarjanje z biološkimi izzivi, ki so zgolj z laboratorijskim pristopom pogosto nedosegljivi (obratno je seveda tudi možno), hkrati pa je procesorska moč v primerjavi z dragimi laboratorijskimi reagenti običajno relativno poceni.

3.1.2 Diskusija celotnega dela

Ko smo pričeli z doktorskim delom na tematiko bakterijskih lakaz, smo začeli povsem novo zgodbo, in to ne le v naši raziskovalni skupini, ampak širše. Res je obstajalo nekaj izvirnih raziskovalnih člankov, ki so opisovali bakterijske lakaze ali pa vsaj lakazno aktivnost, najdeno v različnih bakterijskih sevih, in peščica preglednih člankov (Alexandre in Zhulin, 2000; Claus, 2003; Sharma in sod., 2007), ki so navajali iste vire in se nadejali velike raznolikosti teh encimov, ki bi očitno morala slediti iz prepoznane raznolikosti bakterijskega sveta, vendar o njih ni mogel nihče ničesar določenega zaključiti. Mi smo zato želeli natančneje opisati raznolikost lakaz. Študija Haralda Kellnerja in sodelavcev (2008), v kateri so preučevali delčke bakterijskih genov za lakazam podobne encime

neposredno v tleh, je prva prepričljivo pokazala to raznolikost v gozdnih in travniških tleh. Pomen teh rezultatov za ekologijo (če že ne kar takoj tudi za biotehnologijo) je bil navdihujoč. Kljub temu sva se spomladi 2010 izmed 40 doktorskih in podoktorskih raziskovalcev, prisotnih na mednarodni delavnici *Laccase Academy* (COST868/602), le dva udeleženca nameravala ukvarjati z bakterijskimi encimi, ostalim (vključno z mentorji) pa se je to tedaj zdela potrata časa in denarja.

Povod raziskave je bilo poznavanje in dostop do zanimivih habitatov na Ljubljanskem barju. Tega danes sestavljajo izsušena travišča in kmetijske površine z redkimi zaplatami gozda. Nekdaj debela plast šote je večinoma porezana in propadla in obstaja le še nekaj ostankov barja. Preučevali smo dva tipa tal, ki sta se najbolj razlikovala po izvoru (šota iz ločja in šašev na nizkem barju, šota iz šotnega mahu na visokem barju), rastju (travišče ali brezovo-borov gozd), pH (7,6 v nizkem in 4,4 v visokem barju) in v vsebnosti organske snovi (16 % v nizkem in 45 % v visokem barju). V dveh študijah (Kraigher in sod., 2006; Ausec in sod., 2009) smo ugotovili, da sta bakterijski združbi v obeh tipih tal zelo pestri in da se razlikujeta med seboj. Posebno skupina *Acidobacteria* je pritegnila našo pozornost, saj smo kar 27-42 % vseh DNA sekvenc uvrstili v to deblo bakterij. V tistem obdobju se je pojavilo več študij, ki so navajale visok delež acidobakterij v združbah različnih habitatov, in različni avtorji so ugotavljali, da metabolna raznolikost teh bakterij primerljiva s celotnim deblom *Proteobacteria* (Barns in sod., 2007), čemur so pritrjevali tudi prvi trije sekvencirani genomi acidobakterij (Ward in sod., 2009).

Takrat z iskanjem homologov znanih lakaz v genomih acidobakterij nismo našli lakazam podobnih genov, vendar je bil vzorec genomov skromen, naša metoda iskanja, ki je temeljila na algoritmu BLAST (Altschul in sod., 2000), pa tudi ni bila optimalna. V tleh Ljubljanskega barja je ogromno organskega materiala, veliko je bolj ali manj razgrajenega lignina in težko razgradljivih huminskih kislin, torej dovolj fenolnih snovi, zato naše pričakovanje lakaz v tem ekosistemu ni bilo neosnovano. Poleg tega smo v tleh visokega barja zaznali visoko aktivnost lakaznih encimov, ki je bila večja od primerjanih gozdnih ali travniških tal (Ausec in sod., 2011a). S to metodo ni mogoče določiti, ali so encimi glivnega ali bakterijskega izvora niti kako raznoliki so. Vseeno se je Ljubljansko barje zdelo še zanimivejše.

