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Multiplasmiidne süsteem naftaleeni ja fenooli lagundamiseks *Pseudomonas fluorescens* tüves PC20

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KASUTATUD LÜHENDID

2,4-D – 2,4-diklorofenoksüatsetaat

bp – aluspaar

C12O – katehhooli 1,2-dioksügenaas

C23O – katehhooli 2,3-dioksügenaas

CAA – kasaminohapped

CFU – kolooniat moodustav ühik

EDTA – dinaatriumetüleendiamiintetraatsetaat

Glc – glükoos

IPTG – isopropüül-β-tiogalaktosiid

kb – kiloaluspaar

LB – Luria-Bertani sööde

MGE – mobiilne geneetiline element

NADH – redutseeritud nikotiinamiidadeniindinukleotiid

Nah – naftaleen

PAH – polütsükliline aromaatne süsivesinik

PC34O – protokatehhuaadi 3,4-dioksügenaas

p-Cre – *p*-kresool

PCR – polümeraasi ahelreaktsioon

Phe – fenool

PMO – fenooli monooksügenaas

Sal – salitsülaat

SDS – naatriumdodetsüülsulfaat

Sm – streptomütsiin

SSC – standardne naatriumsulfaadi puhver

TAE – Tris, atsetaat, EDTA

Tris – tris(hüdroksümetüül)aminometaan

Trp – trüptofaan

U – ühik

X-gal – 5-bromo-4-kloro-3-indolüül-β-D-galaktopüranosiid

Xyl – ksüleen

SISSEJUHATUS

Paljud monoaromaatsed ja polütsüklilised aromaatsed süsivesinikud satuvad keskkonda peamiselt tööstustootmise tulemusena ja need on enamikele elusorganismidele toksilised. Eesti mastaabis on üheks probleemsemaks piirkonnaks Kirde-Eesti. Seal paiknevatest põlevkivitööstuse ettevõtetest satub keskkonda hulgaliselt erinevaid fenoolseid ning polüaromaatseid ühendeid. Selliseid ühe või mitme benseenituumaga ühendeid on võimelised lagundama bakterid, kasutades erinevaid degradatsiooniradu. Väga paljud degradatiivsed geenid või terved lagundamisrajad asuvad bakteriaalsetel plasmiididel ning on ümbritsetud mobiilsete geneetiliste elementidega, andes bakterite kataboolsete tunnuste levikule horisontaalse suuna.

Bioremediatsiooni efektiivsuse suurendamiseks ning looduses esinevate biodegradeerivate mikroobikonsortsiumite „täiustamiseks“ on aromaatsete ühendite degradatsioonirajad ning neis sisalduvad ensüümid olnud uurijate erilise tähelepanu all juba aastakümneid. Väga oluline roll bioremediatsioonis ning mikroobide kohanemisel fenoolidega reostatud keskkonna tingimustes on täita kataboolsetel plasmiididel. Plasmiidide molekulaarse struktuuri uurimisel peab otseste DNA analüüsidega kaasnema ka bakteritüvede isoleerimine ning nende kataboolsete plasmiidide tüübi, hulga ja ülekandumise tuvastamine.

Käesoleva töö eesmärkideks olid:

- Selgitada bioaugmentatsioonis edukalt kasutatud bakteritüve *P. fluorescens* PC20 plasmiidne koostis seoses nende poolt määratud kataboolsete funktsioonidega;
- Määrata tüves PC20 leiduva kahe plasmiidi pNAH20 ja pPHE20 mittesobivusgrupid ja konjugatiivsus;
- Võrrelda plasmiidide pNAH20 ja pPHE20 kataboolsete operonide nukleotiidseid järjestusi teiste tuntud kataboolsete plasmiidide analoogsete operonidega;
- iseloomustada tüves PC20 naftaleeni ja fenooli lagundamise võtme-ensüümide ekspressiooni.

Tänan Eeva Heinaru juhendamise, nõuannete ning mõistva suhtumise eest, Merike Merimaad ja Eve Vedlerit koostöö ning alati lahke abi eest, dr Inga Sarandit võrdlusplasmiidi pIS110 ning dr. E.L. Madsenit Cornelli Ülikoolist *P. putida* tüvede NCIB9816-4 ja Cg1 kasutusvõimaluse eest.

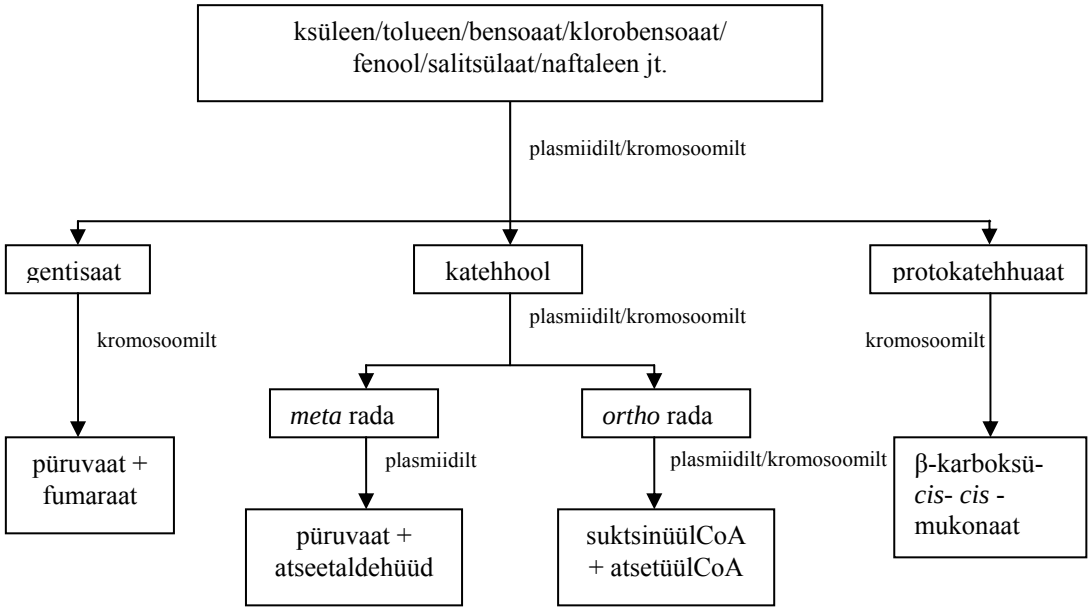
1. KIRJANDUSE ÜLEVAADE

1.1. Aromaatsete ühendite bakteriaalse lagundamise üldmehhanismid

Aromaatsed ja polütsüklilised aromaatsed süsivesinikud (*Polycyclic Aromatic Hydrocabons* - PAHid) moodustavad olulise osa keskkonda sattuvatest saasteainetest, millest suur hulk tööstusliku ning põllumajandusliku tegevuse tagajärjel tekkinud ühenditest on elusorganismidele toksilised, kantserogeensed ja/või mutageensed. Paljusid selliseid ühendeid nagu fenoolid, kresoolid, kloro- ja nitroaromaatsed, aga ka polütsüklilised aromaatsed ühendid nagu naftaleen, antratseen, fenantreen ja püreenid, on lagundatavad mikroorganismide, peamiselt bakterite ja seente poolt (Mishra *et al.*, 2001; Nojiri et *al.*, 2004). Aromaatsete ühendite degradeerimiseks kasutavad bakterid erinevaid kataboolseid radu. Ühendite lagundamine toimub kas osaliselt või täielikult, sõltuvalt benseeni tuumade arvust, asendusrühmade tüübist ning mikroorganismi poolt kodeeritavatest ensüümidest.

Kuigi erinevates katabolismiradades kasutatakse aromaatsete ühendite degradeerimiseks väga erinevaid ensüüme, muudetakse need ühendid piiratud hulgaks vaheühenditeks nagu näiteks (asendustega) katehhoolid ning protokatehhuaat (van der Meer *et al.*, 1992). Edasine lagundamine Krebsi tsükli vaheühenditeni toimub peamiselt kas mööda *meta* või *ortho* rada (joonis 1), v.a. üksikud erandid. Mõningad aromaatsed ühendid, näiteks salitsülaat ja üle salitsülaadi degradeeritavad polüaromaatsed ühendid (naftaleen, fenantreen, antratseen jt.) võidakse metaboliseerida lisaks katehhooli rajale ka üle gentisaadi raja (Lal *et al.*, 1995).

Aromaatsete ühendite kataboliseerimiseks vajalike ensüümide geenid paiknevad bakterites kas kromosoomis või plasmiidis. Üldiselt toimub paljude aromaatsete ühendite, näiteks tolueeni, ksüleeni, bensoaadi, fenooli jt. lagundamine kromosoomsetelt geenidelt *ortho* raja kaudu ning plasmiidselt kodeeritud geenidelt mööda *meta* rada (Lal *et al.,* 1995) (joonis 1).

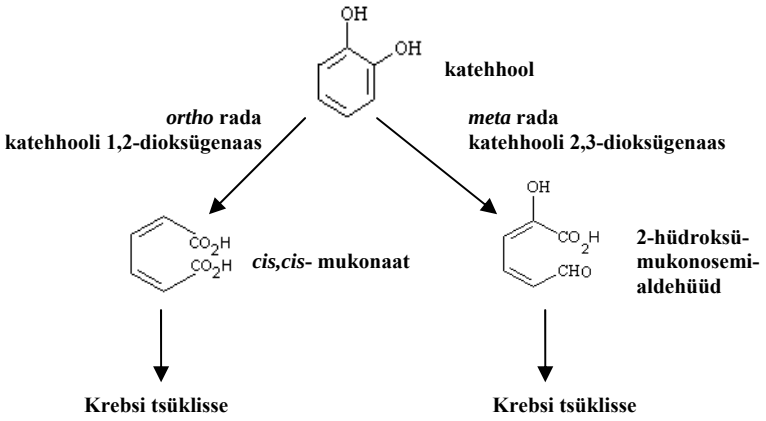


Joonis 1. Aromaatsete ühendite aeroobse lagundamise enamlevinud rajad mikroorganismides (Lal *et al.*, 1995).

Aromaatsete substraatide aeroobsed lagundamisrajad bakterites saab põhimõtteliselt jagada kolme etappi (Williams, Sayers, 1994):

1. Esmalt läbib substraat rea muutusi asendusrühmade osas nii, et selle etapi produktiks on dihüdroksüaromaatne vaheühend (katehhool, gentisaat, protokatehhuaat või nende derivaadid). Selle etapi ensüümid, mono- ja dioksügenaasid, katalüüsivad vastavalt ühe või kahe hapniku aatomi sisestamist aromaatsesse ühendisse hüdroksüülrühmana.
2. Teises etapis toimub dihüdroksüleeritud vaheühendi benseenituuma lõhustamine dioksügenaaside toimel. Rõnga avamisel lõhuvad dioksügenaasid ühe süsinik-süsinik sideme ning lisavad molekulaarse hapniku (2 hapniku aatomit), mille tulemusena tekib küllastamata alifaatne hape.
3. Aromaatse tuuma lagundamise käigus tekkinud produktid konverteeritakse madalama molekulmassiga alifaatseteks ühenditeks, mis lülitatakse põhiainevahetusse.

Aromaatset rõngast avavaid dioksügenaase saab jagada kahte funktsionaalsesse klassi: intradioolsed ehk *ortho*-tüüpi dioksügenaasid ja ekstradioolsed ehk *meta*-tüüpi dioksügenaasid (van der Meer *et al.*, 1992) (joonis .2).



Joonis 2. Intradioolne (*ortho* rada) ja ekstradioolne (*meta* rada) aromaatse tuuma lõhustamine.

Intradioolsed dioksügenaasid on Fe^{3+}-sõltuvad ensüümid, mis lõhuvad benseenituumas oleva süsinik-süsinik sideme kõrvuti paiknevate hüdroksüülrühmade vahelt (hüdroksüülrühmade suhtes *ortho* asendis) (van der Meer *et al.*, 1992; Williams, Sayers, 1994). Intradioolsete ensüümide perekonna võib grupeerida kolmeks:

1) protokatehhuaadi 3,4-dioksügenaasid (PC34O-d), näiteks PcaHG;

2) katehhooli 1,2-dioksügenaasid (C12O-d), näiteks CatA;

3) klorokatehhooli 1,2-dioksügenaasid, näiteks TcbC, TfdC ja ClcA (van der Meer *et al.*, 1992).

ortho raja ensüümid osalevad selliste aromaatsete ühendite degradatsioonis, mille lagundamise keskseteks vaheproduktideks on katehhool või protokatehhuaat (Mishra *et al.*, 2001). Need ühendid transformeeritakse β-ketoadipaadi enoollaktooniks, mis edasiste reaktsioonide käigus konverteeritakse suktsinaadiks ja atsetüül-CoA-ks (van der Meer *et al.*, 1992). Üldiselt paiknevad *ortho* raja geenid bakteri kromosoomis.

Ekstradioolsed ehk *meta*-tüüpi dioksügenaasid on Fe^{2+}-sõltuvad ensüümid, mis lõhuvad benseenituuma hüdroksüülrühmade kõrvalt (hüdroksüülrühmade suhtes *meta* asendis), andes produktiks 2-hüdroksümukonosemialdehüüdi või selle derivaadid. Need metaboliseeritakse järgnevalt Krebsi tsükli võtmeühenditeks (Williams, Sayers, 1994). Põhiainevahetusse sisenevad *meta* rajas tekkinud vaheproduktid püruvaadi ja atsetüül-CoA-na (Murray, Williams, 1974). *meta* raja ensüümide geenid tolueeni, naftaleeni või fenooli lagundavates organismides on klasterdunud üheks operoniks (Harayama, Rekik, 1993). Fülogeneetilise suguluse järgi on ekstradioolsed dioksügenaasid jagatud erinevatesse tüüpidesse (Eltis, Bolin, 1996). C23O-d, mis eelistatult lagundavad

monotsüklilisi substraate, kuuluvad dioksügenaaside perekonda 1.2.A ja neid kodeerivad geenid on enamlevinud pseudomonaadides aromaatse tuuma lagundamisel.

1.2. Kataboolsed plasmiidid

1.2.1. Üldiseloomustus

Paljusid looduslikust keskkonnast isoleeritud biodegradatiivsete omadustega bakteritüvesid iseloomustab kataboolsete plasmiidide esinemine. Plasmiidid on autonoomselt replitseeruvad ekstrakromosomaalsed DNA replikonid, mis etendavad olulist rolli bakterite geneetilises mitmekesisuses ning mikroorganismide kiires kohanemisvõimes pidevalt muutuvates keskkonnatingimustes (Dennis, 2005). Plasmiidselt kodeeritud kataboolsed rajad tagavad geneetiliselt paindliku süsteemi biodegradatiivsete omaduste säilumiseks ja ülekandumiseks populatsioonis (Sayler *et al*., 1990). Keemilise ühendi degradatsiooniks vajalike ensüümide sünteesi kodeerivaid geene sisaldavad kataboolsed plasmiidid on suhteliselt suured (50-500 kb), kandes endas hulgaliselt mobiilseid geneetilisi elemente nagu insertsioon-järjestused ja transposoonid. Need bakterirakkudes esinevad kovalentselt suletud tsirkulaarsed molekulid on tavaliselt madala koopia-arvuga, kusjuures suuremad plasmiidid võivad esineda rakus vaid 1-2 koopiana ja väikestel plasmiididel on koopia-arv kõrgem.

Väike koopia-arv rakus vähendab küll plasmiidi replikatsiooniks vajaminevat energiakulu, kuid samas vähendab ka tõenäosust, et raku jagunemisel saab iga tütar-rakk plasmiidi koopia. Seega on rakkude jagunemisel oluline tagada plasmiidide sattumine igasse tütar-rakku ehk õige segregatsioon. Korrektne segregatsioon võib olla häiritud mitme koopia-arvuga plasmiidide puhul, kus identsed plasmiidi koopiad rekombineeruvad, moodustades multimeere. Plasmiidide multimeeride lahutamise süsteem (*mrs – multimer resolution system*) tagab rakkude jagunemisel iga plasmiidi koopia funktsioneerimise eraldi üksusena, lahutades rekombineerunud multimeerid monomeerideks. IncP gruppi kuuluvatel plasmiididel tagavad selle peamiselt *incC* ja *korB* geenide poolt kodeeritud valgud (Thomas, 2000).

Sarnaselt teistele suurtele plasmiididele sisaldab kataboolsete plasmiidide selgroog olulisi modulaarseid geneetilisi regioone, mis on välja kujunenud selleks, et vähendada metaboolset koormust peremeesrakule ning kindlustada plasmiidi reproduktsioon ja ülekandumine teistesse rakkudesse (Thomas, 2000). Plasmiidide säilumine ning levik on tagatud plasmiidselt kodeeritud replikatsiooni-, stabiilsuse- ja ülekandesüsteemidega. Oma

eksistentsiks ning enese paljundamiseks kasutavad plasmiidid suuremalt jaolt peremeesraku ainevahetust läbi plasmiidse kontrollsüsteemi. Replikatsiooni kontrollsüsteemi moodustavad replikatsiooni alguspunkt (*oriV*), initsiatsiooniks vajalik Rep valk, selle seondumissaidid *ori* piirkonnas (iteronid) ning initsiatsioonifaktorid (Helsinki *et al.*, 1996). Tsirkulaarsete plasmiidide puhul on kirjeldatud 3 peamist replikatsiooni mehhanismi: *theta type, strand replacement* ja *rolling circle* (del Solar *et al.*, 1998).

Stabiilsuse ning jätkusuutlikkuse tagamiseks on plasmiidides koopiaarvu kontrolliv süsteem garanteerimaks raku jagunemise käigus plasmiidi koopiaarvu püsimist.. Selleks kasutavad plasmiidid negatiivse tagasiside regulatsioonisüsteemi, mille plasmiidselt kodeeritud kontroll-elemendid mõjutavad replikatsiooni initsiatsiooni (del Solar, Espinosa, 2000, Chattoraj, 2000).

Kataboolsete geenide klastrid asuvad plasmiidides sageli operonides, mis vastutavad teatud keemiliste ühendite metaboliseerimiseks vajalike funktsioonide eest. Need kataboolsed rajad on lülitatud plasmiidi selgroo vähe-olulisematesse regioonidesse, et mitte häirida plasmiidi normaalseks funktsioneerimiseks vajalike geenide tööd. Sellised vähe-olulised saidid on ka kataboolseid lagundamisradu kandvate transposoonide jt. MGEde „randumispaigad". Plasmiidide järjestuste analüüs on näidanud, et mida konserveerunumad on kataboolsed rajad, seda tihedamalt on nende geenid ülesehituslikult klasterdunud (Dennis, 2005). Sarnaselt plasmiidi selgroo geneetilisele ülesehitusele, toimub kataboolsete radade geneetilise organiseerituse pidev täienemine saavutamaks optimaalset tasakaalu geeniekspressiooni ja maksimaalse lagundamisvõime vahel (Thomas, 2000).

1.2.2. Mittesobivusgrupid

Plasmiide klassifitseeritakse mittesobivusgruppide alusel (*Incompatibility* e. Inc grupid), kus kriteeriumiks on võetud DNA *rep* piirkonna järjestused (Helsinki *et al.*, 1996). Sarnast replikatsiooni kontrollsüsteemi omavad plasmiidid ei saa koos eksisteerida ühes ja samas peremeesrakus, kuna sel puhul tekib konkurents piiratud arvu replikatsiooni või alalhoidmise saitide pärast ning pole kindlustatud kõigi plasmiidide säilumine. Niisiis, samasse mittesobivusgruppi kuuluvad plasmiidid ei saa ühes rakus koos eksisteerida (Miller, Cohen, 1993). IncP plasmiididel osaleb geeniekspressiooni regulatsioonis tsentraalne kontrollregioon (*ccr - central control region*), millelt kodeeritud regulaatorid

KorA, KorB ja KorC koordineerivad enamusi plasmiidi ellujäämiseks vajalikke funktsioone (Balzer *et al.*, 1992; Williams *et al.*, 1993; Jagura-Burdzy, Thomas, 1995).

Kuigi perekonnast *Pseudomonas* isoleeritud plasmiidid on jagatud vähemalt 14 mittesobivusgruppi (Thomas, Haines, 2004), kuuluvad nende senikirjeldatud kataboolsed plasmiidid gruppidesse IncP-1, -2, -7 või -9 (Williams *et al.*, 2004; Ogawa *et al.*, 2004). Tänu oma laiale peremeeste ringile ning levikule keskkonnas on mittesobivusgrupi IncP-1 plasmiide kõige põhjalikumalt uuritud. Mitmete IncP-1 perekonda kuuluvate kataboolsete plasmiidide nukleotiidne järjestus on täielikult määratud nagu atrasiini lagundav pADP1 (Martinez *et al.*, 2001), haloatsetaati kataboliseeriv pUO1 (Sota *et al.*, 2003), kloro-aromaatikat lagundav pJP4 (Trefault *et al.*, 2004) ning 2,4-D kataboolne plasmiid pEST4011 (Vedler *et al.*, 2004).

Oletatakse, et plasmiidid, mis sisaldavad geene lagundamaks looduslikku päritolu keemilisi ühendeid erinevad plasmiididest, mis suudavad lagundada alles hiljuti inimtegevuse tagajärjel loodusesse viidud ksenobiootilisi ühendeid. Nii on välja pakutud, et IncP-1 tüüpi plasmiidid nagu pJP4 ja pSS60 (Layton *et al.*, 1992) kannavad endas geene pigem ksenobiootiliste ühendite lagundamiseks ning IncP-9 ja IncP-2 tüüpi plasmiidid sisaldavad looduslikku päritolu ühendite lagundamisradu (Top, Springael, 2003).

IncP-9 tüüpi plasmiidid on teadaolevalt isoleeritud vaid pseudomonaadidest. Nende seast on täielikult sekveneeritud naftaleeni lagundavad plasmiidid pDTG1 (GenBank: AF491307) (Dennis, Zylstra, 2004) ja kaua uuritud, kuid alles nüüdseks sekveneeritud, NAH7 (GenBank: AB237655) (Sota *et al*., 2006) ning ksüleeni ja tolueeni lagundav plasmiid pWW0 (Greated *et al.*, 2002). IncP-9 gruppi kuulub ka näiteks osaliselt järjestatud antibiootikumi resistentsusplasmiid pM3 (Greated *et al.*, 2000).

Senikirjeldatud IncP-7 tüüpi plasmiidide seast on põhjalikult kirjeldatud ja täielikult sekveneeritud *P. resinovorans* tüvest CA10 eraldatud plasmiid pCAR1, mis sisaldab geene karbasooli/dioksiini (*car*) ning antranilaadi (*ant*) täielikuks degradatsiooniks (Maeda *et al.*, 2003). Lagundamist läbi viivad geenid asuvad suurel transposoonil Tn*4676*. pCAR1 replikatsiooni jt. plasmiidi ellujäämiseks vajalikud geenid osutusid sarnasteks tolueeni plasmiidi pL6.5 *P. fluorescens*'ist (GenBank: AJ250853) ning naftaleeni plasmiidi pND6-1 *Pseudomonas* sp. tüve ND6 vastavate geenidega (Li *et al.*, 2004). Hiljuti on Yano jt. (2007) poolt sekveneeritud ~108 kb suurune mitte-konjugeeruv tolueeni plasmiid pWW53 *P. putida* tüvest MT53, mille elutähtsad osad osutusid sarnasteks plasmiididega pCAR ja pND6-1.

IncP-2 gruppi kuuluvad plasmiidid on suhteliselt suured (~500 kb) ning võimelised kergesti moodustama rekombinantseid molekule (Dennis, 2005). Kataboolsetest plasmiididest kuuluvad perekonda IncP-2 kampri degradatsioonirada kodeeriv plasmiid CAM (Tan, 1999) ning oktaani ja dekaani degradeeriv OCT plasmiid (Nojiri *et al.*, 2004). IncP-2 tüüpi plasmiide sisaldavate tüvede ristamisel saadakse sageli rekombinantseid hübriidseid plasmiide. Hübriidsed megaplasmiidid, mis sisaldavad nii *cam*- kui ka antibiootikumi resistenstusgeene on võimelised neis sisalduvaid omadusi kõrge sagedusega üle kandma retsipient-tüvedesse (Jacoby, 1983). Fenoole ja metüleeritud fenoole degradeerivast *P. putida* tüvest CF600 on isoleeritud fenooli plasmiid pVI150, mille mittesobivusgrupiks on samuti IncP-2 (Bartilson *et al.*, 1990).

1.2.3. Evolutsioon

Plasmiidide DNA järjestuste võrdlemine nii selgroo kui ka kataboolsete operonide osas on andnud võimaluse heita pilk kataboolsete plasmiidide evolutsioneerumisele. Nii on plasmiidide DNA järjestuste ning geneetilise ülesehituse võrdlemine viinud järeldusteni, et paljud ülekandevõimelised biodegradatiivsed plasmiidid, mis kodeerivad kataboolseid geene erinevate ühendite lagundamiseks, on selgroo osas homoloogsed. Seega esineb looduses grupp sarnase selgroo osaga plasmiide, erinedes üksteisest vaid neis sisalduvate kataboolsete radade poolest (Lehrbach *et al.*, 1983). Näitena võib tuua IncP-9 tüüpi naftaleeni plasmiidi pDTG1 ja TOL plasmiidi pWW0. Oletatakse, et pDTG1 on kujunenud tänu ülemise naftaleeni lagundamise raja (*nah* operon) ning alumise salitsülaadi lagundamise raja (*sal* operon) integratsioonile eellas-plasmiidi IncP-9 selgroogu läbi mitmete rekombinatsiooni sündmuste. Plasmiidi pWW0 puhul toimus aga vaid üks transpositsioon, kus eellas-plasmiidi koosseisu lülitati ksüleeni (*xyl*) lagundamiseks vajalikke geene sisaldav transposoon Tn*4653* (Dennis, 2005). Enne nende sündmuste toimumist võis eellas-IncP-9 plasmiidi näol tegemist olla umbes 25 kb suuruse DNA rõngaga, mis sisaldas replikatsiooniks, alalhoiuks ning ülekandeks vajalikke geene. Kui plasmiidide pDTG1 ja pWW0 selgroo-osade GC-sisaldused on väga sarnased, siis suuremad erinevused GC-sisalduse protsentides kataboolsete alade osas lubab oletada, et antud regioonid pärinevad erinevatest organismidest (Dennis, Zylstra, 2004; Greated *et al.*, 2000; Greated *et al.*, 2002). Kataboolsete geenide osas omab pDTG1 väga kõrget sarnasust plasmiidiga pND6-1, ülejäänud selgroo osas on pND6-1 homoloogne IncP-7 plasmiidide pCAR1 ja pL6.5-ga, mis lubab oletada, et plasmiid pDTG1 on kombinatsioon kahest plasmiidist (Dennis, 2005).

Plasmiidis NAH7 olevad *nah* geenid kodeeritakse defektselt transposoonilt Tn*4655* ning need on sarnased plasmiididel pDTG1 ja pND6-1 leiduvate kataboolsete geenidega (Tsuda, Iino, 1990). Selle põhjal võiks oletada, et ka pDTG1 tekkel võidi *nah*-geenid mobiliseerida pND6-1-sarnaselt plasmiidilt otse IncP-9 selgroogu. MGE-d, mis ümbritsevad plasmiidide pDTG1 ja pND6-1 ülemist ning alumist degradatsioonirada on aga liialt erinevad. Seega on *nah* geenide põlvnemine kummaski plasmiidis toimunud iseseisvalt läbi transpositsioonide või mittehomoloogse rekombinatsiooni teel ning pärinevad tõenäoliselt ka erinevatest allikatest (Dennis, Zylstra, 2004; Dennis, 2005; Li *et al.*, 2004).

1.2.4. Konjugatiivse ülekande mehhanismid

Bakteriaalse evolutsiooni käigus bakterite võime asustada uusi keskkondi on pigem tingitud uute geenide omandamisest horisontaalsel geeniülekandel kui punktmutatsioonide akumuleerumisest tingitud geeni funktsiooni modifitseerumisest (Francia *et al*., 2004). Bakteritel on unikaalseimaks mehhanismiks geeniülekandel konjugatsioon, mille käigus toimub plasmiidse DNA ülekanne doonortüvelt retsipiendile rakk-rakk kontaktil.

Paljud kataboolsed plasmiidid on konjugatiivsed ehk võimelised levima ühelt bakterilt teisele. Üldiselt kodeerivad suured plasmiidid tervet konjugatiivse ülekandumise masinavärki, samas kui paljud väikesed plasmiidid kodeerivad ainult *cis*- ja *trans*-funktsioone, mida on vaja, et iseseisvalt ülekanduvad plasmiidid neid mobiliseeriks. Konjugatiivse ülekande süsteemi plasmiidis moodustavad *oriT* piirkond, kust DNA ülekanne initsieeritakse, ning geenid, mis vastutavad doonor-raku ja retsipiendi omavahelise füüsilise kontakti moodustamise ja plasmiidse DNA ülekande eest (Zatyka, Thomas, 1998). Osa plasmiide on võimelised replitseeruma ainult ühe perekonna eri liikides, seega saab nende plasmiidide puhul konjugatsioon toimuda ainult ühe perekonna piires. Suur osa plasmiide on aga laia peremeesspetsiifikaga, võimaldades horisontaalset geenide levikut erinevate perekondade ja isegi sugukondade esindajate vahel (Thomas, 2000).

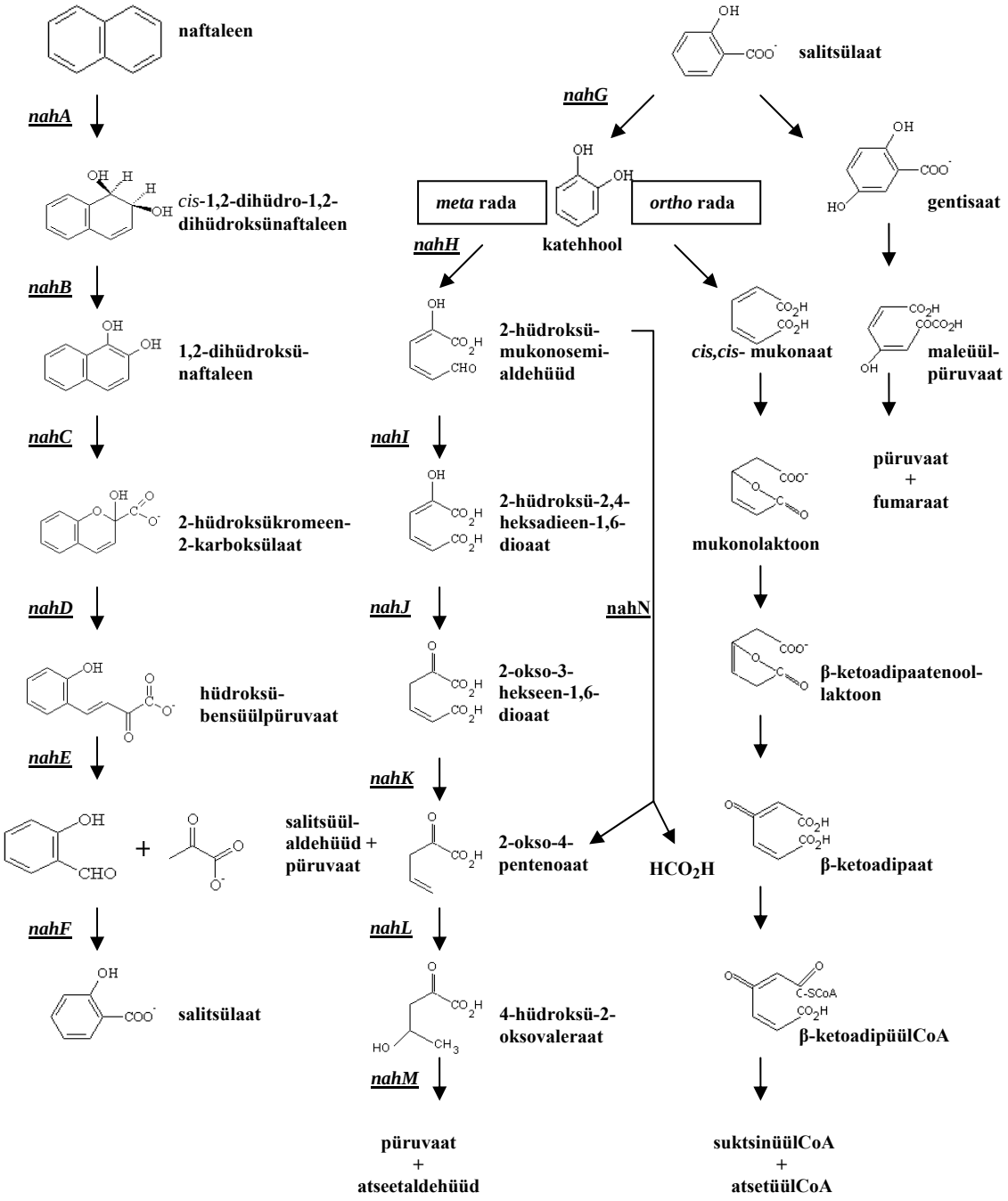
IncP-9 tüüp-plasmiidide pWW0 ja pDTG1 puhul vastutavad konjugatiivse plasmiidiülekande eest 19 geeni 20 kb suurusel selgroo fragmendil. Need geenid on grupeerunud 3 operoni, millest 2 paiknevad kahel pool *oriT*'st ning süntees neilt toimub erinevates suundades, andes produktideks TraA, TraB, TraC ning TraD, lisaks 2 hüpoteetilist valku, mis kotranskribeeritakse *traD*-ga. Tra-valgud osalevad *oriT* spetsiifilises äratundmises ning protsessingus. Kolmas operon (*mpf*A-J), millelt kodeeritud

10 valku vastutavad rakkudevahelise füüsilise kontakti loomise (*mating pair formation*) ja konjugatiivse piiluse moodustumise eest (Greated *et al.*, 2000; Dennis, Zylstra, 2004). Kuna ülekande geenide pidev ekspressioon oleks peremeesrakule metaboolseks koormaks, hoitakse plasmiidis pWW0 konjugatiivsete geenide süntees madalal tasemel konstitutiivsena läbi negatiivse tagasiside kontrollmehhanismi (Lambertsen *et al.*, 2004). Leitud on ka kontrollmehhanisme, kus ülekanne toimub vastusena teatud keskkonna ja füsioloogilistele tingimustele. Näiteks kirjeldasid Beaber jt. (2004) plasmiidi ülekande induktsiooni rakkudes, kus kutsuti esile SOS vastus. Plasmiidi pWW0 konjugatsioonil on täheldatud jämedate painduvate piilide moodustumine *P. putida* tüves mt-2 (Bradley, Williams, 1982), teiste *P. putida* tüvede puhul oli piiluse ekspressioon raku pinnal represseeritud, lubades oletada, et ülekande funktsioonide avaldumine sõltub suuresti ka peremeesrakust (Bradley, Williams, 1982). pWW0 ülekandesagedus on positiivses korrelatsioonis doonorrakkude kasvuga (Smets *et al.*, 1995), substraadi kontsentratsiooniga ja bakterite metaboolse aktiivsusega (Lambertsen, 2001). pWW0 ülekandesagedus võib olla väga kõrge, ulatudes optimaalsete tingimuste korral isegi 1 transkonjugandini doonor-raku kohta (Ramos-Gonzalez *et al.*, 1991).

1.3. Naftaleeni plasmiidid ja naftaleeni kataboolse raja geneetiline ülesehitus

1.3.1. Plasmiid NAH7

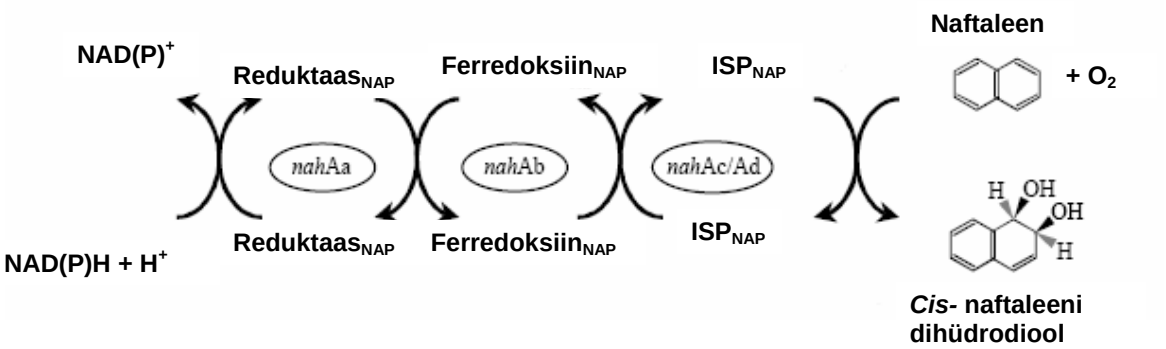
Naftaleeni plasmiidid sisaldavad kataboolseid geene naftaleeni degradatsiooniks üle salitsülaadi. *Pseudomonas putida* tüvest G7 (isoleeritud USA-st) eraldatud arhetüüpse naftaleeni plasmiidi NAH7 (83 kb) geenid on organiseeritud kahte operoni (Dunn, Gunsalus, 1973; Yen, Gunsalus, 1982). Neist esimene, *nahABCDEF* kodeerib ensüüme, mis on vajalikud naftaleeni konverteerimiseks salitsülaadiks (*nah* operon), ning teine, *nahGTHINLJKM* on vajalik salitsülaadi edasiseks konversiooniks põhiainevahetuse vaheühenditeni üle *meta* raja (*sal* operon) (Yen, Gunsalus, 1982) (joonis 3). Kõik need kataboolsed geenid asuvad 30 kb suurusel NAH7 plasmiidi fragmendil (Lal *et al.*, 1995). Süntees mõlemalt operonilt toimub ühes ja samas suunas ning on positiivselt reguleeritud LysR-tüüpi transkriptsiooni aktivaatori NahR valgu poolt (Yen, Gunsalus, 1985), mida ekspresseeritakse konstitutiivselt madalal tasemel (Cebolla *et al.*, 1997). *nahR* geeni 5' regioon kattub *nahG* geeni promootoralaga ning transkriptsioon sellelt toimub vastupidises suunas võrreldes kataboolsetelt geenidelt toimuva transkriptsiooniga.