V prvi fazi raziskovanja raznolikosti lakaz na Barju smo uporabili pristope do tedaj edine študije, v kateri so preučevali bakterijske gene za lakaze v naravnem okolju (Kellner in

sod., 2008). Pripravili smo genske knjižnice kratkih fragmentov (<150 baznih parov) lakaznih genov med prvim in drugim vezavnim mestom za baker (Slika 1) in s filogenetskimi metodami skušali prepoznati izvorne skupine bakterij. Filogenija lakaznih genov je bila relativno nezanesljiva, saj je bilo referenčnih DNA zaporedij malo in je bil filogenetski signal omejen zaradi kratkosti preučevanih odsekov. Kasneje smo ob pripravi bioinformatske študije spoznali, da geni za lakazam podobne encime niso dobri filogenetski markerji, predvsem zaradi mobilnosti teh genov in horizontalnih prenosov. Kljub temo smo v tleh Ljubljanskega barja opazili več konsistentnih skupin in smo velik delež zaporedij (16 % v genskih knjižnicah nizkega barja, 35 % v knjižnicah visokega barja) znova povezali s skupino *Acidobacteria,* kar je bilo v skladu z rezultati pridobljenimi na podlagi genov za 16S rRNA.

Bistven del te ekološko usmerjene raziskave pa je bil razvoj novih začetnih oligonukleotidov, s katerimi smo lahko pomnoževali velik del gena za lakazo, torej fragmente med 1. in 4. vezavnim mestom za baker (Slika 1). Začetni oligonukleotidi so močno degenerirani, da bi z njimi lahko zajeli kar največjo raznolikost, zato nas ni presenetilo, da poleg pričakovanega PCR-produkta dobimo tudi dodatne pomnožke. Na koncu se je izkazalo, da so to večinoma kar pravi pomnožki, le da so nepričakovanih velikosti, kar dodatno kaže na raznolikost lakazam podobnih encimov. Ugotovili smo tudi, da lahko pomnožujemo tudi gene za dvodomenske lakaze. Čeprav je bil obstoj teh encimov na podlagi redkih nukleotidnih zaporedij predviden že pred desetletjem (Nakamura in sod., 2003) in imamo sedaj že dve trodimenzionalni strukturi (Komori in sod., 2009; Lawton in sod., 2009), vemo o aktivnosti in fiziološki vlogi dvodomenskih lakaz izredno malo. Zato je bilo pomembno odkritje naše raziskave, da so geni za take encime pogosti, raznoliki in splošno prisotni.

Bistvena težava z uporabljenim pristopom je v tem, da je nemogoče trditi, da so vsi zaznani geni dejansko funkcionalni in da torej kodirajo dejanske encime, ki imajo običajne lastnosti lakaz. S takim pristopom je tudi nemogoče pridobiti celotne gene, ki bi jih lahko preučevali s heterologno ekspresijo. Sklepali smo lahko le o potencialu, ki obstaja v teh tleh, vendar bi za njegovo dejansko izrabo potrebovali nove metode metagenomike. Pri tem pristopu se ukvarjamo s celokupno okoljsko DNA, ki jo v fragmenth bodisi neposredno sekvenciramo ali pa večje vključke kloniramo v vektorje (npr. 10 kbp v plazmide ali 40 kbp v fozmide) in jih kasneje presejamo za želene lastnosti glede na zaporedje DNA ali aktivnost izraženih beljakovin. Znano je, da je izkoristek pri iskanju novih encimov s takim pristopom izrazito majhen, torej je treba preiskati zelo veliko

metagenomskih klonov, da bi našli kaj uporabnega (Suenaga, 2011). Zato je ključno, da so preučevani geni v izbranem okolju ne le raznoliki, ampak tudi dovolj pogosti. Ker smo v barjanskih tleh konsistentno našli velik delež (podobnih) acidobakterij, smo kasneje v sodelovanju z Univerzo v Groningenu (Nizozemska) pristopili k iskanju novih lakaz z metagenomiko. Pri presejanju 12000 klonov smo bili uspešni in našli celoten gen za lakazo, ki je zelo podoben prej odkritim fragmentom, kar dokazuje uspešnost te metode. Ta raziskava ni potekala v okviru doktorskega dela in trenutno še traja, zato jo na tem mestu omenjamo zgolj kot zanimivost.