Joonis 3. Naftaleeni ja salitsülaadi kataboolsed rajad pseudomonaadides (Yen, Serdar, 1988). ***nahA***- naftaleeni dioksügenaas; ***nahB***- *cis*-naftaleendihüdrodiooli dehüdrogenaas; ***nahC***- 1,2-dihüdroksünaftaleeni dioksügenaas; ***nahD***- 2-hüdroksükromeen-2-karboksülaadi isomeraas; ***nahE***- 2-hüdroksübensaalpüruvaadi aldolaas; ***nahF***- salitsüülaldehüüdi dehüdrogenaas; ***nahG***- salitsülaadi hüdroksülaas; ***nahH***- katehhooli 2,3-dioksügenaas; ***nahI***- 2-hüdroksümukonosemialdehüüdi dehüdrogenaas; ***nahJ***- 2-hüdroksümukonaadi tautomeraas; ***nahK***- 4-oksalokrotonaadi dekarboksülaas; ***nahL***- 2-okso-4-pentenoaadi hüdrataas; ***nahM***- 4-hüdroksü-2-oksovaleraadi aldolaas ***nahN***- hüdroksümukonosemialdehüüdi hüdrolaas.

Kui regulaatorvalk NahR seostub indutseerijaga, milleks on salitsülaat, aktiveerib see transkriptsiooni mõlemalt *nah* operonilt, seostudes vastavate operonide DNA enhaanserjärjestustele. NahR seondumine *nahG* geeni –60 bp *upstream* regioonile transkriptsiooni startsaidist indutseerib *nahG* ekspressiooni, kuid represseerib oma enda sünteesi negatiivse autoregulatsiooni kaudu (Schell, Poser, 1989).

Naftaleen on suletud bitsükliline aromaatne süsivesinik, mida leidub rohkesti toornafta ja nafta produktides. PAHide mikroobses lagundamisprotsessis on esmasteks ensüümideks dioksügenaasid, mis seavad aromaatse tuuma teiste ensüümide jaoks nn. ründevalmis. Dioksügenaasid on multimeersed, koosnedes kolmest komponendist: reduktaas, ferredoksiin ning raud-väävelvalk (*iron-sulfur protein* –ISP_{NAP}) (Simon *et al.*, 1993) (joonis 5). *P. putida* tüve G7 plasmiidilt NAH7 kodeeritud ISP_{NAP} koosneb kahest mitte-identsest subühikust, α ja β, mis on sünteesitud geenidelt *nahAc* ja *nahAd* (Sanseverino *et al.*, 1993; Stuart-Keil *et al.*, 1998; Mavrodi *et al.*, 2003). Need geenid on reguleeritud LysR-tüüpi regulaatorvalgu NahR poolt.



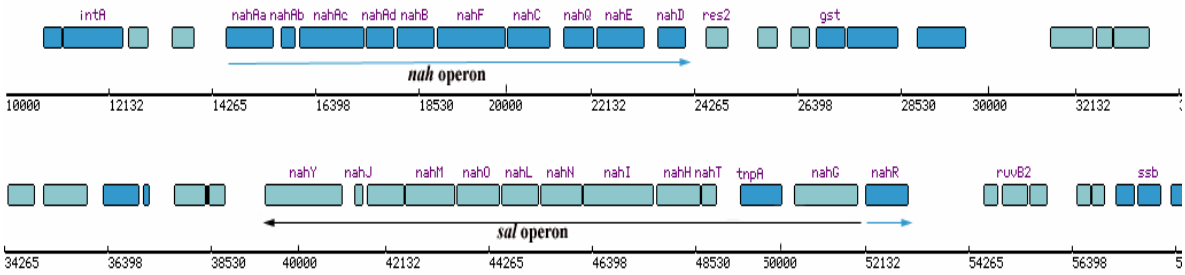
Joonis 5. Skemaatiline ülevaade naftaleeni dioksügenaasi funktsioonist.

Naftaleeni dioksügenaas vahendab lisaks naftaleeni degradatsioonile ka selliste polüaromaatsete ühendite lagundamist nagu fenantreen, antratseen, dibensotiofeen, fluoriin ning metüleeritud naftaleenid (Ahn *et al.*, 1999). *nahAc* geen on Gram-negatiivsete bakterite seas kõrgelt konserveerunud ning lisaks *Pseudomonas*'e liikidele on leitud sarnase nukleotiidse struktuuriga naftaleeni dioksügenaase ka perekondadest *Mycobacterium, Gordona, Sphingomonas, Rhodococcus,* ja *Xanthomonas* (Hamann et al., 1999).

1.3.2. Plasmiid pDTG1

Pseudomonas putida tüvest NCIB 9816-4 (isoleeritud Wales'ist) eraldatud 83 kb suurune naftaleeni plasmiid pDTG1 (Serdar, 1985) on täielikult sekveneeritud Dennis ja

Zylstra (2004) poolt. Plasmiidi pDTG1 kataboolsed geenid paiknevad kahes operonis, kuid erinevalt NAH7 plasmiidist, toimub süntees *nah* ja *sal* operonidelt erinevates suundades (You *et al*., 1988) (joonis 4). Kataboolsete geenide ekspressioon on LysR-tüüpi transkriptsiooni aktivaatori NahR positiivse kontrolli all. *P. putida* NCIB 9816-4 ning veel seitsme naftaleeni lagundava bakteri *nahR*-sarnaste geenide ning *nahG-nahR* intergeense piirkonna omavahelisel võrdlemisel leiti, et intergeensed alad olid 100% identsed pDTG1 vastava piirkonnaga, samas kui NahR valku kodeerivad alad olid märkimisväärselt erinevad. Järeldati, et *nahG-nahR* regulaatorgeenid naftaleeni lagundavate bakterite hulgas on kõrgelt konserveerunud (Park *et al.*, 2002).



Joonis 4. Skemaatiline ülevaade pDTG1 plasmiidi kataboolsete geenide asetusest ja transkriptsiooni suundadest. Ensüümid: *nahAa*, naftaleeni 1,2-dioksügenaasi reduktaasi komponent; *nahAb*, naftaleeni 1,2-dioksügenaasi ferredoksiini komponent; *nahAc*, naftaleeni 1,2-dioksügenaasi raud-väävelvalgu suur subühik; *nahAd*, naftaleeni 1,2-dioksügenaasi raud-väävelvalgu väike subühik; *nahB*, 1,2-dihüdroksü-1,2-dihüdronaftaleeni dehüdrogenaas; *nahF*, salitsüülaldehüüdi dehüdrogenaas; *nahC*, 1,2-dihüdroksünaftaleeni dioksügenaas; *nahQ*, funktsioon teadmata; *nahE*, trans-o-hüdroksübensüülideenpüruvaadi hüdrataas-aldolaas; *nahD*, 2-hüdroksükromeen-2-karboksülaadi isomeraas; *nahY*, kemotaksise valk; *nahJ*, 4-oksalokrotonaadi tautomeraas; *nahK*, 4-oksalokrotonaadi dekarboksülaas; *nahM*, 4-hüdroksü-2-oksovaleraadi aldolaas; *nahO*, atseetaldehüüdi dehüdrogenaas; *nahL*, 2-oksopent-4-pentenoaadi hüdrataas; *nahN*, 2-hüdroksümukonosemialdehüüdi hüdrolaas; *nahI*, 2-hüdroksümukonosemialdehüüdi dehüdrogenaas; *nahH*, katehhooli 2,3-dioksügenaas; *nahT*, ferredoksiin; *nahG*, salitsülaadi hüdrogenaas; *nahR*, LysR-tüüpi transkriptsiooni aktivaator.

1.3.3. Plasmiid pND6-1

Naftaleeni lagundavast tüvest *Pseudomonas* sp. ND6 (isoleeritud Hiinast) (Zhang *et al.*, 2000) eraldatud plasmiidi pND6-1 täisjärjestuse määramine näitas plasmiidi suuruseks 102 kb (Li *et al.*, 2004). Kataboolsete geenide organisatsioon on sarnane plasmiididest NAH7 ning pDTG1 leiduvate naftaleeni geenidega. Sarnaselt varemkirjeldatutele plasmiididele leidub ka plasmiidis pND6-1 *nah* operon, *sal* operon ning regulaatorgeen *nahR*, mis on nukleotiidse järjestuse osas väga kõrge identsusega plasmiidi pDTG1 vastavate kataboolsete aladega. Sarnaselt plasmiidile pDTG1 asub regulaatorgeen *sal* operoni ees ning transkriptsioon *nah* ja *sal* operonilt toimub erinevates suundades. pND6-1 plasmiidi puhul esines *sal* operoni ning *nahR* geeni vahel 6 hüpoteetilist valku või

transposaasi kodeerivat geeni rohkem kui plasmiidis pDTG1 ning plasmiidis pND6-1 ei esine transposaasi *tnpA*, mis paikneb plasmiidis pDTG1 *nahT* ja *nahG* geenide vahel. Lisaks leiti plasmiidist pND6 kaks isofunktsionaalset operonidest eraldi paiknevat geeni kodeerides vastavalt NahG ja NahW sarnast valku. Zhao jt. (2005) näitasid, et isofunktsionaalselt geenilt kodeeritud salitsülaadi hüdroksülaas NahU on palju kõrgema afiinsusega salitsülaadi ning kofaktorite suhtes ning on suurema katalüütilise efektiivsusega kui alumises lagundamisrajas olevalt geenilt kodeeritud NahG.

Plasmiidi pND6-1 selgroo-osa on sarnane plasmiidi pCAR1 selgroole, mille alusel paigutati kõnealune plasmiid IncP-7 gruppi (Maeda *et al.*, 2003).

TÖÖ EESMÄRGID

Käesoleva töö eesmärkideks oli:

1. Selgitada *P. fluorescens* tüve PC20 plasmiidne koostis ja plasmiidide kataboolne roll.
2. Määrata tüve PC20 fenooli ja naftaleeni lagundamist läbiviivate kataboolsete operonide lokalisatsioon ja geneetiline struktuur.
3. Määrata tüve PC20 plasmiidide mittesobivusgrupid ja konjugatiivse ülekande sagedused.
4. Iseloomustada tüves PC20 naftaleeni lagundamist läbiviivate võtme-ensüümide ekspressiooni.

2. MATERJAL JA METOODIKA

2.1. Töös kasutatud bakteritüved ja plasmiidid

Töös kasutatud bakteritüved, plasmiidid ning nukleotiidsed järjestused on kirjeldatud tabelis 1.

Tabel 1. Töös kasutatud bakteritüved ja plasmiidid ning Geenipangast saadud nukleotiidsed järjestused

Bakteritüvi	***Iseloomustus***	***Plasmiid*** ***(GenBank No.); IncP***	***Viide / allikas***
Bakteritüved			
Pseudomonas fluorescens F PC20	Phe^+ *p-*$Cre^+$$Nah^+$ Sal^+	pNAH20; pPHE20	Heinaru *et al.*, 2000
Pseudomonas fluorescens C PC24	Phe^+ *p-Cre*$^+$	pPC24	Heinaru *et al.*, 2000
Pseudomonas putida G7	Nah^+ Sal^+	NAH7 (AB237655); IncP-9	Ghosal *et al.*, 1987
Pseudomonas putida NCIB9816-4	Nah^+ Sal^+	pDTG1 (AF491307); IncP-9	Serdar *et al.*, 1985
Achromobacter xylosoxidans subsp. *denitrificans* EST4002	2,4-D	pEST4011; IncP-1	Mäe *et al.*, 1993
Pseudomonas putida PaW340	Nah^- Sal^- Phe^- Sm^R Trp^-	-	Jeenes, Williams, 1982
Pseudomonas putida PaW85	Nah^- Sal^- Phe^- Sm^S	-	
Pseudomonas putida TKSal23 PaW340	Nah^+ Sal^+ Phe^- Sm^R Trp^-	pNAH20	Käesolev töö
Pseudomonas putida TK3Phe PaW340	Nah^- Sal^- Phe^+ Sm^R Trp^-	pPHE20	Käesolev töö
Pseudomonas putida mt-2	Tol^+	pWW0 (AJ344068); IncP-9	Nakai *et al*., 1983
Pseudomonas putida CF600	Phe^+	pVI150; IncP-2	Shingler *et al.*, 1989
Pseudomonas putida Cg1	Nah^+ Sal^+	pCg1	Herrick *et al.*, 1997
Pseudomonas putida EST1026	Phe^+ Tol^-	-	Kivisaar *et al.*, 1990
Escherichia coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* φ80d *lacZ*	-	Hanahan, 1985
Plasmiidid			
	PCR produkti kloneerimisvektor	pTZ57R	Fermentas, Leedu
		pIS110	Inga Sarand (TÜTI)
Nukleotiidsed järjestused Geenipangast			
Pseudomonas putida MT53	Tol^+ Xyl^+	pWW53 (AB238971); IncP-7	Keil *et al.*, 1985
Pseudomonas putida	Sm^R	pM3; IncP-9	Greated *et al.*, 2000
Pseudomonas species ND6	Nah^+Sal^+	pND6-1 (AY208917); IncP-7	Li *et al.*, 2004

2.2. Bakteritüvede säilitamine ja kasvatamine

Bakteritüvesid säilitati 30%-lises glütseroolis -75°C juures või agarkultuurina 4°C juures minimaalsöötmel, mis sisaldas mineraalainete segu M9 (Adams, 1959), 0,25% mikroelemente (Bauchop, Elsden, 1960) ning süsiniku- ja energia-allikana vastavalt tüvedele kas 2,5 mM fenooli või 5 mM salitsülaati. Bakterimassi saamiseks kasutati kas eelpoolkirjeldatud minimaalsöödet või siis sama substraadi kontsentratsiooniga vedelat

minimaalsöödet. Vedelkultuurid kasvatati termostateeritud loksutil (180 pööret min^{-1}) 30°C juures. Naftaleeni lagundamise võime tuvastamiseks eksponeeriti kultuuri minimaalsöötmes naftaleeni aurudes (naftaleeni kristallid asetati kaanele ja tassi eksponeeriti ümberpööratud asendis).

2.3. DNA manipulatsioonid

2.3.1. Totaalse DNA eraldamine

Totaalse DNA eraldamiseks kasvatati rakke eelnevalt 5 ml LB söötmes termostateeritud loksutil (180 pööret min^{-1}) 30°C juures üleöö. DNA eraldamiseks kasutati *UltraClean* DNA isoleerimise komplekti (Mo Bio Laboratories, Inc., USA) ning sellele vastavat protokolli.

2.3.2. Plasmiidse DNA eraldamine

Plasmiidse DNA eraldamiseks kasutati aluselise lüüsi modifitseeritud meetodit (Connors, Barnsley, 1982). Saadud plasmiidse DNA proovid elektroforeesiti 0,9% agaroosgeelil ning visualiseeriti etiidiumbromiidiga (0,5 μg ml^{-1}). Kui vastavat DNA-d sooviti järgnevalt hübridiseerida, denatureeriti see geelis SOL A-ga [1,5 M NaCl; 0,5 M NaOH] 45 minutit ja neutraliseeriti SOL B-ga [1,5 M NaCl; 0,5 M Tris-HCl, pH 7,2; 1 mM EDTA] 2 x 30 minutit ning kanti geelist vaakumblotteriga (*Appligene*, Boekel Scientific, USA) nailonmembraanile (*Hybond^{TM}-N*, Amersham Biosciences UK Ltd., UK). DNA seoti kovalentselt filtri membraanile UV-valgusega 245 nm juures (*Stratalinker® 1800*; Stratagene, USA) ning hübridiseeriti.

Restriktsiooni jaoks eraldati plasmiidne DNA kasutades *Qiagen MidiKit*'i (Qiagen Inc., USA) lähtudes tootja soovitustest protokollis.

2.3.3. Radioaktiivse DNA proovi valmistamine ja hübridisatsioon

Hübridisatsioonil kasutatud radioaktiivse märkega DNA proovid valmistati *pheA* geenist (947 bp) ja C23O geeni *nahH* fragmendist (924 bp), mis on amplifitseeritud vastavalt *P. putida* tüvest EST1026 ning *P. putida* tüvest G7. DNA märgistamiseks kasutati *random priming* meetodit. Märgisena viidi DNA-sse [α^{32}P]dCTP (DuPont/NEN Company, USA), kasutades *DecaLabel^{TM} DNA Labelling Kit*'i vastavalt firma standardprotokollile (Fermentas, Leedu). Töödeldud nailonmembraanid asetati hübridisatsioonitorudesse, lisati prehübridisatsioonilahust, mis sisaldas järgmisi ühendeid:

6x SSC; 5x Denhardti lahus [1 g l^{-1} Ficolli (*Type 400*; Amersham Biosciences UK Ltd., UK); 1 g l^{-1} polüvinüülpürrolidooni; 1 g l^{-1} veise seerumi albumiini (BSA) (*Fraction V*; Sigma-Aldrich, USA)], 0,5% SDS ning 20 µg ml^{-1} denatureeritud fragmenteeritud lõhe sperma DNA-d. Hübridisatsioonitorusid inkubeeriti 1 tund 65°C juures hübridisatsiooniahjus. Seejärel lisati denatureeritud märgistatud geeniproov ja inkubeeriti üleöö samades tingimustes. Peale hübridiseerimist pesti membraane hübridisatsiooniahjus (65°C juures) 2 x SSC (17,53 g l^{-1} NaCl; 8,82 g l^{-1} Na-tsitraati) – 0,1% SDS lahuses 2 x 15 minutit, siis 1x SSC (8,765 g l^{-1} NaCl; 4,41 g l^{-1} Na-tsitraati) - 0,1% SDS lahuses 1x15 minutit ja 0,1x SSC (0,876 g l^{-1} NaCl; 0,441 g l^{-1} Na-tsitraati) – 0,1% SDS-ga 1x 15 minutit. Peale pesemist filtrid kuivatati peaaegu kuivaks ja asetati kile vahele. Filtrid eksponeeriti *Molecular Dynamics PhosphoImager*'i (Amersham Biosciences UK Ltd., UK) kassetis ning saadud kujutist töödeldi arvutis, kasutades tarkvara *Image Quant* (ITC-Academic Computing Health, USA).

2.3.4. Restriktsioon

TKSal23 plasmiidse DNA restriktsioon viidi läbi reaktsioonisegus, lõppmahuga 50 µl, mis sisaldas ensüüme EcoRI (lõppkontsentratsiooniga 0,4 U μl^{-1}) O^+ puhvris või HindIII (lõppkontsentratsiooniga 0,4 U μl^{-1}) R^+ puhvris ning 10 µl plasmiidset DNA-d. Ensüümid ja puhver pärinesid firmast Fermentas, Leedu ning reaktsioon viidi läbi vastavalt firma protokolli soovitustele. Restriktsioon toimus 37°C juures 2 tundi, misjärel kanti restriktsioonisegu 2%-le agaroosgeelile. DNA fragmentide suuruste hindamiseks kasutati elektroforeesil 1 kb suurusmarkerit (Fermentas, Leedu).

Täielikult sekveneeritud plasmiidide pDTG1, pND6-1 ning NAH7 restriktsioon ensüümidega EcoRI ja HindIII viidi läbi virtuaalselt, kasutades plasmiidsete järjestuste töötlusprogrammi pDRAW32 (http://www.acaclone.com/) ning visualiseeriti virtuaalsel geelil samas programmis. Restriktsioonipiltide ühildamiseks rekonstrueeriti TKSal23 geelelektroforeesi pilt virtuaalgeeli joonisele.

2.3.5. Kloneerimine ja transformatsioon

Transformatsioonil kasutatud *E. coli* DH5α kompetentsed rakud valmistati Inoue jt. (1990) poolt väljatöötatud meetodil.

Puhastatud PCR-i fragmendid kloneeriti *InsT/Aclone* PCR produkti kloneerimiskomplekti pTZ57R vektorisse kasutades vastavaid protokolle (Fermentas, Leedu). Ligaasisegu lisati *E. coli* DH5α kompetentsetele rakkudele vahekorras 1:10,

misjärel hoiti neid 20 minutit jääl. Sellele järgnes temperatuurišokk (1,5 minutit 41°C juures) ning eksponeerimine jääl 10-15 minutit. Transformeerunud rakud külvati selektiivsele LB-ampitsilliin tardsöötmele, mis sisaldas ampitsilliini (0,15 mg ml^{-1}), IPTG-d (0,048 mg ml^{-1}) ja X-gal-i (0,08 mg ml^{-1}) ning kasvatati 37°C juures üleöö.

Vektorisse kloneeritud fragmentide õigsust kontrolliti PCR-ga, kasutades vektorspetsiifilisi M13 Forward ja M13 Reverse praimereid (tabel 2).

2.3.6. Polümeraasi ahelreaktsioon (PCR)

PCR-i reaktsioonisegu, lõppmahuga 25 µl, sisaldas 1x PCR puhvrit [75 mM Tris-HCl, pH 8,8; 20 mM $(NH_4)_2SO_4$; 0,01% Tween 20], 0,2 mM lõppkontsentratsioonis igat nukleotiidi (dATP, dGTP, dCTP, dTTP), praimerite paari (tabel 2), kumbagi 20 pmol, 2,5 mM lõppkontsentratsiooniga $MgCl_2$ ja 2 µl rakkude lüsaati või 1 µl totaalset või plasmiidset DNA-d. Termoresistentse polümeraasina kasutati 0,5 U *Thermus aquaticus*'e *Taq* DNA polümeraasi (Fermentas, Leedu).

Reaktsioonid viidi läbi *Mastercycler® personal* PCR masinaga (Eppendorf AG, Saksamaa), järgneva programmiga: initsiatsioon 96°C juures 3 minutit, millele järgneb 35 tsüklit denaturatsioon 94°C juures 45 sekundit kuni 1 minut, praimerite seondumine praimeritele vastava seondumistemperatuuri juures 45 sekundit ja DNA ahela süntees 72°C juures sõltuvalt produkti suurusest. Lõppekstensioon toimus 8 minuti jooksul 72°C juures.

Tabel 2. Töös kasutatud PCR praimerid

Nimi	5'→3' järjestus	t°C**	Suurus (bp)	Positsioon	Viide
pheA geeni amplifitseerimiseks (Hübridisatsioon, konjugatsiooni kontroll)					
pheA1	CAGGATCGAATATCGGTGGCCTCG	61	947	Forward, 734-757 *P. putida* pEST1226 *pheA*	Heinaru *et al.*, 2000
pheA2	CTTCACGCTGGCGTAACCAATCGC			Reverse, 1680-1703 *P. putida* pEST1226 *pheA*	Heinaru *et al.*, 2000
C23O geeni amplifitseerimiseks (Hübridisatsioon, konjugatsiooni kontroll, kataboolse ala sekveneerimine)					
C23O-ORF-F	AGGTGWCGTSATGAAMAAAGG*	60 →50 (10 tsükl.)	934	Reverse, 48604-48624 pDTG1 järgi	Junca, Pieper, 2003
C23O-ORF-R	TYAGGTSAKMACGGTCAKGAA*	55 (25 tsükl.)		Forward, 47691-47711 pDTG1 järgi	Junca, Pieper, 2003
rep-regiooni amplifitseerimseks (IncP gruppide määramine)					
korA3Fa	GCAGACCCATTCCATGACCACC	53	2108		Krasowiak *et al.*, 2002
Rep3Rc	CCACCGACACTGATGGTCTG				Krasowiak *et al.*, 2002
rep9F	CGCGGYACWTGGGTWCAGAC*	58	447		Käesolev töö
rep9R	GGYGGWTCCATRCCWGGRCC*				Käesolev töö
IncP-7-U	CCCTATCTCACGATGCTGTA	52	524		Izmalkova *et al.*, 2005
rep-reg-L	GCACAAACGGTCGTCAG				Izmalkova *et al.*, 2005
Kataboolsete geenide ja geenide-vaheliste alade amplifitseerimiseks					
nahAF	CCCTAGCGCGTAACTACCCC	42	1029	Forward, 16006-16025 pDTG1 järgi	Simon *et al.,* 1993
nahAR	GGTCCAGACCTCGGTGGTG			Reverse, 17016-17034 pDTG1 järgi	Simon *et al.,* 1993
onahF	ATGGAACTGCGTGATCTGGA	60	904	Forward, 52104-52123 pDTG1 järgi	Park *et al.*, 2002
onahR	TCAATCAGAAAACAGGTCGAAC			Reverse, 52988-53006 pDTG1 järgi	Park *et al.*, 2002
proF	GGTCAGGCCCAGGTTCTC	60	284	Reverse, 52185-52202 pDTG1 järgi	Park *et al.*, 2002
proR	ACCGATGCGCAAGCCAGGTTTATT			Forward, 51920-51943 pDTG1 järgi	Park *et al.*, 2002
nahHF	GTATCGAGCGTTATTCAAAGCCG	58	979	Reverse, 48687-48666 pDTG1 järgi	Ghosal *et al.,* 1987
nahHR	CGGCCTTTAGGTCATAACGGTCATG			Forward, 47686-47709 pDTG1 järgi	Ghosal *et al.,* 1987
nahG1F	GGTACGTCCACCAAGTGCTCG	57	480	Forward, 51965-51082 pDTG1 järgi	Käesolev töö
nahG1R	GTCAACTGTAAACCATCATGAG			Reverse, 51487-51503 pDTG1 järgi	Käesolev töö
nahG2F	CGTATAACTCGCCGGTCTCC	57	561	Forward, 50812-50831 pDTG1 järgi	Käesolev töö
nahG2R	GGTCGACAGCCTACACCTG			Reverse, 51371-51353 pDTG1 järgi	Käesolev töö
nahG3F	GAAGCCTACGACGACCTGC	57	1090	Reverse, 50887-50869 pDTG1 järgi	Käesolev töö
nahG3R	CTGCCAGGCTTTGAATAACG			Forward, 48660-48679 pDTG1 järgi	Käesolev töö
Vektorspetsiifilised praimerid					
M13F	CATTTTGCTTGCCGGTCA	52			Fermentas
M13R	AACAGCTATGACCATG				Fermentas

*Nukleotiidide tähistused: **W**, A või T; **S**, C või G; **M**, A või C; **Y**, C või T; **K**, G või T; **R**, A või G

****t°C,** praimerite seondumistemperatuur

2.3.7. PCR produktide kontroll, visualiseerimine ning geelist puhastamine

PCR produktid kontrolliti elektroforeetiliselt 0,8%-lisel agaroosgeelil 1 kb DNA suurusmarkeriga (*GeneRuler^{TM} 1 kb DNA Ladder*, Fermentas, Leedu) võrdlemisel. Naftaleeniraja geenide amplifitseerimisel kasutati positiivse kontrollina *P. putida* tüve G7 DNA-d. Geel valmistati TAE [50 mM Tris-atsetaat; 1 mM EDTA; pH 8,2] puhvris ja pärast agaroosi täielikku lahustumist lisati etiidiumbromiidi (0,5 μg ml^{-1}). DNA proovid segati 6x elektroforeesi värviga (0,2% broomfenoolsinine, 0,2% ksüleen-tsüanool, 60%

glütserool, 60 mM EDTA). Elektroforees toimus 100 V juures. DNA fragmendid visualiseeriti UV valguses.

Amplifitseeritud DNA produktid puhastati 2% agaroosgeelist, kasutades *QIAquick* (Qiagen Inc., USA) või *UltraClean* (Mo Bio Laboratories, Inc., USA) geelist ekstraheerimise kommertsiaalset komplekti ning vastavat protokolli.

2.4. Sekveneerimine ja järjestuste analüüs

2.4.1. Sekveneerimine

PCR-ga saadud fragmentide sekveneerimiseks teostati eelnevalt PCR reaktsioonisegule töötlus praimerite katkilõikamiseks ja nukleotiidide inaktiveerimiseks. Selleks kasutati ensüümi ExoI (eksonukleaas I) (USB Corporation, USA) lõppkontsentratsiooniga 0,4 U μl^{-1} ja SAP-i (kreveti aluseline fosfataas) (USB Corporation, USA), lõppkontsentratsiooniga 0,2 U μl^{-1}. Töötlus viidi läbi 37°C juures 15 minutit ja ensüümid inaktiveeriti 80°C juures 15 minutit.

Sekveneerimiseks kasutati *DYEnamic^{TM} ET Terminator Cycle Sequencing Kit*'i (Amersham Biosciences UK Ltd., UK). Sekveneerimisreaktsioonid viidi läbi PCR masinas järgmistel tingimustel: matriits-DNA kaksikahela denaturatsioon 95°C 25 sekundit, praimeri seondumine 15 sekundit, DNA ahela ekstensioon 60°C 1 minut. Vastavat tsüklit korrati 40 korda. PCR meetodil saadud puhastatud fragmendid sekveneeriti, kasutades vastavaid PCR praimereid (5 pmol). Kui sekveneeriti eelnevalt vektorisse viidud geeni fragmente, siis kasutati vektorspetsiifilisi praimereid (tabel 2). Sekveneerimine viidi läbi automaatsekvenaatoriga MegaBACE.

2.4.2. Sekveneeritud järjestuste analüüs

Sekveneeritud nukleotiidsed järjestused analüüsiti programmiga BioEdit (versioon 5.0.9) (Hall, 1999) ning järjestuste võrdlemiseks geenipangas (GenBank) olevate järjestustega kasutati programme BLASTN ja BLASTX (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Konjugatsioon

Konjugatsiooni läbiviimiseks kasvatati eelnevalt doonortüve PC20 (Phe^{+} Sm^{S} Trp^{+}) ja retsipienttüve PaW340 (Phe^{-} Sm^{R} Trp^{-}) rakud 5 ml LB puljongis üleöö. Seejärel tehti

ümberkülv uude LB söötmesse ning kasvatati 0,8 optilise tiheduse ühikuni 580 nm juures. Rakud koguti tsentrifuugimise teel ja võeti üles 100-200 µl LB söötmes. Filterkonjugatsiooniks segati kokku 200 µl doonortüve ja 50 µl retsipienttüve rakususpensiooni (10^6-10^8 CFU ml^{-1}) ja 50 µl kanti Sartoriuse steriilsele atsetaattselluloos-filtrile diameetriga 25 mm ning poori suurusega 0,2 µm, mis asetati LB tassile. Filtritel olevaid kultuure kasvatati tassil üleöö 30°C juures. Üleskasvanud rakkude massi eemaldamiseks filtrilt kanti see steriilselt 50 ml koonilisse kolbi ja raputati intensiivselt Vortexil 1 ml steriilse M9 lahusega. Saadud rakususpensioonid tsenrifuugiti, pesti steriilse M9-ga ja pärast rakkude suspendeerimist 200 µl M9-s valmistati kolooniate loendamiseks parajad lahjendused. Nendest plaaditi 100 µl erineva koostisega minimaalsöötmetele: (1) Sal^+ Trp^+ Sm^+; (2) Phe^+ Trp^+ Sm^+; (3) Nah^+ Trp^+ Sm^+, kus lõppkontsentratsioonid olid salitsülaadil 5 mM, fenoolil 2,5 mM ja trüptofaanil 8 mg ml^{-1}. Streptomütsiini kontsentratsioon söötmetes oli 1 mg ml^{-1}. Konjugandid kasvatati üles 30°C juures (3-5 päeva). Doonorrakkude arvukus loendati fenooli või glükoosi (0,2%) minimaalsöötmel. Spontaansete mutantide teket doonorrakkudel testiti $Glc^+Sm^+Trp^-$ minimaalsöötmel, mis sisaldas 0,2% glükoosi ja streptomütsiini (1 mg ml^{-1}). Konjugatsiooni protsess oli analoogne ka juhul, kui kasutati doonorrakkudena transkonjugante ja retsipiendina prototroofset *P. putida* PaW85 tüve. Sel juhul hinnati konjugatsiooni toimumist salitsülaadi ja fenooli minimaalsöötmetel.

2.6. Ensüümiaktiivsuste määramine

Induktsioonikatsed viidi läbi 250-ml Erlenmeyeri kolbides minimaalsöötmega, mis sisaldas kasaminohapete hüdrolüsaati (CAA) ja indutseeriva süsinikuallikana kas fenooli (2,5 mM), salitsülaati (5 mM) või naftaleeni kristalle (0,01%). Ensüümiaktiivsused määrati rakuvabades ekstraktides, mille saamiseks pesti eksponentsiaalse kasvufaasi keskosast kogutud rakke 2x külma 100 mM fosfaatpuhvriga (K_2HPO_4-KH_2PO_4; pH 7.5), resuspendeeriti samas puhvris ning lüüsiti ultraheliga. Purustamata rakud ja rakukestad eemaldati tsentrifuugimise teel (12000 x g 4°C juures 25 minutit). Saadud supernatanti kasutati rakuvaba ekstraktina ensüümide eriaktiivsuste määramisel.

C23O aktiivsus mõõdeti spektrofotomeetriga (*Ultrospec II*, Pharmacia LKB Biochrom Ltd., UK) 375 nm juures produkti, 2-hüdroksümukonosemialdehüüdi moodustumise järgi (Hegeman, 1966). Reaktsioon toimus 100 mM fosfaatpuhvris (pH 7,5), kuhu oli lisatud rakuvaba ekstrakt ning substraadina katehhool (0,3 mM).

C12O aktiivsus mõõdeti spektrofotomeetriga 260 nm juures produkti, *cis,cis*-mukonaadi tekke järgi (Feist, Hegeman, 1969). Reaktsioonisegu oli sama, mis C23O puhulgi, kuid C23O inaktiveerimiseks töödeldi seda eelnevalt 5 minutit 0,1% H_2O_2-ga.

Salitsülaadi hüdroksülaasi aktiivsus määrati spektrofotomeetriliselt NADH oksüdeerimise järgi 340 nm juures. Reaktsioon viidi läbi stabiliseerivas 100 mM fosfaatpuhvris (pH 7,6), millele oli lisatud EDTA (1 mM) ning substraadina Na-Sal (133 μM). Lisaks sellele koosnes reaktsioonisegu (2 ml) NADH-st (147 μM) ning rakuvabast ekstraktist. Endogeense NADH oksüdatsiooni määramiseks viidi reaktsioon läbi substraadita variandis.

Ensüümide eriaktiivsuste arvutamiseks leiti lisaks optilise tiheduse muutusele minutis valgu kontsentratsioon (mg ml^{-1} inkubatsioonisegus). Valgu kontsentratsioonid määrati Bradfordi meetodil (Bradford, 1976). Ensüümiaktiivsused väljendati mikromoolides minutis mg valgu kohta.

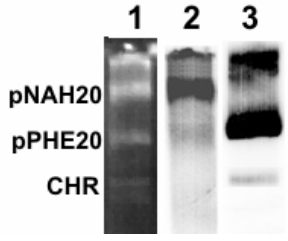
3. TULEMUSED JA ARUTELU

3.1. *P. fluorescens* tüve PC20 plasmiidid pNAH20 ja pPHE20

3.1.1. Üldiseloomustus

Käesoleva töö uurimisobjekt *Pseudomonas fluorescens* biotüüp F tüvi PC20 valiti fenooli ja *p*-kresooli lagundavate tüvede kollektsioonist lähemaks iseloomustamiseks eelkõige põhjusel, et lisaks kahe alternatiivse katabolismiraja (*meta* ja *ortho*) ekspresseerumisele aromaatse tuuma lagundamisel (Heinaru *et al.*, 2000), on see tüvi ka tugev biodegradeerija õlirikastes mikrokosmides (LISA I). Veelgi enam, *meta* raja võtme-ensüüm C23O grupeerus fülogeneetiliselt samasse rühma kirjanduses iseloomustatud naftaleeni lagundavate bakterite *P. putida* G7 ja *P. putida* NCIB9816-4 C23O-dega (LISA II). Tüvi PC20 on võimeline kasutama ainsa süsiniku- ja energia-allikana lisaks fenoolile ja *p*-kresoolile veel rida teisi toksilisi aromaatseid ja polüaromaatseid ühendeid, s.h. dimetüülfenoole ja naftaleeni. Nimetatud erisused ja varemtuvastatud kahe plasmiidse replikoni olemasolu (Heinaru *et al.*, 2000) olid põhjusteks antud tüve plasmiidse koostise lähemaks uurimiseks.

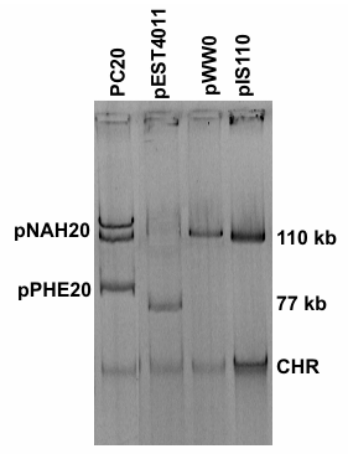
Tüve PC20 plasmiidse DNA hübridisatsioonil kahe erineva kataboolse geeni prooviga saadi suuremas plasmiidis signaal C23O geeniga *nahH* ning väiksemas fenooli lagundamise raja PMO geeniga *pheA* (joonis 6). Saadud tulemustest võib järeldada, et aromaatse tuuma lagundamise *meta* ja *ortho* rada asuvad erinevates plasmiidides, kusjuures suurem neist on seotud naftaleeni ja väiksem fenooli katabolismiga. Sellest lähtuvalt nimetati uuritavad plasmiidid vastavalt naftaleeni pNAH20 ja fenooli pPHE20 plasmiidideks.



Joonis 6. *P. fluorescens* tüve PC20 plasmiidse DNA agaroosgeelelektroforees (rada 1) ning hübridisatsioon *P. putida* G7 NAH7 C23O (*nahH*) geeni fragmendiga (rada 2) ja *P. putida* EST1026 PMO (*pheA*) geeni fragmendiga (rada 3). CHR – kromosoomne DNA.

Plasmiidide ligikaudse suuruse üle otsustamiseks võrreldi neid elektroforeetilise liikumise järgi sekveneeritud TOL plasmiidiga pWW0 (117 kb) (Greated *et al.*, 2002), dr.

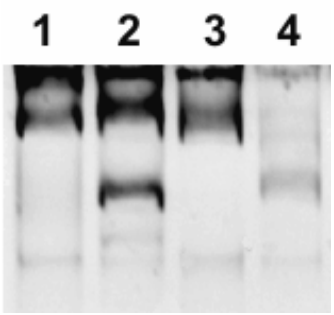
Inga Sarandilt (Tartu Ülikool, Tehnoloogia Instituut) saadud plasmiidiga pIS110 (110 kb) ning 2,4D plasmiidiga pEST4011 (77 kb) (Vedler *et al*., 2004) (joonis 7). pNAH20 ja pPHE20 ligikaudseteks suurusteks hinnati vastavalt 100 kb ja 80 kb.



Joonis 7. Plasmiidse DNA agaroosgeelelektroforeesi pilt *P. fluorescens* tüvel PC20 (pNAH20 ja pPHE20), *Achromobacter xylosoxidans* subsp. *denitrificans* tüvel EST4002 (pEST4011) (77 kb), *P. putida* tüvel mt-2 (pWW0) (117 kb) ning plasmiidist pIS110 (110 kb). CHR – kormosoomne DNA.