Preučevanja raznolikosti lakaz smo se nato lotili tudi *in silico*, saj nam bioinformatika omogoča izrabo vse znane raznolikosti, zajete v zaporedjih genomske DNA najrazličnejših bakterij. V resnici ta nabor še zdaleč ni nepristranski, saj so nekatere skupine izrazito slabo zastopane, druge pa imajo v javnih podatkovnih bazah zaradi medicinske ali zgodovinske pomembnosti nesorazmerno velik delež, zato si nekatere svetovne pobude prizadevajo za uravnoteženje teh zbirk s sekvenciranjem genomov predstavnikov manj raziskanih skupin bakterij (Wu in sod., 2009). V naši raziskavi ne moremo sklepati o deležu bakterijskih genomov v posamezni skupini (deblu), ki vsebujejo gene za lakazam podobne encime, saj tak delež ne bi bil smiseln. Toda dovolj zgovoren je že, da smo našli take gene v vseh debelih, za katere so bili v času raziskave na voljo zaporedja genomov.

Kot najpomembnejše zaključke te raziskave je potrebno izpostaviti dokaze o mobilnosti lakaznih genov med bakterijami. Podali smo prve pokazatelje, da so bakterijski geni za lakaze kodirani na plazmidih, poleg tega pa smo našli več primerov horizontalnega prenosa genov. Obstajajo tudi drugi dokazi o horizontalnem prenosu teh genov, na primer homologov *copA* (Behlau in sod., 2013). To razloži, zakaj lakaze niso dober filogenetski marker, kar smo ugotavljali že v prejšnji raziskavi (Ausec in sod., 2011a). Poleg tega nam dejstvo, da smo morali uporabiti pet verjetnostnih modelov za lakaze (pHMM), kaže, da obstaja najbrž še dosti več evolucijsko in funkcionalno ločenih skupin genov in njihovih produktov. Sirim in sod. (2011) so uporabili kar 56 modelov pHMM za nabiranje teh genov, nekateri avtorji (Reiss in sod., 2013) pa ravno zaradi velike raznolikosti teh genov in encimov priporočajo uporabo izraza lakazam podobni encimi (ang. laccase-like enzymes).

Poleg splošnih dognanj o lastnostih genov in deloma proteinov lakazam podobnih encimov pa je seveda sam nabor najdenih genov tisto, kar omogoča neposredno nadaljevanje

raziskav, saj le študij na nivoju beljakovine preseže ugibanja o funkcionalnosti gena in aktivnosti encima. Poleg tega lahko premišljena izbira izvornega organizma bistveno pripomore k verjetnosti, da bo imel izbrani encim želene lastnosti. V zadnjem delu doktorske disertacije, ki je trenutno še v postopku recenzije, dokazujemo ravno to: z izražanjem gena za lakazo iz ekstremofilnega organizma *Thioalkalivibrio* sp., ki živi pri pH 10, smo v *E. coli* proizvedli alkalifilno lakazo, ki je zmožna oksidacije fenolnih snovi v bazičnih pogojih, in sicer pri pH vrednostih, ki večinoma presegajo poročane vrednosti v znanstveni literaturi. Kljub temu, da je ThioLacc zagotovo alkalifilen encim in da lahko oksidira tipične lakazne substrate, pa ne moremo trditi, da tudi *in vivo* oksidira organske substrate. Na osnovi 3D modela tega encima in njegove podobnosi z encimom CueO iz *E. coli* lahko ugibamo, da ThioLacc v organizmu opravlja funkcijo oksidacije kovinskih ionov. To bo sicer težko potrditi zaradi počasne rasti izvornega organizma in še nerazvitih genetskih metod za ta ekstremofilni organizem. Zato bi bilo nemara ustrezneje, da bi encim po priporočilih Reiss in sod. (2013) poimenovali lakazi podobna oksidaza.