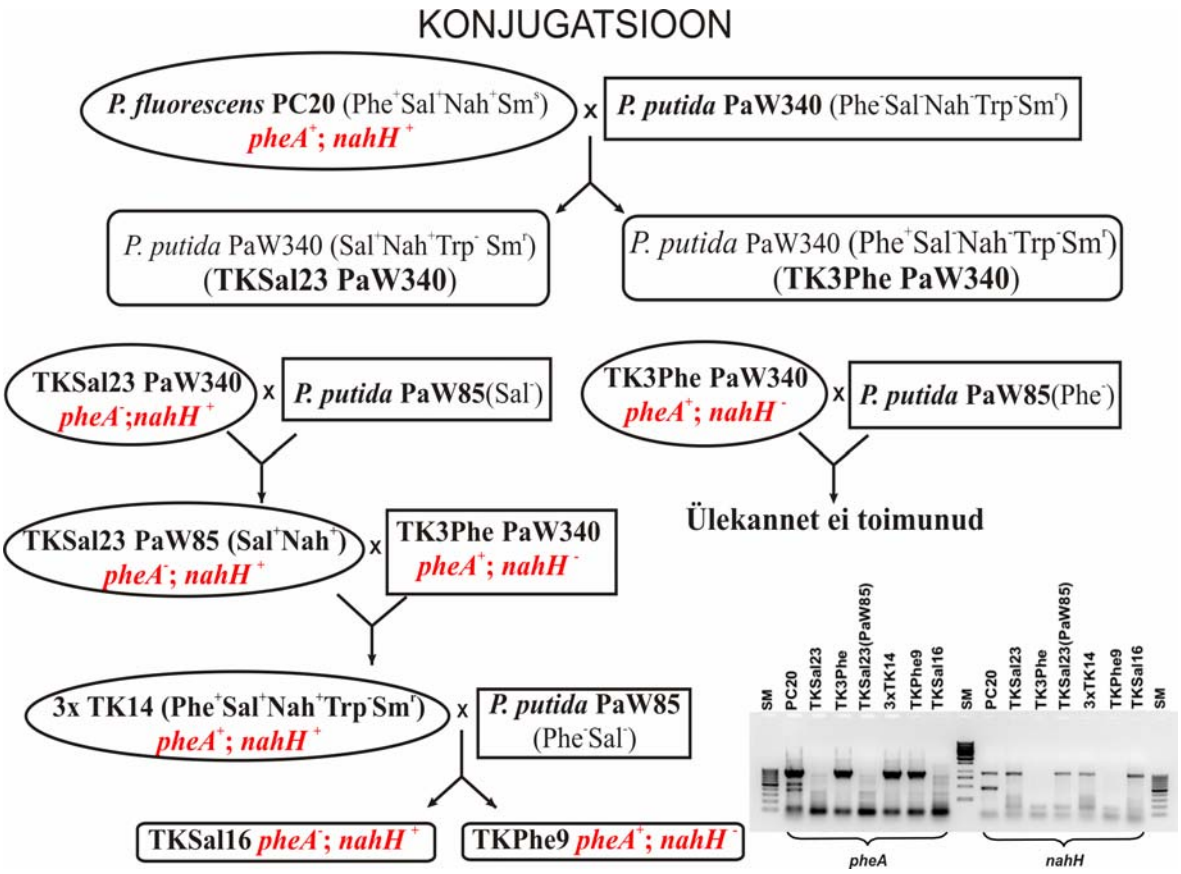
3.1.2. Konjugatiivne ülekanne

Selleks, et asuda tüve PC20 kahte plasmiidi eraldi uurima, testiti nende ülekandumise võimet. Esmalt õnnestus näidata doonortüvelt PC20 naftaleeni plasmiidi pNAH20 konjugatiivne ülekanne trüptofaani suhtes auksotroofsesse ja streptomütsiini suhtes resistentsesse retsipienttüvesse *P. putida* PaW340. Täiendavad konjugatsioonikatsed näitasid, et ka fenooli plasmiid pPHE20 on ülekandevõimeline retsipienttüvesse PaW340, kuigi ülekandesagedus ($6{,}4 \times 10^{-5}$) osutus 2 suurusjärku madalamaks kui pNAH20 ülekandesagedus ($1{,}5 \times 10^{-3}$) (tabel 3). Kõik uuritud kolooniad osutusid õigeteks transkonjugantideks mitte streptomütsiini suhtes resistentseteks mutantideks. Lähemaks uurimiseks valiti välja kumbagi plasmiidi omav transkonjugant, TKSal23 (pNAH20 PaW340-s) ja TK3Phe (pPHE20 PaW340-s). Salitsülaadil ja naftaleenil kasvava transkonjugandi TKSal23 PaW340 ning fenoolil kasvava transkonjugandi TK3Phe PaW340 plasmiidne DNA eraldati ning joonisel 8 esitatud plasmiidse DNA agaroosgeelelektroforeesi pilt kinnitab plasmiidide ülekandumist.



Joonis 8. Plasmiidse DNA agaroosgeelelektroforeesi pilt transkonjugantidel TKSal23 PaW340 (rada 3) ja TK3Phe PaW340 (rada 4). Võrdlusena on toodud *P. putida* tüve mt-2 (rada 1) ja doonortüve *P. fluorescens* PC20 plasmiidne DNA (rada 2).

Tüve PC20 plasmiidide omavahelise seotuse uurimiseks teostati ristamine mõlema transkonjugandiga, kasutades retsipienttüvena prototroofset *P. putida* tüve PaW85 (joonis 9).



Joonis 9. Skemaatiline ülevaade *P. fluorescens* tüve PC20 plasmiidide pNAH20 ja pPHE20 ülekandumise testimisest. Joonise alumises paremas nurgas on toodud kontrollmaterjalina *pheA*- (947 bp) ning *nahH*- (924 bp) spetsiifiliste praimeritega teostatud PCR-i agaroosgeelelektroforeesi pilt.

Ülekandevõimeliseks osutus vaid naftaleeni plasmiid pNAH20, mida tõestas saadud rekombinantide streptomütsiinitundlikkus ning võime kasvada nii salitsülaadi ja

naftaleeni söötmel ilma trüptofaani juuresolekuta. Ülekandesagedus oli pNAH20-l veidi väiksem (1,1 x 10^{-4}) kui ülekande korral algselt doonortüvelt PC20 (tabel 3). Ristamiste õigsust kontrolliti nii transkonjugantide kasvuga vastavatel söötmetel kui ka fenooli ja naftaleeni plasmiidis olevate kataboolsete radade markergeenide (*pheA* ja *nahH*) amplifitseerimisega PCR-i abil (tabel 2), mille tulemused on esitatud joonis 9 taskus.

Tabel 3. Plasmiidide pNAH20 ja pPHE20 ülekandumise ja „edasikandumise" sagedused määratuna transkonjugantide suhtearvuna doonorrakkudesse pärast 24-tunnist ristamist

Plasmiid	***Doonor***	***Retsipient***	***Ülekandesagedus***
pNAH20	*P. fluorescens* PC20	*P. putida* PaW340	1,5 x 10^{-3}
pPHE20	*P. fluorescens* PC20	*P. putida* PaW340	6,4 x 10^{-5}
pNAH20	TKSal23 PaW340	*P. putida* PaW85	1,1 x 10^{-4}
pPHE20	TK3Phe PaW340	*P. putida* PaW85	puudub

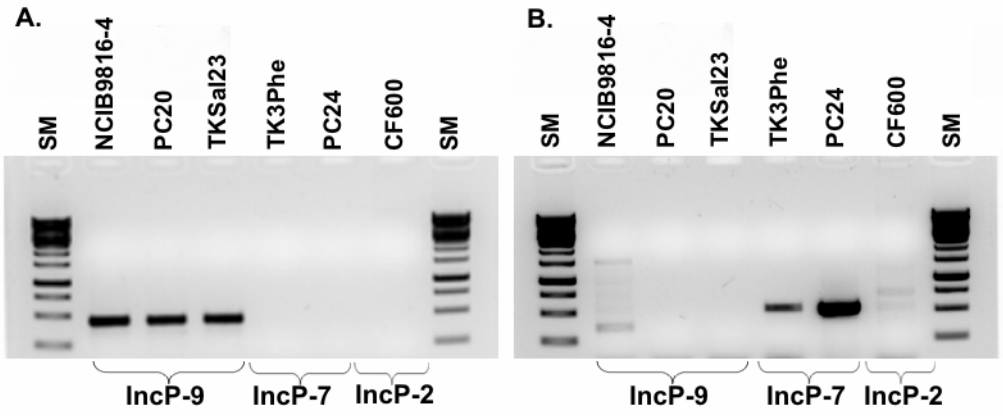
Transkonjugandis TK3Phe PaW340 olev fenooli plasmiid pPHE20 taasülekandevõimeliseks ei osutunud ning see lubab oletada, et fenooli plasmiidi pPHE20 konjugatsiooni toimumiseks on vajalik plasmiidi pNAH20 poolt kodeeritud valkude olemasolu.

Kui konjugatsiooni doonortüvena kasutati naftaleeni plasmiidi pNAH20 sisaldav TKSal23 PaW85 ning retsipienttüvena fenooli plasmiidi pPHE20 sisaldav TK3Phe PaW340, saadi ristamise tulemusena „ema"doonortüvega PC20 sarnaste kataboolsete omadustega auksotroofsed rekombinandid, näiteks 3xTK14 PaW340. Nii *pheA* geeni kui ka *nahH* geeni olemasolu transkonjugandis 3xTK14 lubab väita, et fenooli plasmiidi sisaldavasse retsipienttüvesse oli üle kandunud naftaleeni plasmiid pNAH20. Kui edasisel ristamisel *P. putida* PaW85'ga, saadi kahesuguseid transkonjugante, millest testiti TKSal16 (*pheA*$^-$ *nahH*$^+$) ja TKPhe9 (*pheA*$^+$ *nahH*$^-$), leidis kinnitust eelpool mainitud fakt, et tüve PC20 naftaleeni plasmiid pNAH20 on ülekandevõimeline, samas kui fenooli plasmiidi pPHE20 konjugatsiooni toimumiseks on vajalik plasmiidi pNAH20 poolt kodeeritud valkude juuresolek.

3.1.3. Määramine mittesobivusgruppidesse

Plasmiidide mittesobivusgruppide määramiseks on disainitud hulk praimereid replikatsiooni, stabiilsuse ja ülekandegeenide piirkonnast (Krasowiak *et al.,* 2002). Nii on valitud IncP-9 plasmiidide testimiseks kõrgelt konserveerunud alad: *oriV*, *rep*, *parB* ja *korA* geenid. Kahjuks ei sobi kõik kirjanduses toodud praimerite paarid, näiteks, *korA3*Fa

ja *rep3*Rc (Krasowiak *et al.*, 2002) IncP-9 plasmiidi gruppide detekteerimiseks ning iseloomustamiseks isolaatidest. Nimelt ei saadud spetsiifilist amplifikatsiooni produkti ka tüüpiliste IncP-9 plasmiidi omavate tüvedega nagu *P. putida* G7 ja *P. putida* NCIB9816-4, samas aga *P. putida* mt-2 selle andis. Seetõttu koostati käesoleva töö käigus IncP-9 mittesobivusgrupi määramiseks PCR praimerid rep9F ja rep9R põhinedes kolme täielikult sekveneeritud IncP-9 plasmiidi *repA* geeni nukleotiidsele järjestusele. Disainitud praimerite paar töötas efektiivselt ja andis spetsiifilised produktid kõigi meie poolt kasutatud IncP-9 tüüpi plasmiidi sisaldavate tüvedega ja ka naftaleeni plasmiidi pNAH20-ga sõltumata sellest, kas PCR-is kasutati märklaud-DNA-na plasmiidset või totaalset DNA-d, mis oli eraldatud nii transkonjugandist kui ka algtüvest PC20 (joonis 10). Produkt puudus nii fenooli plasmiidi omaval transkonjugandil TK3Phe, *P. fluorescens* tüvel PC24 kui ka *P. putida* tüvel CF600. Viimase tüve plasmiid kuulub IncP-2 gruppi ning antud juhul kasutati seda negatiivse kontrollina.



Joonis 10. Agaroosgeelelektroforees PCRi produktidest plasmiidide mittesobivusgruppide IncP-9- (A osa) ja IncP-7- (B osa) spetsiifiliste praimeritega *repA* geeni regioonidest. Lühendid: **SM** suurusmarker, **NCIB9816-4** *P. putida* NCIB 9816-4 (plasmiid pDTG1); **PC20** *P. fluorescens* PC20; **TKSal23** naftaleeni plasmiidi pNAH20 sisaldav transkonjugant *P. putida* TKSal23 PaW340; **TK3Phe** fenooli plasmiidi pPHE20 sisaldav transkonjugant *P. putida* TK3Phe PaW340; **PC24** *P. fluorescens* PC24; **CF600** *P. putida* CF600 (plasmiid pVI150).

IncP-9-spetsiifiliste praimeritega saadud 406 bp suurune produkt 570 bp suurusest *repA* geenist plasmiidis pNAH20 sekveneeriti ning saadud nukleotiidset järjestust võrreldi teiste teadaolevalt IncP-9 gruppi kuuluvate plasmiidide vastavate järjestustega. Tabelis 4 esitatud kõrged identsusprotsendid kinnitavad eeltoodud väidet, et *P. fluorescens* tüve PC20 plasmiid pNAH20 kuulub mittesobivusgruppi IncP-9 ja annab 100%-lise identsuse naftaleeni plasmiidi pDTG1 *repA* geeniga

Tabel 4. 570 bp suuruse *repA* geeni 406 bp pikkuste nukleotiidsete järjestuste identsuse võrdlus transkonjugandi TKSal23 PaW340 (**pNAH20**) ning teistel teadaolevalt IncP-9 gruppi kuuluvatel plasmiididel: **pNAH7** *P. putida* tüves G7; **pCg1** *P. putida* tüves Cg1; **pDTG1** *P. putida* tüves NCIB 9816-4; **pWW0** *P. putida* tüves mt-2; **pM3** *P. putida*'s

	pNAH20	**pNAH7**	**pCg1**	**pDTG1**	**pWW0**
pM3	74,8%	73,8%	74,8%	74,8%	83,5%
pWW0	75,1%	74,9%	75,1%	75,1%	
pDTG1	100%	92,3%	100%		
pCg1	100%	92,3%			
pNAH7	92,3%				

IncP-7 tüüpi plasmiidid ei oma kuigi kõrgelt konserveerunud plasmiidi selgroogu, vaid alluvad kergesti horisontaalse geeniülekandega seotud muutustele (Dennis, 2005). Selline selgroo ebatavaline mosaiiksus lubab spekuleerida, et IncP-7 tüüpi plasmiidid, alludes mitmesugustele geneetilistele ümberkorraldustele, annavad küll rakule eelise spetsiifiliste keskkonnatingimustega kohanemiseks, kuid nende määramine spetsiifiliste PCR-i produktide amplifitseerimise järgi pole lihtne. Tüve PC20 fenooliplasmiidi mittesobivusgrupi määramiseks kasutati IncP-7 gruppi kuuluvate plasmiidide detekteerimiseks kirjanduses toodud *repA* geeni regiooni praimereid (Upper ja Lower) (Izmalkova *et al.*, 2005). Joonisel 10 (B paneel) toodud agaroosgeelelektroforeesipilt näitab, et eeltoodud praimereid kasutades saadi produkt transkonjugandi TK3Phe ja juhuslikult meie fenooli ja *p*-kresooli lagundavate tüvede muuseumist valitud plasmiidse replikoniga tüve *P. fluorescens* C PC24 kasutamisel märklaud DNA-na (tabel 1). Produkti ei saadud ei tüve NCIB 9816-4, transkonjugandi TKSal23 ega tüve CF600 (negatiivne kontroll) puhul ning üllatuslikult ka tüve PC20-ga.

PCR-i produktide spetsiifilisust kontrolliti nukleotiidsete järjestuste põhjal. Plasmiidi pPHE20 ja tüve PC24 plasmiidi 524 bp suurune *rep*- regioon sekveneeriti ning saadud nukleotiidset järjestust võrreldi teiste teadaolevalt IncP-7 gruppi kuuluvate plasmiidide vastavate järjestustega (tabel 5). Selgus, et fenooli plasmiidi *repA geeni* järjestus oli 100%-liselt identne naftaleeni plasmiidi pND6-1, TOL plasmiidi pWW53 ja meie tüve PC 24 plasmiidi *repA* geeniga.

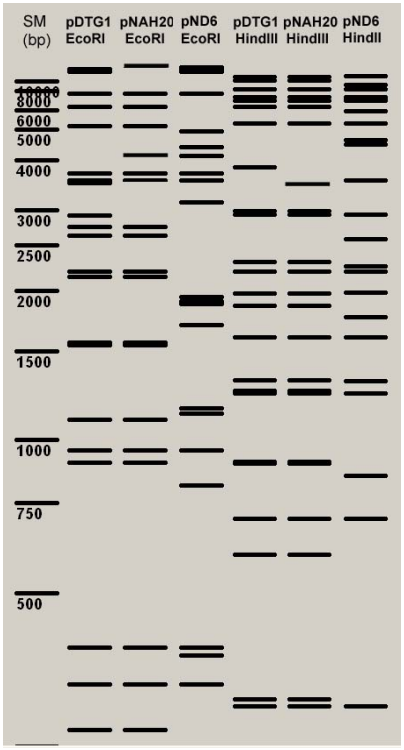
Tabel 5. 524 bp suuruste *repA* geeni regiooni fragmentide nukleotiidsete järjestuste identsuse võrdlus transkonjugandi TK3Phe PaW340 (**pPHE20**) ning teistel IncP-7 gruppi kuuluvatel plasmiididel: **PC24** plasmiid *P. fluorescens* tüves PC24; **pWW53** *P. putida* tüves MT53; **pND6-1** *Pseudomonas* sp. tüves ND6; **pCAR1** *P. resinovarans* tüves CA10

	pPHE20	**PC24**	**pWW53**	**pND6-1**
pCAR1	97%	97%	97%	97%
pND6-1	100%	100%	100%	
pWW53	100%	100%		
PC24	100%			

3.2.. pNAH20 võrdlemine teiste naftaleeni plasmiididega

3.2.1. Restriktsioonanalüüs

Transkonjugandist TKSal23 PaW340 eraldatud plasmiidne DNA restrikteeriti ensüümidega EcoRI ja HindIII. Joonisel 11 on toodud programmis pDRAW32 restrikteeritud plasmiidide pDTG1 ja pND6-1 restriktsioonipildid võrrelduna rekonstruktsiooniga plasmiidi pNAH20 restriktsioonipildist. Plasmiidide pNAH20 ja pDTG1 restriktsioonipiltide suur sarnasus lubab väita, et antud plasmiidid on nii geneetiliselt kui ka ülesehituselt lähedased. Märksa suurem on aga erinevus IncP7 gruppi kuuluval naftaleeni plasmiidil pND6 plasmiidide pNAH20 ja pDTG1-ga.



Joonis 11. Plasmiidse DNA restriktsioon ensüümidega EcoRI ja HindIII *P. putida* tüve NCIB9816-4 plasmiidi pDTG1 ning *Pseudomonas* sp. ND6 plasmiidi pND6 puhul tarkvaraprogrammis pDRAW32 ning *P. fluorescens* tüve PC20 puhul rekonstruktsioon agaroosgeelelektroforeesi pildist

Kui IncP9 naftaleeni plasmiidide ja isegi TOL plasmiidi pWW0 selgroo regioon 40 kb ulatuses on sama struktuuriga (Greated *et al.,* 2002; Dennis, Zylstra, 2004; Sota *et al.,* 2006), siis kataboolne operon on sarnane erinevatesse IncP gruppidesse kuuluvatel naftaleeni plasmiididel (pND6, pDTG1 ja pNAH7) (Li *et al.,*2004).

3.2.2. Kataboolne ala

Multimeerse naftaleeni dioksügenaasi üks geenidest *nahAc* on looduses kõrgelt konserveerunud ning leidnud rakendust potentsiaalse biomarkerina polütsükliliste aromaatsete süsivesinike (PAH-ide) lagundamise aktiivsuse määramisel keskkonnas. Naftaleeni 1,2-dioksügenaasi raud-väävelvalgu suurt subühikut kodeeriv geen *nahAc* plasmiidis pNAH20 amplifitseeriti, kloneeriti ning määrati nukleotiidne järjestus 940 bp ulatuses geeni algusosast (GenBank EF680322). Identsused nukleotiidses jäjestuses võrdlusplasmiidide pNAH7, pDTG1 ning pND6-1 vastava alaga saadi vastavalt 94,5%, 99,6% ning 99,3%.

Täielikult on sekveneeritud pNAH20 kataboolse ala regulaatorvalku kodeeriv geen *nahR*, *sal* operoni regulaatorala ning selle operoni algusosa geenid *nahG*, *nahT*, *nahH* ja nende geenide vahelised piirkonnad (GenBank AY887963), kokku 4120 bp. Identsusprotsendid 99-100 plasmiidi pDTG1 vastavate aladega (tabel 6) lubavad väita, et antud kataboolsed geenid on väga kõrgelt konserveerunud ka geograafiliselt kaugetes tüvedes. Kui pDTG1 identifitseeriti *P. putida* tüves NCIB9816-4 Serdari ja Gibsoni poolt (1989) Wales'is, siis teine naftaleeni plasmiid, pND6-1, homoloogse ülemise ja alumise kataboolse operoniga, isoleeriti Hiinas (Zhang *et al.*, 2000). Erinevalt meie poolt uuritud plasmiidist, mis kasutab naftaleeni lagundmisel *sal* operoni katehhooli *meta* rada, toimub katehhooli lagundamine *P. putida* tüves NCIB9816-1 kromosoomselt kodeeritud *ortho* raja kaudu (Dennis, Zylstra, 2004). See on tingitud 1196 bp pikkuse IS elemendi insertsioonist salitsülaadi hüdroksülaasi (*nahG*) ja katehhooli 2,3-diokügenaasi (*nahH*) geeni vahele, mis plasmiidis pNAH20 puudub.

Tabel 6. Plasmiidi pNAH20 geenide ning geenide vaheliste alade nukleotiidse järjestuse sarnasusprotsendid plasmiidide NAH7, pDTG1 ning pND6-1 vastavate aladega. Protsentide alla on toodud antud regiooni puudutavad märkused

	NAH7	***pDTG1***	***pND6-1***
nahR	90% Kodeeritav ala 222 bp võrra geeni lõpuosas pikem	99%	99%
nahR...nahG	77,9%	100%	100% 5635 bp suurune insertsioon
nahG	93,1%	100%	100%
nahG...nahT	88%	100% 1196 bp suurune IS element	100%
nahT	89,1% Kodeeritav ala 12 bp võrra geeni lõpus pikem	99%	99%
nahT...nahH	87,5%	100%	100%
nahH	91%	99%	99%

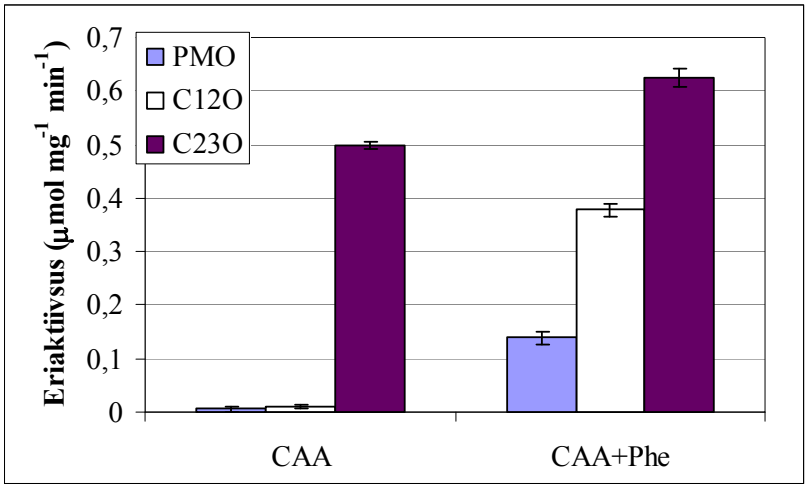
Plasmiidilt NAH7 kodeeritav regulaatorvalk NahR on 74 aminohappe võrra pikem kui plasmiididelt pNAH20 ja pDTG1 (tabel 6), mis on tingitud raaminihkest 281. aminohappe juures. Seega on regulaatorvalkude järjestuste lõpuosad 20 aminohappe osas täiesti erinevad. Kas ja kuidas sellised erinevused mõjutavad ekspressiooni NahR valgu poolt kontrollitud operonidelt, vajab täiendavaid võrdluskatseid. Ka väike ferredoksiini-sarnast valku kodeeriv geen *nahT* on plasmiidis pNAH20 12 bp võrra lühem ning sellevõrra on pikenenud geenide *nahT* ja *nahH* vaheline ala. Tõenäoliselt on valgu lühenemise põhjustanud stop-koodoni kaasa toonud mutatsioon. Sarnasusprotsendid eelnimetatud geenide ja geenivaheliste alade puhul plasmiididides pNAH20 ja NAH7 jäävad vahemikku 77,9 kuni 93,1%.

Plasmiidis pND6-1 on *nahR* geeni ja *sal* operoni vahel 5635 bp suurune insertsioon (6 transposaasi sarnast geeni) võrreldes teiste kõnealuste plasmiididega. Erinevalt pDTG1 plasmiidist sisaldab pND6-1 isofunktsionaalset salitsülaadi hüdroksülaasi geeni *nahU*, mis paikneb naftaleeni lagundamise alumise raja geenidest eraldi. NahU valgul on suurem seondumisvõime salitsülaadi ja kofaktoritega ning kõrgem katalüütiline efektiivsus (Zhao *et al.,* 2005).

3.3. Fenooli ja naftaleeni kataboliseerivate võtme-ensüümide ekspressioon

Tüve PC20 fenooli lagundamisel osalevate ensüümide ekspressiooni uuriti induktorivabas (CAA) ja fenooliga indutseeritud tingimustes (CAA+Phe) (joonis 12). PMO ja C12O, mida kodeerib *pheBA* operon, ekspresseeruvad vaid fenooli juuresolekul, C23O süntees aga on tugeva konstitutiivse tasemega. Siit järeldub, et tüves PC20 võib

fenooli katabolismis samaaegselt funktsioneeruda kaks alternatiivset aromaatse rõnga lagundamises osalevat dioksügenaasi.



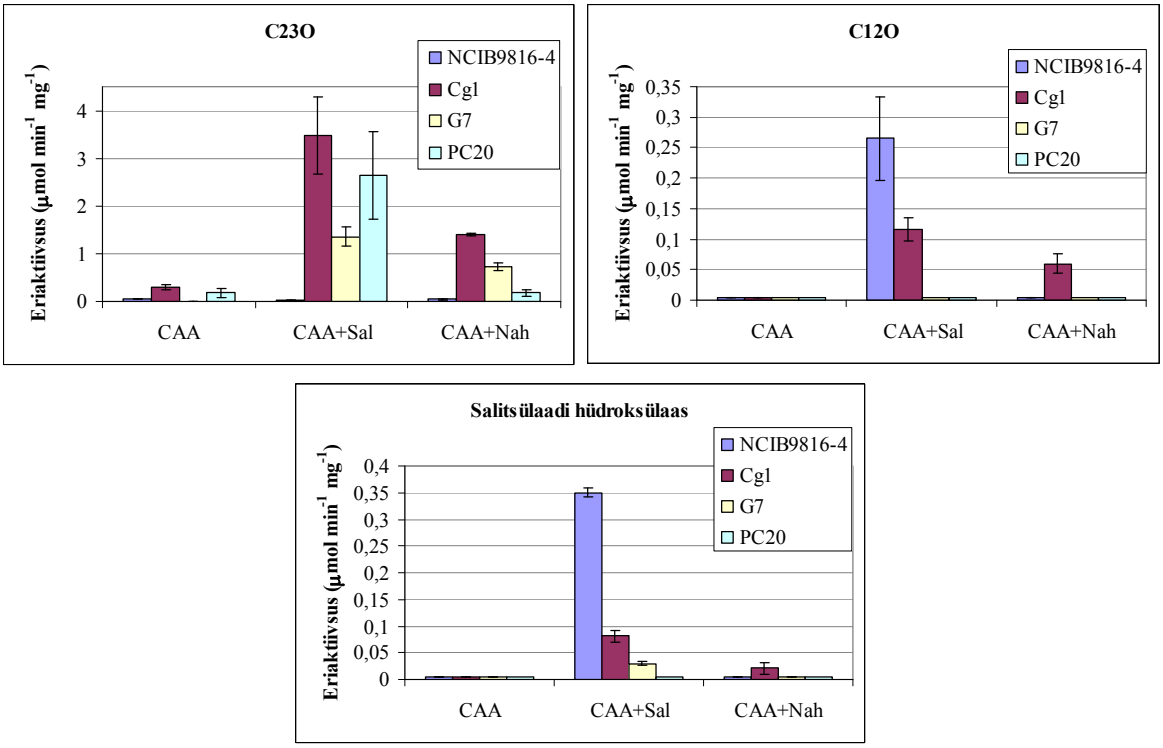
Joonis 12. Kataboolsete ensüümide induktsioon fenooliga rakuvabades *P. fluorescens* PC20 ekstraktides.

Naftaleeni katabolismis osalevate ensüümide induktsiooni tüves PC20 uuriti võrdlevalt ka teistes meile kättesaadavates naftaleeni lagundavates tüüptüvedes. C23O-i, C12O-i ning salitsülaadi hüdroksülaasi aktiivsused määrati järgmistes tüvedes: *P. putida* NCIB9816-4, USA-st isoleeritud ning tüvega NCIB9816-4 sarnaseks märgitud (Stuart-Keil, *et al.*, 1998) *P. putida* Cg1 ja *P. putida* G7 (joonis 13). Leidis kinnitust väide, et *P. putida* tüves NCIB9816-4 toimub aromaatse tuuma lõhustamine üle katehhooli *ortho* raja, kuna induktsioonil salitsülaadiga saadi C12O-i aktiivsus ning C23O-i aktiivsust ei õnnestunud detekteerida. Samuti on salitsülaadi hüdroksülaasi aktiivsus tüves NCIB 9816-4 võrreldes teiste tüvedega tugevalt indutseeritud salitsülaadi poolt. Tuleb siiski rõhutada fakti, et *meta* rada ei puudu selles tüves, vaid *nahH* geen on nõrgalt ekspresseerunud. Uurides selle tüve kasvu salitsülaadil kui ainsal C- ja energiaallikal, saime C12O ja C23O eriaktiivsused vastavalt 1,124 ± 0,038 ja 0,472 ± 0,006 µmooli min^{-1} mg^{-1}. Kasvukeskkonna pruunistumine ja tüve NCIB 9816-4 madal kasvukiirus salitsülaadil kui C-allikal võib-olla tingitud vaheprodukti katehhooli pöörduvast akumulatsioonist C23O madala aktiivsuse tõttu. Seda võimalust kinnitab ka Park kaastöölistega (2004).

P. putida tüves Cg1 oli täheldatav C23O-i sünteesi konstitutiivne tase sarnaselt tüvele PC20 (joonis 13). Salitsülaat ja isegi naftaleen indutseerivad tüves Cg1 erandlikult ka C12O-i sünteesi. Kuna kirjandusest on vähe teada tüve Cg1 geneetilise ning kataboolse ülesehituse kohta, puudub hetkel seletus, miks antud tüves sünteesitakse mõlemad aromaatse tuuma lõhustamise võtme-ensüümid, samal ajal kui induktorivabas

kasvukeskkonnas see puudub. Oletuslikult paikneb tüves Cg1 C12O-i kodeeriv geen kromosoomis, tüves PC20 asub C12O-i geen aga teises plasmiidis (pPHE20) ning selle süntees pole salitsülaadi poolt indutseeritav.

Erinevalt teistest vaatluse all olnud tüvedest, ei sünteesitud *P. putida* tüves G7 C23O-i konstitutiivselt. Võimalik, et konstitutiivsuse puudumine on kuidagi seotud regulaatorvalgu ehitusega. Nagu kirjeldatud peatükis 3.2.2 erines plasmiid NAH7 teiste plasmiidide regulaatorvalkudest primaarstruktuuri poolest 20 aminohappe ulatuses järjestuse lõpuosas ning oli 74 aminohappe võrra pikem. Vastavalt ootustele, C12O-i aktiivsust tüves G7 ei detekteeritud (joonis 13).



Joonis 13. Naftaleeni katabolismiensüümide ekspressioon induktsioonil salitsülaadi (CAA+Sal) ja naftaleeniga (CAA+Nah) erinevates naftaleeni lagundavates tüvedes: *P. putida* NCIB9816-4, *P. putida* Cg1, *P. putida* G7, *P. fluorescens* PC20.

Joonistelt 12 ja 13 on näha, et kui fenooli katabolismis esinevad tüves PC20 korraga mõlemad aromaatse tuuma lagundamise rajad, siis salitsülaadi lagundamisel osaleb vaid C23O (2,7 µmol min^{-1} mg^{-1}). Selline kahe raja kooseksisteerimine on tingitud C23O sünteesi konstitutiivsest baastasemest (0,2-0,5 µmol min^{-1} mg^{-1}).

KOKKUVÕTE JA JÄRELDUSED

Käesolevas töös uuriti bioaugmentatsioonil kasutatud fenooli ja naftaleeni lagundava bakteritüve *P. fluorescens* biotüüp F tüve PC20 plasmiidide poolt määratud geneetilisi erisusi ja kataboolseid omadusi. Tüvi PC20 on vastupidav fenoolse reostusega keskkonnatingimustes, tema plasmiidselt määratud kataboolsed omadused on looduskeskkonnas ülekanduvad sealsetele bakteritele ning plasmiidne ülekanne on kergesti jälgitav tänu naftaleeniraja alumise operoni võtme-ensüümi katehhooli 2,3-dioksügenaasi konstitutiivsele ekspressioonile. Töö pearõhk asetati tüve PC20 multiplasmiidse süsteemi uurimisele.

Tulemuste põhjal tehti järgmised järeldused:

1. *P. fluorescens* F tüvi PC20 sisaldab kahte erineva kataboolse funktsiooniga plasmiidset DNA replikoni, millest ~100 kb suurune plasmiid pNAH20 määrab naftaleeni lagundamist ja väiksem ~80 kb suurune plasmiid pPHE20 fenooli lagundamist.
2. Naftaleeni plasmiid pNAH20 kodeerib aromaatse tuuma lõhustamist üle *meta* raja ning fenooli plasmiid pPHE20 üle *ortho* raja. *ortho* raja induktsioonil säilub bakteritüves *meta* raja võtme-ensüümi katehhooli 2,3-dioksügenaasi konstitutiivselt sünteesitud kõrge tase.
3. Naftaleeni plasmiid pNAH20 on konjugatiivne ja fenoooli plasmiid pPHE20 mittekonjugatiivne.
4. Naftaleeni plasmiidi pNAH20 esinemisel on fenooli plasmiid pPHE20 konjugatiivselt ülekantav e. mobiliseeritav retsipienttüvedesse.
5. Naftaleeni plasmiid pNAH20 kuulub plasmiidsesse mittesobivusgruppi IncP-9 ja fenooli plasmiid pPHE20 gruppi Inc-P7.
6. Naftaleeni plasmiidi pNAH20 selgroog, aga ka rida kataboolseid geene on kõrgelt homoloogsed bakteritüve *P. putida* NCIB9816-4 plasmiidi pDTG1 vastava ala geenidega vaatamata sellele, et nende plasmiidide poolt määratud naftaleeni degradatsioonil kasutatakse erinevaid katehhooli lõhustamise radu.
7. Fenooli plasmiidi pPHE20 mobiliseeritavus naftaleeni plasmiidi pNAH20 vahendusel, plasmiidide kuuluvus erinevatesse plasmiidsetesse mittesobivusgruppidesse ning mõlema plasmiidi geenide üheaegne ekspressioon näitavad, et bakteritüves PC20 esineb funktsioneeriv multiplasmiidne süsteem.

SUMMARY

Genetic distinctness and catabolic properties of plasmids of the phenol and naphthalene degrading bacterial strain *P. fluorescens* F PC20 were studied. Strain PC20 is able to survive in environmental conditions polluted with phenolic compounds and was used in bioaugmentation studies. The plasmid encoded catabolic properties of this strain have been shown to be transferred horisontally to other bacteria in natural environments, which is easily traceable by virtue of constitutive synthesis and expression of catechol 2,3-dioxygenase, the key enzyme of the lower pathway of naphthtalene degradation. The main emphasis of this study was directed to multiplasmid system of the strain PC20. The following conclusions were made:

1. *P. fluorescens* F strain PC20 comprises two replicons of plasmid DNA with different catabolic functions. Plasmid pNAH20 with average size of 100 kb encodes genes for degradation of naphthalene and plasmid named as pPHE20 with average size of 80 kb encodes genes for phenol degradation.
2. Aromatic ring of the respective compounds are cleaved through *meta* cleavage pathway in case of naphthalene plasmid pNAH20 and through *ortho* cleavage pathway in case of phenol plasmid pPHE20. Induction of *ortho* cleavage pathway does not alter the high level of constitutive synthesis of catechol 2,3-dioxygenase.
3. Naphthalene plasmid pNAH20 is conjugative while the phenol plasmid pPHE20 is non-conjugative.
4. Phenol plasmid pPHE20 is mobilisable to other bacterial strains in the presence of naphthalene plasmid pNAH20.
5. Naphthalene plasmid pNAH20 belongs to incompatibility group IncP-9 while phenol plasmid pPHE20 belongs to IncP-7.
6. The backbone and also many of the catabolic genes of naphthalene plasmid pNAH20 are highly homologous to the respective genes encoded in plasmid pDTG1 of *P. putida* strain NCIB9816-4 despite of the fact that these plasmids use different catechol cleavage pathways.
7. All the aforementioned facts indicate that the strain PC20 comprises a functional multicomponent plasmid system for degradation of phenol and naphthalene.

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Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area

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Abstract

Denaturing gradient gel electrophoresis of amplified fragments of genes coding for 16S rRNA and for the largest subunit of multicomponent phenol hydroxylase (LmPH) was used to monitor the behaviour and relative abundance of mixed phenol-degrading bacterial populations (*Pseudomonas mendocina* PC1, *P. fluorescens* strains PC18, PC20 and PC24) during degradation of phenolic compounds in phenolic leachate- and oil-amended microcosms. The analysis indicated that specific bacterial populations were selected in each microcosm. The naphthalene-degrading strain PC20 was the dominant degrader in oil-amended microcosms and strain PC1 in phenolic leachate microcosms. Strain PC20 was not detectable after cultivation in phenolic leachate microcosms. Mixed bacterial populations in oil-amended microcosms aggregated and formed clumps, whereas the same bacteria had a planktonic mode of growth in phenolic leachate microcosms. Colony hybridisation data with catabolic gene specific probes indicated that, in leachate microcosms, the relative proportions of bacteria having *meta* (PC1) and *ortho* (PC24) pathways for degradation of phenol and *p*-cresol changed alternately. The shifts in the composition of mixed population indicated that different pathways of metabolism of aromatic compounds dominated and that this process is an optimised response to the contaminants present in microcosms.