Ta del naših raziskav ima najbolj neposredno biotehnološko uporabnost, saj pridobljena heterologna lakaza ThioLacc izkazuje lastnosti, ki so pomembne v biotehnoloških procesih. Kljub temu ThioLacc še ni primerna za takojšnjo aplikacijo, saj bi bilo treba večino korakov primerno optimizirati. Najpomembnejši je redoks potencial encima. Običajno imajo bakterijske lakaze nižji redoks potencial od glivnih, zato je nabor snovi, ki jih lakaza lahko oksidira, primerno manjši (Mate in sod., 2011). Nekatere raziskave z mutagenezo lakaznih genov kažejo, da je možno to lastnost z umetnim izborom pomembno izboljšati (Gupta in Farinas, 2010). Izolacija z Ni-NTA afinitetno kromatografijo, ki je enostavna in zanesljiva možnost izolacije v laboratorijskem merilu, je predraga za uporabo v večjih sistemih, zato bi bilo treba uporabiti drugačne načine čiščenja lakaze (na primer obarjanje in ultrafiltracija, ionsko-izmenjevalna in gelska kromatografija). Še bolje bi bilo, če bi podobno kot pri glivah dosegli, da bi producentski organizem izločal lakazo iz celic, saj je bistveno lažje pridobivati encim iz izrabljenega gojišča kot pa iz lizata celic. Poleg tega je izrednega pomena zagotoviti stabilnost encima, kar dosežemo s pravilno formulacijo encimskega pripravka (Fernández-Fernández in sod., 2012). Trenutno je veliko raziskav povezanih z imobilizacijo lakaz na različne nosilce, kar omogoča trajnejšo rabo in boljšo izrabo encima. Kljub tem kritičnim pomislekom pomen našega dela ni zanemarljiv, saj nakazuje možnosti izrabe velikega potenciala, ki smo ga našli z našo bioinformatsko študijo.

V pričujočem doktorskem delu smo tako prehodili pot bakterijskih lakaz od gena do

encima. Identificirali smo okolje, ki je bilo zanimivo za raziskovanje teh encimov, odkrili smo raznolikost genov za lakaze v izbranih tleh Ljubljanskega barja ter prisotnost teh genov v vseh razpoložljivih bakterijskih genomih. Iz tega nabora smo načrtno izbrali gen, za katerega se je zdelo verjetno, da kodira alkalifilno lakazo, kar smo kasneje tudi potrdili na heterologno izraženem encimu.

3.2 SKLEPI

- V šotnih tleh Ljubljanskega barja obstajajo zelo raznolike bakterijske združbe. Vsebnost in izvor organske snovi ter pH tal vplivata na strukturo združb. V kislih tleh visokega barja in v nevtralnih tleh nizkega barja je bila pestrost bakterijske združbe podobna.

- V obeh preučevanih tipih barjanskih tal smo zaznali visok delež bakterij iz debla *Acidobacteria*, v tleh visokega barja smo to opazili z analizo ribosomalnega (16S rRNA) in funkcionalnega (lakaze) gena, kar kaže na velik a slabo razumljen ekološki pomen teh bakterij.

- V **hipotezi 1** smo predpostavili splošno razširjenost genov za lakazam podobne encime, kar smo potrdili z bioinformatsko analizo, s katero smo našli take gene v predstavnikih vseh večjih bakterijskih debel.

- V **hipotezi 2** smo predpostavili, da bomo z molekularnimi metodami v izbranem okolju našli nove gene za lakaze. To smo pokazali s klonskimi knjižnicami kratkih in dolgih fragmentov genov za lakazam podobne encime, v katerih smo zabeležili povsem nove skupine genov, ki se močno razlikujejo od že znanih v podatkovnih bazah, kar potrjuje predvidevanja, da je ta skupina encimov bolj raznolika, kot smo sprva predvidevali.

- Bakterijske lakaze imajo očitno raznolike, čeprav večinoma še nezane biološke funkcije. Naši rezultati kažejo, da se velik delež the encimov v celicah izloča iz citoplazme, kar je v nasprotju s prejšnjim razumevnjem. Geni, ki kodirajo lakazam podobne encime, se aktivno prenašajo s horizontalnim prenosom, najdemo pa jih tudi pri avtotrofih (ki lakaz torej ne uporabljajo pri izrabi substrata) in anaerobih (čeprav lakaze potrebujejo kisik).