Keywords: Biodegradation; Mixed cultures; Phenol hydroxylase; PAHs; PCR-DGGE

1. Introduction

Intrinsic biodegradation as well as bioremediation by selected biodegradative microorganisms are the key factors in restoration of polluted environments [1]. Bioavailability of pollutants and survival and catabolic activity of introduced microorganisms play important roles in bioremediation technologies. Therefore, before applying bioaugmentation it is necessary to isolate, identify and characterise pollutant-degrading indigenous bacterial strains and analyse their activity in situ. Laboratory experiments with pure cultures are also essential for detailed study of the physiology and genetics of microorganisms.

Catabolic pathways operating in natural communities reflect interactions between microbial species under mixed culture conditions where extensive sharing of nutritional resources is common [2] and interaction of two or several strains is often a prerequisite for growth and biodegradation [3]. It has already been shown that a mixed culture of strains with different catabolic types overcomes incompatibilities in degradation of divergent substrate mixtures [4]. Among multitude of microbial

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species in the environment, few populations that can use specific substrates become dominant, while others exist as minorities [5]. One caveat of microbial remediation systems is the fact that a high concentration of phenolic and other pollutants may be toxic to the microbes within the biological treatment compartment [6]. Therefore, a rapid method is needed to monitor the bacterial community during the treatment process.

Traditional microbiological methods, including plating onto selective minimal agar to enumerate viable bacteria, are known to underestimate the actual population values by one to two orders of magnitude [7]. Direct microscopic counts of bacteria often suffer from a high level of background staining and are very tedious due to accumulation of microorganisms at interfaces (development of a biofilm). On the other hand, molecular techniques have been successfully used to assess microbial activity of different strains and to detect specific functional genes [8,9]. The gene probes have been developed for particular microbial groups biodegrading common phenolic contaminants, including naphthalene [10].

The initial conversion of phenol carried out by either a monocomponent [11,12] or a multicomponent phenol hydroxylase [13,14] leads to central intermediates that are further degraded by either *ortho*- or *meta*-cleavage. The fragments of genes responsible for the monohydroxylation of the *ortho* position of the aromatic ring have been PCR-amplified from environmental DNA. The gene encoding the largest subunit of a multicomponent phenol hydroxylase (LmPH) has been used for the determination of genetic variability among the isolates of phenol-degrading bacteria [8].

The genes of ring-cleavage dioxygenases are good targets for monitoring of biodegrading populations involved in ring-cleavage of aromatics and in early steps of degradation of some polycyclic aromatic hydrocarbons (PAHs), which are very common in crude oil [15–17]. Aerobic biodegradation of PAHs with three or less rings (phenanthrene, anthracene, naphthalene) proceeds through a dioxygenase attack on an aromatic ring resulting in formation of a *cis*-dihydrodiol [18]. Salicylate formed after rearomatisation reactions is further catabolised via catechol or gentisate by dioxygenases. Catechol 2,3-dioxygenase (C23O) genes are among the most informative genetic markers in evaluation of the efficacy of bioremediation [10,19–23].

Our previous work demonstrated a multiplicity of catabolic types of degradation of phenol and *p*-cresol and the existence of characteristic assemblages of species and specific genotypes among the strains isolated from river water continuously polluted with phenolic compounds of oil shale ash leachate [24]. In the present investigation we have concentrated on functional analyses of these bacteria in removing phenols and PAHs from phenolic leachate and crude oil. For optimisation of bioremediation in the field, the dynamics of functionally dominant bacterial populations in laboratory microcosms was studied.

2. Materials and methods

2.1. Bacterial strains and their characterisation

Species identification according to analysis of BIOLOG GN data, rep-PCR genomic fingerprints and sequences of 16S rRNA genes revealed that bacterial strains used in this study belong to *Pseudomonas mendocina* PC1, *P. fluorescens* biotype G PC18, *P. fluorescens* biotype A PC20 and *P. fluorescens* biotype C PC24 [24,25]. Genomic DNA from following *P. putida* reference strains: mt-2 [26], CF600 [27] and EST1026 [28] was extracted and used as positive control for PCR detection of genes coding for C23O, LmPH and phenol monooxygenase (*pheA*), respectively. Approximately 1500-bp sequences of the 16S rRNA genes of strains PC1, PC18, PC20 and PC24 have been deposited in the GenBank data library under accession numbers AF232713, AF228366, AY538264 and AF228367, respectively.

2.2. Microcosm experiments, growth media and cell enumeration

Inocula for microcosms were pregrown in $1 \times$ M9 medium [24] containing 2.5 mM phenol. Cells of individual strains in the late logarithmic phase of growth were harvested by centrifugation at 12,000*g* for 10 min at 4 °C, washed and suspended in sterile $1 \times$ M9 medium and used to inoculate microcosms at final concentration of cells of approximately $1–1.5 \times 10^7$ colony forming units (CFU) ml^{-1}. Mixed cultures contained approximately equal numbers of cells of each strain added.

Microcosm experiments with oil were carried out in 250 ml Erlenmeyer flasks containing 60 mg of crude oil in 100 ml of mineral medium. M9 mineral medium was sterilised before addition of crude oil as the sole source of carbon and energy. Microcosm experiments with phenolic leachate were performed in 250 ml Erlenmeyer flasks containing 50 ml filter-sterilised (0.22 μm pore size Millipore filter) oil shale leachate. The pH was adjusted to 7.5 with HCl and $20 \times$ M9. Uninoculated flasks were included as reference for abiotic losses. Both microcosms were incubated for 30 days at 25 °C with rotary shaking (120 rpm) and analysed periodically. Bacterial populations were enumerated on phenol agar plates using the spread-plate technique. After incubation at 30 °C for 4 days the number of phenol-degrading bacterial strains (CFU ml^{-1}) was determined. The plates were prepared in triplicate and mean values and standard deviations were calculated.

2.3. Chemical analyses of leachate samples

After inoculation of microcosms, samples for substrate analysis were periodically withdrawn from abiotic control variant and leachate microcosms, filtered through a 0.22 μm Millipore filter and stored frozen until the analysis with HPLC [24] or GC-MS. In the latter case phenolic compounds were extracted by vigorous shaking with 1 ml of diethylether. Phenols and PAHs were identified using a GC-MS system consisting of Varian Star 3400 Gas Chromatograph (Varian Inc., USA) and the Finnigan MAT Magnum™ Mass spectrometer (Finnigan MAT Inc., USA) operated in electron impact ionisation mode. The capillary column Econo-Cap EC-5, 30 m in length with inside diameter of 0.25 mm and a stationary-phase film thickness of 0.25 μm (Alltech, USA) was used for the separation of compounds. Helium was used as carrier gas. The injection volume was 1 μl, injector temperature and split ratio were 270 °C and 1:10, respectively. The total run time was 20 min and the following temperature program was used: 100–200 °C (8 °C min^{-1}), 200–280 °C (30 °C min^{-1}), 280 °C for 5 min. Substrates were quantified using calibration graphs with internal standard diphenylmethane, which was added to the extracts at the end of the extraction. All results are given as recovery-corrected. The recoveries of the studied compounds varied from 49% (SD% 4.3) to 90% (SD% 4.6%) for benzoate and naphthalene, respectively. The recoveries of the phenolic compounds were in the range 70–90% (except for 2,3-dimethylphenol: 52%). Combined standard uncertainty of the results is uncertainty of the result that takes into account all significant uncertainty sources and is expressed as a standard deviation, i.e. at approximately 68% confidence level [29]. Combined standard uncertainties of the results were between 6% and 10%. The uncertainties include contributions from sample preparation, instrumental measurement (both samples and calibration standards) and peak integration.

2.4. Chemical analyses of oil-amended microcosm samples

Samples were collected from oil-amended microcosms 30 days after inoculation with single strain PC20 and a mixture of strains and compared with abiotic controls. All samples for chemical analyses were stored at −20 °C until use. Hydrocarbons in oil-amended microcosms were extracted and analysed by GC-MS. For extraction from oil-amended microcosms, samples were transferred to the separation funnel. The sample bottle was washed with 3 ml of carbon tetrachloride (CCl_4), which was thereafter transferred directly to the extracts bottle. The sample bottle was then washed with an additional 3 ml of CCl_4, and transferred to the separation funnel, which was shaken vigorously for 1 min with periodic venting to release the excess pressure. The organic layer was allowed to separate from the water layer for 10 min and the process repeated four more times. All solvent extracts were combined.

2.5. DNA extraction

Template DNA was isolated from leachate and oil-amended microcosms inoculated with mixed microbial populations at different time points. DNA was extracted from samples (2 ml) using UltraClean microbial DNA isolation kits (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions. Three serial 10-fold dilutions of samples (10^0–10^{-2}) were made in sterile water and used for DNA extraction. The extracted DNA was stored at −20 °C. DNA was quantified spectrophotometrically and samples were diluted to give final concentration of approximately 15 ng μl^{-1}.

2.6. Primers and PCR conditions

The primers and PCR conditions used for the amplification of fragments of genes coding for C23O, the LmPH and phenol monooxygenase (*pheA*), are listed in Table 1. Time-dependent changes in mixed populations were detected by amplification of 16S rRNA gene

Table 1
Characteristics of DNA probes in the study

Probe, primer		Primer sequence (5′ → 3′)	Size bp	References
16S rDNA	PRBA338f	ACTCCTACGGGAGGCAGCAG	236	[30]
	PRUN518r	ATTACCGCGGCTGCTGG		[31]
C23O	E3F	GGTATGGCGGCTGTGCGTTTCGACCA	356	[25]
	E2R	AGAACACTTCGTTGCGGTTACC		
PheA	pheA1	CAGGATCGAATATCGGTGGCCTCG	947	[24]
	pheA2	CTTCACGCTGGCGTAACCAATCGC		
LmPH	PheGC	CRATYGACGARCTGCGYCA	209	This study
	Phe212	GTTGGTCAGCACGTACTCGAAGG AGAA		[8]
GC[a] clamp		CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGG		[31]

[a] The GC clamp was attached to the end of the 5′ end of the PRBA338f and PheGC primers.

products using universal bacterial primers targeting the V3 variable region (Table 1). The GC-clamp (40 bp) was added to the F338GC primer to enable denaturing gradient gel electrophoresis analysis (DGGE). PCR amplification was performed in a total volume of 50 μl. DNA (15 ng) of extracted from samples was added as a template to a 50 μl reaction mixture. The PCR mixture included 1 × PCR buffer provided in the kit (with $(NH_4)_2SO_4$), 200 μM concentrations of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 20 pmol of each primer and 0.5 U of *Taq* DNA polymerase (Fermentas). Step cycles for probe reactions were as follows: the V3 region of 16S rRNA genes were amplified at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min; C23O genes were amplified at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 20 s; *pheA* genes were amplified at 94 °C for 1 min, 61 °C 45 s and 72 °C for 1 min; LmPH genes were amplified at 94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min. All amplifications were performed for 32 cycles, and 72 °C for 10 min was employed as a single final extension step.

2.7. Analysis of PCR products

PCR was carried out as described above to assess fluctuations in catabolic potential with time. PCR mixes (5 μl) were subjected to agarose gel (2%) electrophoresis for approximately 15 min at 100 V in 1 × TAE buffer, pH 8.3. DNA fragments were stained for 20 min in 1 × TAE buffer with ethidium bromide (final concentration, 0.5 $\mu g l^{-1}$) and de-stained twice in MilliQ water for 20 min prior to UV transillumination. A molecular weight marker (100 bp DNA ladder, Fermentas) was included at both sides of each gel and PCR products were quantified by comparison with a standard using E.A.S.Y Win32 Software (Herolab GmbH, Germany).

2.8. DGGE analysis

Population dynamics were determined in microcosms for 30 days by DGGE analysis of PCR-amplified 16S rRNA and LmPHs genes. Genes encoding the LmPH of strains PC1 and PC18 were used as templates to characterise the functionally dominant population in leachate and oil-amended microcosms. The phenol-hydroxylating activity followed by C23O activity has been shown earlier in these bacteria [24]. *Pseudomonas* sp. CF600 was used as a positive reference strain possessing the LmPH and the strains PC20 and PC24, which lack LmPH and thus did not give a positive amplification reaction.

Approximately 500 ng of a PCR product was applied for DGGE analysis, using the method of Muyzer et al. [31]. The DCode DGGE system (Bio-Rad Laboratories, Hercules, CA) was used for electrophoresis as recommended by the manufacturer. A 10% (w/v) polyacrylamide (37.5:1 acrylamide:bis-acrylamide in 1 × TAE buffer) gels with a gradient of DNA-denaturant agent (100% denaturant agent is 7 M urea and 40% deionised formamide) was performed. The linear gradients of the denaturant used for separation were 35–70% and 40–60% for the 16S rRNA gene and LmPH gene fragments, respectively. To ensure well-polymerised slots, a 3 ml top gel containing no denaturant was added. Wells were loaded with equal amounts of DNA, and electrophoresis was performed in 1 × TAE buffer for 13 h at a constant temperature of 60 °C and a voltage of 100 V. Gels were stained in MilliQ water containing 0.5 $\mu g l^{-1}$ ethidium bromide and de-stained twice in MilliQ water prior to UV transillumination.

2.9. Colony hybridisation analysis

Samples for colony hybridisation were taken from leachate microcosms inoculated with strains PC1, PC18 and PC24. Colonies of both strains of PC1 and PC18 harbouring the C23O gene were distinguishable by different colony morphology and the colonies of strain PC24 were detected by a *pheA* gene probe. Cells were enumerated by standard plating and the relative proportions of each strain were compared by hybridisation of colonies with specific gene markers. Cells from colonies grown on phenol media were hybridised with radioactively labelled gene probes as described previously [32]. Probes for the *pheA* and C23O genes were generated by PCR amplification followed by purification from 2% agarose gels using the QIAquick gel extraction kit (Qiagen Inc.) [25]. The amplified products were subsequently random-prime labelled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Inc.) using a DecaLabel™ kit, MBI Fermentas. Autoradiograms were generated using PhosphorImager (Molecular Dynamics).

2.10. Microscopy

Bacterial aggregates were stained with L-7007 LIVE/DEAD *Bac* Light stain (Molecular Probes Inc.) and examined using an epifluorescent microscope (Olympus U-RFL-T, Japan). Prior to imaging, samples (1 ml) were filtered through a sterile 0.2 μm Nucleopore polycarbonate black filters (Costar; Cambridge, MA) and washed with MilliQ water. Distilled water (1 ml) and mixed *Bac* Light dyes (3 μl) were poured onto a membrane filter and incubated for 20 min in the dark prior to filtration. After filtration, without any washing, the filter membrane was immersed in *Bac* Light mounting oil on a microscopic glass slide and covered with a cover slip. Using this staining method, bacteria with intact cell membranes show green fluorescence whereas bacteria with damaged membranes have red fluorescence.

3. Results

3.1. The population dynamics in leachate and oil-amended microcosms studied by using 16S rRNA gene analysis

In leachate microcosms the DGGE profiles of 16S rRNA gene products demonstrated that the most drastic changes in population structure occurred already during the first day of incubation (Fig. 1(a)). The band representing the population of strain PC20 was not detectable even after the first day of incubation, indicating that this strain was not important in decomposition of leachate substrates. At the same time, *P. mendocina* PC1 was the predominant species in leachate microcosms and its relative abundance increased during the treatment period. The shifts in microbial composition of phenolic leachate microcosms indicated that populations of strains PC18 and PC24 were present throughout the experiment. DGGE profiles revealed an increase in abundance of strains PC24 and PC18 on the third and the fifth or on the third day, respectively. In contrast, in oil-amended microcosms all added strains were present throughout the experiment and showed only a slight decrease in abundance after treatment for 20 days (Fig. 1(b)). Comparisons of DNA band intensities revealed that strain PC24 was predominant in decomposition of substrates in oil-amended microcosm.

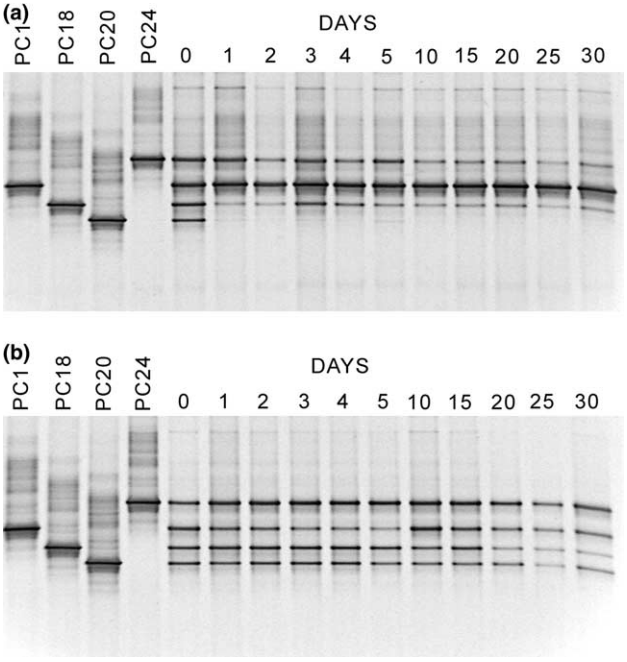

Fig. 1. Negative image of ethidium bromide-stained DGGE gel containing PCR-amplified fragments of 16S rRNA genes from phenolic leachate- (a) and oil-amended (b) microcosms after inoculation with a mixed culture of strains PC1, PC18, PC20 and PC24. Equal quantities (approximately 1×10^7 CFU ml^{-1}) of cell suspensions of each strain were added. Numbers above lanes indicate the days of incubation.

3.2. Monitoring of bacterial populations in leachate- and oil-amended microcosms using LmPH genes as a marker

More precise characterisation of the role of different phenol-degrading bacterial strains in different substrate mixtures was achieved by PCR amplification of phenol hydroxylase (LmPH) genes and DGGE analysis. As expected, comparison of intensities of the PCR amplification products of LmPH DNA bands between strains PC1 and PC18 showed that the dominant population in leachate microcosm throughout the experiment was strain PC1 (Fig. 2(a), see also Fig. 1(a)). In oil-amended microcosms the population of PC1 decreased and a slight increase was detected only between days 10 and 15 (Fig. 2(b)). Conversely, the population of strain PC18 was predominant in oil-amended microcosms. Relative abundance of both populations increased between 5 and 15 days in oil-amended microcosms.

Thus, DGGE analysis of 16S rRNA and LmPH genes indicated that in each microcosm specific populations were selected, and the roles of particular biodegradative bacterial strains may be coordinated in time by substrates available in the mixture.

3.3. Decomposition of phenolic compounds and PAHs in microcosms

Decomposition of phenols, benzoate and PAHs was determined using GC-MS. Fig. 3 shows the biodegradation efficiency of phenol, cresols and benzoic acid in phenolic leachate microcosms. Phenol and benzoic acid were completely removed during the first day and cresols after 10 days. The absence of significant biodegradation of dimethylphenols (3,5-; 2,3-; 2,5-; 2,4- and 2,6-DMP) was observed, with the exception of 3,4-dimethylphenol, which was degraded completely in two days (data not shown).

Only lighter PAHs – naphthalene, anthracene and phenanthrene – were chosen for study. Cresols and naphthalene were totally removed from oil-amended microcosms by mixed populations during 30 days, while degradation of phenanthrene and anthracene was negligible (Fig. 4). A single culture of naphthalene-degrading strain PC20 was less effective in biodegradation of phenols than in removal of PAHs (Fig. 4). This is a good indication for the importance of this strain in decomposition of oil products.