- Iz nabora genov smo enega izrazili in predvidevali (**hipoteza 3**), da bo heterologna lakaza imela drugačne biokemijske lastnosti od že znanih lakaz. Izbrani gen iz obligatnega alkalifila je dejansko kodiral pH-tolerantno lakazo, ki lahko oksidira fenolne spojine pri neobičajno bazičnih pH vrednostih.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

V pričujočem delu smo raziskovali bakterijske lakaze. To je skupina oksidoreduktaz z bakrom v aktivnem centru, ki sklapljajo oksidacijo (običajno fenolnega) substrata z redukcijo molekularnega kisika do vode. V zadnjih letih se je tudi na podlagi naših raziskav pokazalo, da so tovrstni encimi ne le prisotni pri bakterijah, ampak so tudi izjemno raznoliki, tako po svojih strukturnih značilnostih (več skupin dvo- in trodomenskih lakaz) kot tudi po encimski aktivnosti. Lakaze oksidirajo širok spekter različnih spojin, vendar z različnimi preferencami in pri različnih fizikalno-kemijskih pogojih.

Najprej smo utemeljili, zakaj so šotna tla Ljubljanskega barja zanimivo okolje za preučevanje raznolikosti bakterijskih genov za lakaze in njim podobne encime. Z analizo genov za 16S rRNA smo preučevali raznolikost bakterijske združbe v kislih tleh visokega barja in jo primerjali z združbo v nevtralnih tleh nizkega barja. Kljub nizkemu pH je bila združba visokega barja raznolika, predvsem pa je bila zaznamovana z visokim deležem bakterij iz debla *Acidobacteria*. Ljubljansko barje pretežno meliorirano in šota v večji meri odstranjena, vendar je za ta tla še vedno značilna visoka vsebnost fenolnih in huminskih snovi, ki bi bile lahko substrat za lakaze. Poleg tega smo s testi encimske aktivnosti pokazali visoko aktivnos lakaz v teh tleh, in sicer je bila ta višja od preizkušenih gozdnih, travniških in kmetijskih tal. Zato smo v nadaljevanju preučili raznolikost genov za lakaze v barjanskih šotnih tleh tleh. V ta namen smo razvili nove začetne oligonukleotide, s katerimi smo lahko pomnoževali velike odseke genov in tako podrobneje analizirali njihovo strukturno raznolikost, kot je bilo to mogoče pred tem. Pokazali smo, da lahko na ta način neposredno v tleh preučujemo gene za dvo- in trodomenske lakaze in da tako odkrijemo številne povsem nove, ki imajo malo ali nič podobnih zaporedij v javnih podatkovnih bazah. Velik delež genskih fragmentov smo pripisali acidobakterijam, torej je raznolikost preučevanih funkcionalnih genov odslikovala raznolikost genov za rRNA.

Da bi podrobneje analizirali raznolikost genov za lakaze pri bakterijah, smo uporabili bioinformatski pristop in poiskali gene za lakazam podobne encime v vseh razpoložljivih bakterijskih genomih. Za iskanje smo razvili in uporabili verjetnostne profile – skrite modele Markova (ang. profile Hidden Markov Models, pHMM). V 2200 genomih smo

tako našli preko 1200 genov v predstavnikih vseh bakterijskih debel. Poleg tega pa dobili tudi uvid v biologijo teh genov. Pokazali smo, da to večinoma niso intracelularni encimi, pač pa jih je 76 % transportiranih iz citoplazme, da so ti geni mobilni, saj jih pogosto najdemo na plazmidih ali na genomskih otokih, ki kažejo značilnosti horizontalnega prenosa. Bakterijski geni za lakazam podobne encime imajo verjetno zelo različne funkcije, saj jih najdemo pri bakterijah z različnim načinom življenja (ekstremofili, avtotrofi, anaerobne bakterije).

Da bi pokazali na biotehnološki pomen take zbirke domnevnih genov za lakazam podobne encime, smo en gen izrazili v gostiteljskem organizmu *Escherichia coli* ter očiščeno rekombinantno lakazo delno biokemijsko opredelili. Izbrali smo gen iz bakterije *Thioalkalivibrio* sp., ki je obligatno alkalifilen organizem, saj smo želeli proizvesti encim, ki bi dobro deloval v bazičnem pH. Rekombinantna lakaza je oksidirala večino tipičnih substratov za lakaze, in sicer fenolne spojine pri pH > 8. Tako smo preko raziskovanja splošne raznolikosti bakterijskih genov za lakaze raziskavo privedli do študija specifičnega encima, ki bo morebiti v prihodnje zanimiv tudi za biotehnološko uporabo.

4.2 SUMMARY

This thesis is about bacterial laccases. They form a heterogenous group of copper oxidoreductases that couple the oxidation of a (usually phenolic) substrate with the reduction of molecular oxygen to water. Recently, these enzymes were not only discovered in bacteria but were shown to be very diverse, both in structure (many groups of two- and three-domain laccases) and in activity – although they have a broad substrate spectrum, different groups of laccase-like enzymes prefer different substrates and different physical and chemical conditions.

The first part of the thesis establishes the peat soils of the Ljubljana marsh as a suitable environment to study laccase genes. The bacterial diversity of the low-pH bog soil was analysed using 16S rRNA genes and was compared to the bacterial community of the neutral fen soil of the Ljubljana Marsh. The bog soil community was diverse in spite of the low pH, and had a markedly high proportion of bacteria from the *Acidobacteria* phylum. The peat soils are drained and the peat is mostly removed and degraded. For this reason, the soil is rich in fenolic and humic substances that are potential laccase substrates. Moreover, enzymatic activity tests in soil showed higher laccase activity in the bog soil compared to tested forest, grassland and agricultural soils. For these reasons, we proceeded

to investigate the diversity of laccase genes in the bog soil of the Ljubljana Marsh. New PCR primers were developed to amplify large fragments of the laccase genes to study their diversity in greater depth than before. This approach allowed retrieval of genes for both two- and three-domain laccases directly from soil. Many sequences were discovered that had few or no similar sequences in the public databases. A large proportion of sequences was again affiliated to acidobacteria, the functional diversity thus reflecting the diversity observed at the 16S rRNA level.

To analyze the diversity of genes for laccase-like enzymes across *Bacteria, in silico* approach was used to search for such genes in all available bacterial genomes. Profile Hidden Markov Models (pHMM) were developed and used to screen over 2,200 genomes, and over 1,200 genes were retrieved from all major bacterial phyla. The study revealed insights into this group of enzymes: they were shown to be largely (76%) exported out from the cytoplasm, to be mobile and often present on plasmids or transferred with horizontal gene transfer, and to presumably have many roles in diverse bacteria with very different lifestyles (extremophiles, autotrophs, anaerobic bacteria).

To show the biotechnological potential of this gene collection, one gene was expressed in a heterologous host *Escherichia coli*, purified and partially biochemically characterized. A laccase gene from the obligatelly alkaliphilic bacterium *Thioalkalivibrio* sp. was chosen since the aim was to isolate a laccase that would be active at high pH. The recombinant enzyme could oxidize many of the typical laccase phenolic substrates at pH above 8. In the present work we have thus investigated the general diversity of laccase genes in bacteria and the properties of one particular enzyme that could prove interesting for future biotechnological applications.

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Priloga A: Dovoljenje za objavo članka Ausec in sod. (2009) v tiskani in elektronski obliki

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Bioinformatic Analysis Reveals High Diversity of Bacterial Genes for Laccase-Like Enzymes

Luka Ausec[1], Martha Zakrzewski[2,3], Alexander Goesmann[2], Andreas Schlüter[3], Ines Mandic-Mulec[1]*

1 Department for Food Science and Technology Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia, **2** Center for Biotechnology (CeBiTec), Institute for Bioinformatics, Bielefeld University, Bielefeld Germany, **3** Center for Biotechnology (CeBiTec), Institute for Genome Research and Systems Biology, Bielefeld University, Bielefeld, Germany

Abstract

Fungal laccases have been used in various fields ranging from processes in wood and paper industries to environmental applications. Although a few bacterial laccases have been characterized in recent years, prokaryotes have largely been neglected as a source of novel enzymes, in part due to the lack of knowledge about the diversity and distribution of laccases within Bacteria. In this work genes for laccase-like enzymes were searched for in over 2,200 complete and draft bacterial genomes and four metagenomic datasets, using the custom profile Hidden Markov Models for two- and three-domain laccases. More than 1,200 putative genes for laccase-like enzymes were retrieved from chromosomes and plasmids of diverse bacteria. In 76% of the genes, signal peptides were predicted, indicating that these bacterial laccases may be exported from the cytoplasm, which contrasts with the current belief. Moreover, several examples of putatively horizontally transferred bacterial laccase genes were described. Many metagenomic sequences encoding fragments of laccase-like enzymes could not be phylogenetically assigned, indicating considerable novelty. Laccase-like genes were also found in anaerobic bacteria, autotrophs and alkaliphiles, thus opening new hypotheses regarding their ecological functions. Bacteria identified as carrying laccase genes represent potential sources for future biotechnological applications.

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* E-mail: ines.mandic@bf.uni-lj.si

Introduction

Laccases are members of the multi-copper oxidoreductases that oxidize a variety of phenolic substances including polyaromatic hydrocarbons (PAH), estrogens in wastewater [1,2] and recalcitrant biopolymers such as lignin [3,4]. Due to their broad substrate specificity laccases are of great industrial interest and have been used in paper and wood processing and in the textile industry [5,6,7].

Substrate oxidation by laccases (and subsequent reduction of molecular oxygen) creates reactive radicals which can participate in (i) polymerization (oxidative coupling of monomers), (ii) degradation of polymers or (iii) degradation of phenolics (by cleavage of aromatic rings) [8]. Substrate specificity is broadened by mediators, which are small molecular-mass compounds that are oxidized into radicals by laccases and can subsequently oxidize a variety of other (more complex) substrates such as lignin. Laccases contain four copper atoms held in place in the reaction center by conserved copper-binding regions. Nucleotide sequences specifying the copper-binding sites are suitable for molecular-ecological studies as it is possible to design PCR-primers for these sites [9-11]. Laccases have been found in all domains of life [12] but have been most intensively studied in ligninolytic fungi [13].

The first indication that laccases may be present in bacteria was based on the phenol-oxidase activity observed in *Azospirillum lipoferum* almost 20 years ago [14]. A decade ago, researchers used the BLAST algorithm to find 14 bacterial laccase genes similar to those known from fungi [15]. A few bacterial laccases have been studied since (see [16] for a more recent review). Until recently, fungal laccases have been considered extracellular enzymes while bacterial laccases were assumed to be mostly intracellular or spore-bound. It was speculated [16] that bacteria may have strategies such as rearrangement of the electron transport system to cope with the toxic molecular compounds produced by the oxidation of aromatic substrates within the cell. The simplistic view of fungal laccases as extracellular lignin-degrading enzymes has given way to a more realistic view, in which fungal laccases are involved in various intra- and extracellular developmental processes in morphogenesis and pathogenesis [7,12,17] in addition to their role in degradation of complex substrates. It was suggested that further studies are needed to verify that the diverse fungal laccases retrieved from different environmental studies are indeed extracellular ligninolytic enzymes [3].

In bacteria, the perceived role of laccases has mostly been limited to oxidation of metals and pigment formation [8,16]. The latter function is based on the well studied CotA laccase located in the spore coat of *Bacillus subtilis*, which produces a melanin-like pigment for the protection of the spore against UV-light [18]. The possibility that bacterial laccases play a role in the degradation of recalcitrant biopolymers has been suggested only recently [4,19]. However, bacterial laccases may have several properties that are not characteristic of fungal enzymes. Firstly, the laccase from *Streptomyces lavendulae* [20] shows high thermo resistance and the CotA laccase from *Bacillus subtilis* has a half-life of inactivation at 80°C of about 4 h and 2 h for the coat-associated or the purified enzyme, respectively [18]. The most termophilic laccase from