3.4. Development of bacterial aggregates in microcosms

Enumeration and monitoring of mixed bacterial populations in microcosms using CFU data from spread-plate analyses showed that the number of bacteria was the highest two days after the inoculation in phenol leachate microcosms. In oil-amended microcosms, total CFUs of mixed populations started to decrease from

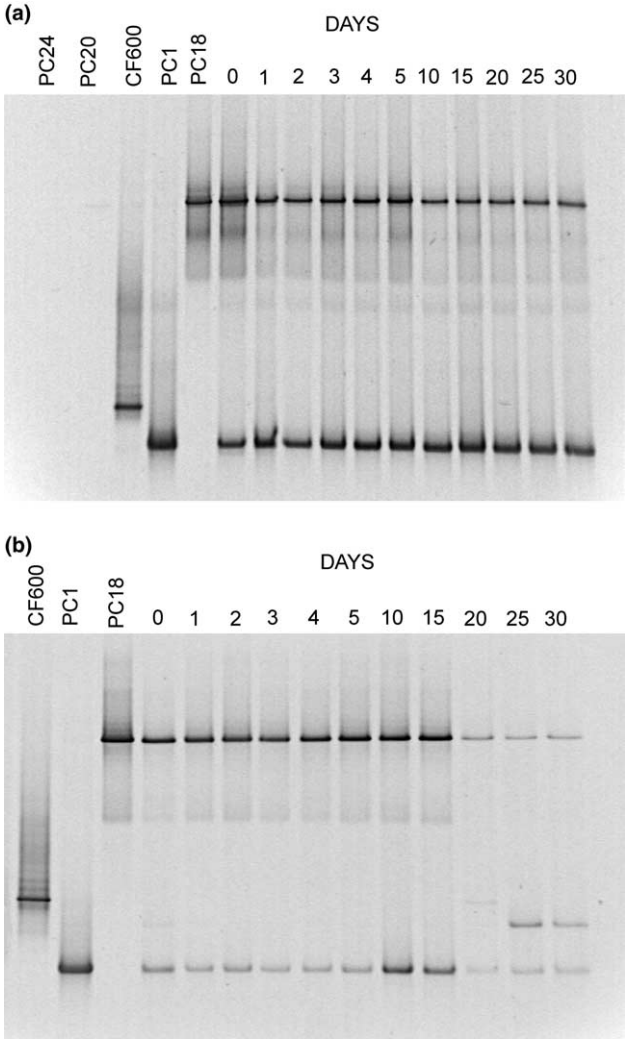

Fig. 2. Negative images of DGGE gels containing PCR-amplified fragments of LmPH genes from strains PC1 and PC18 during incubation for 30 days in phenolic leachate- (a) and oil-amended (b) microcosms. The DNA probes were taken from the same microcosms used in experiments presented in the Fig. 1. The DNA of strains PC20 and PC24 served as negative controls and DNA of *Pseudomonas* sp. CF600 was used as a positive control. Numbers above the lanes indicate the days of incubation.

the beginning of the experiment and showed a slight increase only 5–10 days after inoculation (Fig. 5). CFU data most probably do not reflect the real abundance of bacteria and differences between molecular data and standard plate counts can be explained by clumping of bacteria and formation of microbial aggregates.

Development of bacterial aggregates of single strains and mixed cultures in oil-amended microcosms (Fig. 6(a)–(e)) was followed using the *Bac* Light LIVE/DEAD viability method. Three forms of bacteria were detected: viable, dead and lysed showing green, red and no fluorescence, respectively, among both aggregated and non-aggregated cells. In oil-amended microcosms, aggregated cells of single strain PC24 were not detected one day after the inoculation (Fig. 6(d)), whereas the

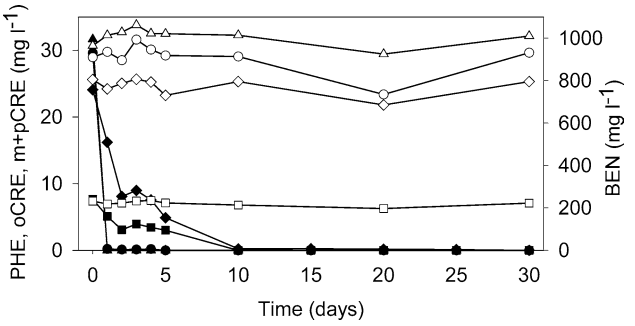

Fig. 3. Removal of phenolic compounds and benzoate by mixed culture of strains PC1, PC18, PC20 and PC24 during incubation for 30 days in leachate microcosms. The concentration of phenol (PHE, ○ control, ● culture), *o*-cresol (*o*CRE, □ control, ■ culture), *m*- and *p*-cresol (*m* + *p*CRE, ◇ control, ◆ culture), benzoate (BEN, △ control, ▲ culture) were determined. Values are the averages of triplicate determinations. The combined standard uncertainties [29] of the results were between 6% and 10%.

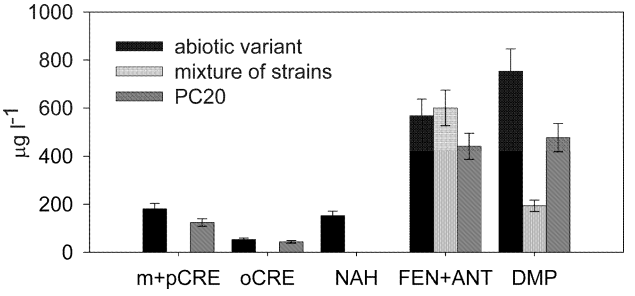

Fig. 4. Comparison of decomposition of aromatic compounds and PAHs in an abiotic control, in a mixture of strains PC1, PC18, PC20 and PC24 and in a single culture of strain PC20 after incubation for 30 days in oil-amended microcosms. Values are the averages of triplicate determinations. Error bars indicate the combined standard uncertainties [29] of the results. Abbreviations: *m* + *p*CRE, *m*- and *p*-cresol; *o*CRE, *o*-cresol; NAH, naphthalene; FEN + ANT, phenanthrene and anthracene; DMP, dimethylphenols.

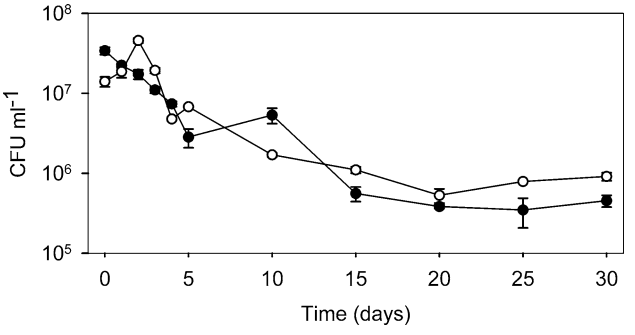

Fig. 5. Enumeration of bacteria in mixed populations from phenolic leachate- (○) and oil-amended microcosms (●) during incubation for 30 days. Values are the averages of triplicate determinations. Error bars indicate standard deviations.

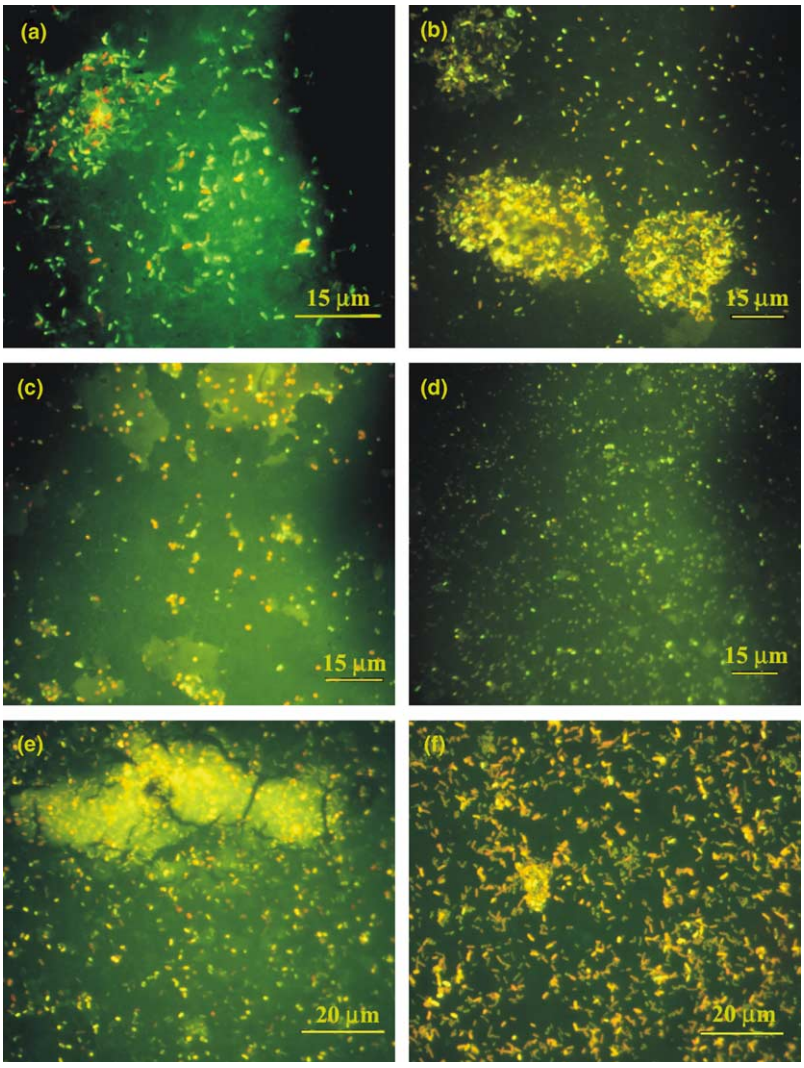

Fig. 6. Formation of bacterial aggregates by single strains in oil-amended microcosms (a – PC1; b – PC18; c – PC20 and d – PC24) and by mixed cultures in oil-amended (e) and leachate microcosms (f). Cells are visualised using the *Bac* Light LIVE/DEAD viability stain. Viable cells are green, whereas dead cells show red fluorescence due to compromised cell membrane. Images were obtained 24 h after cells were inoculated. Magnification 1000×.

strains PC1, PC18 and PC20 grew predominantly as clumps (Fig. 6(a)–(c)). Mixed culture of strains grew in aggregated forms in an oil-amended microcosm and in planktonic mode of growth in phenolic leachate microcosms (Fig. 6(e) and (f)). The cell aggregates were revealed during incubation of oil-amended microcosms. This may explain the artificial data showing decrease in the numbers of CFU during the decomposition of PAH in oil-amended microcosm.

3.5. Dynamics of mixed bacterial cultures in leachate microcosm determined by colony hybridisation

As in leachate microcosms, bacteria grow predominantly planktonically and colony hybridisation was used to compare cell numbers of different strains. Because of the rapid disappearance of strain PC20 the leachate microcosm (see Fig. 1(a)), a mixed culture of three strains (PC1, PC18 and PC24) was studied. Initially, the percentage of PC1 cells increased and those of PC18 and PC24 decreased (Fig. 7). The behaviour of the PC24 population indicated that this strain was not active in degradation of phenol, cresols and dimethylphenols during the first 10 days of incubation. Between days 10 and 15 PC1 abundance decreased and that of PC24 increased (Fig. 7). The third peak in abundance of strain PC1 and the respective decrease in abundance of PC24 was observed on the 25th day. These changes show that relative proportions of bacterial strains having *meta* (PC1) and *ortho* (PC24) pathways and were co-ordinated in a microcosm consortium throughout the experiment. The shifts in the composition of mixed population in leachate microcosms indicated that different pathways of metabolism of aromatics dominated in different stages of decomposition of pollutants.

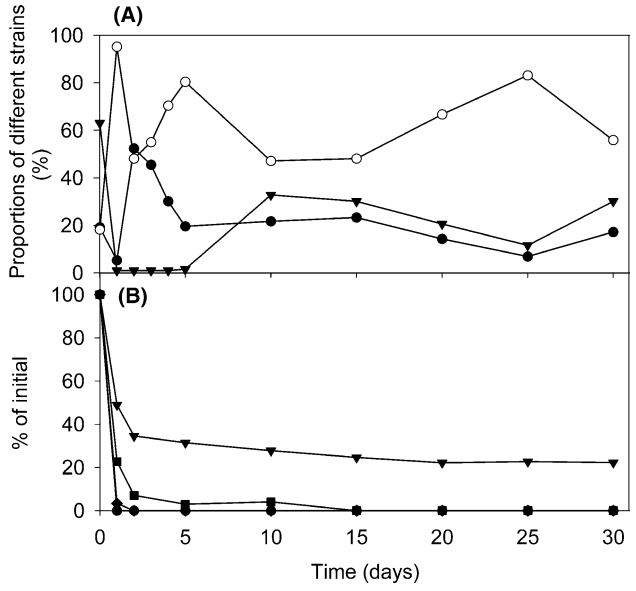

Fig. 7. Relative percentage of each population (○, PC1; ●, PC18 and ▼, PC24) in mixed culture during incubation for 30 days incubation (panel A) and biodegradation curves for phenolic compounds: phenol (●), *m*- and *p*-cresol (◆), *o*-cresol (■) and dimethylphenols (▼) in leachate microcosm (panel B). For strains PC1 and PC18, a C23O gene probe, and in case of the strain PC24, a *pheA* gene probe were used for hybridisation of colonies. At each time point at least 200 colonies were studied. Experiments were repeated with three microcosm cultures and results from a typical experiment from one culture are presented.

4. Discussion

Bacterial growth on pollutant mixtures is an important aspect of bioremediation. In our previous work, interactions between phenol- and *p*-cresol-degrading bacteria in mixtures undergoing biodegradation were described [25]. As reported by Reardon et al. [33] interactions between bacterial species in mixed cultures may be substrate-dependent and cannot be predicted by simple competition models. Phenol- and oil-degrading mixed cultures used in this study comprised four strains (PC1, PC18, PC20, PC24) with known complementary degradative capabilities (defined consortia).

It has been shown that the phenol-degrading strains used have physiological and genetic differences [24]. All of these strains assimilated phenol and *p*-cresol but not *m*-toluate [24]. In addition, strain PC18 is able to degrade salicylate and strain PC20 salicylate and naphthalene. Strains PC1 and PC18 use *meta* cleavage of catechol during growth on phenol. In strain PC20 both *meta* and *ortho* cleavage are induced with phenol and in strain PC24 phenol induces only *ortho* pathway, whereas both strains contain the *pheBA* operon. In strains PC1 and PC20 *p*-cresol is degraded via *meta* cleavage of catechol and in strains PC18 and PC24 via protocatechuate using the *ortho* pathway [24]. To understand interactions within a pollutant-degrading bacterial consortium in situ, functionally dominant populations were identified using analysis of the DNA isolated from laboratory microcosm samples. Molecular techniques are increasingly used in bioremediation studies and Watanabe and coworkers [8] used a combination of molecular and microbiological methods to detect and characterise dominant phenol-degrading bacteria in activated sludge. TGGE and DGGE analyses of 16S rRNA- and LmPH-encoding genes have been used to study functional activity and structural fluctuations of bacterial consortia in a microcosm [9].

In this study, DGGE analysis of PCR products of 16S rRNA genes and of the gene encoding the LmPH showed a shift in composition of bacterial populations during incubation for 30 days on phenolic leachate and crude oil. DGGE analysis of PCR products of 16S rRNA genes showed that *P. mendocina* PC1 became dominant and *P. fluorescens* PC20 disappeared after incubation of a mixed population in leachate microcosms for 30 days (Fig. 1(a) and (b)). Disappearance of a particular population (PC20) from the leachate microcosm and maintenance of the same population in the naphthalene-contaminated oil microcosms throughout the entire experiment indicates that changes in bacterial consortia largely depend on substrate properties. In contrast, no dominant population was detected in oil-amended microcosms by DGGE. Phenol-degrading strain PC20 used in this study is able to metabolise naphthalene. This strain is interesting because it has two large plasmid replicons, a conjugative naphthalene plasmid and a smaller plasmid encoding the degradative *ortho* pathway with *pheBA* operon (data not shown). We believe that the presence of the naphthalene plasmid in the strain PC20 can cause persistence of this strain in oil-amended microcosms. It is likely that carbon sources other than PAHs contributed to microbial growth in phenolic leachate microcosm.

Strain PC20 is most likely unable to degrade phenol in a phenolic leachate microcosm because variable phenol concentrations may affect selection of phenol-degrading bacteria with different kinetic properties [34]. Information on the rate of pollutant biodegradation can be obtained by determination of the half saturation constant, K_s. High concentrations of phenol promote expression of phenol degradation activities with high K_s (low affinity), while strains with low K_s (high affinity) are enriched in the case of low phenol concentrations in the medium [35]. Additionally, high concentrations of phenol cause inefficient growth of low-K_s bacteria, which are outcompeted by high-K_s bacteria [36]. Strains used in this study exhibit kinetically different catabolic activities and have different affinities for phenol. Strain PC1 grows rapidly on phenol and its K_s value for phenol is almost one order of magnitude lower than that of the strain PC20 (data not shown).

To understand the catabolic significance of the strains used in bioremediation of pollutant mixtures, bacterial consortium structure was characterised using catabolic genes as markers. A similar approach has been used to analyse the diversity of functionally dominant populations by Watanabe and Hamamura [5]. Although the C23O-specific primers are often used for identification of dominant bacteria in degradation of one- and two-ringed aromatic hydrocarbons, [19,21–23], detection of phenol-degrading genes was used to characterise catabolic activity in microcosms. Different cleavage pathways were determined among the strains studied using primers specific for multicomponent phenol hydroxylase (LmPH) (Fig. 2) and single component phenol monooxygenase (PheA) genes (Fig. 7). It has been previously reported that multicomponent phenol hydroxylases are predominant in bacteria isolated from phenol-polluted area [37]. The multicomponent phenol hydroxylases are classified into two types according to genetic organisation of the operons: *dmp* type [38] and *mop* type [14], linked to genes for C23O or catechol 1,2-dioxygenase, respectively. In strains PC1 and PC18 used in this study initial phenol degradation is performed by multicomponent phenol hydroxylase into catechol, the aromatic ring of which is further cleaved by C23O. In contrast, in strains PC24 and PC20 the *pheBA* operon encoding phenol degradation and *ortho* pathway is induced [24]. Phenol hydroxylase (PH) and C23O genes from strains PC1 and PC18 were constantly present in phenolic leachate microcosms for 30 days. A PH + C23O-positive strain PC18 dominated in oil-amended microcosms, possibly because of the ability of this strain to degrade salicylate, an intermediate in naphthalene degradation. Accumulation of intermediates in a mixed culture may stimulate enrichment of bacteria that can further metabolise these substrates [39]. In oil-amended microcosms, C23O-positive bacterial populations decreased 20 days after the inoculation, possibly due to disappearance of growth-promoting substrates. The observed fluctuations in the composition of microcosm consortia appear to be driven by carbon and energy sources liberated from crude oil. Wikström et al. [19] also detected C23O in various soil types and recorded a relationship between the abundance of this gene and PAHs concentration.

Molecular monitoring of genes coding for catabolic enzymes of pollutant degradation pathways can show which population has major importance in a specific polluted ecosystem. The behaviour of the strains PC1 and PC18, in which *meta* pathway is induced by phenol, is not very similar in phenolic leachate microcosms (Fig. 7). Throughout this study, the *pheBA* operon (*ortho* pathway) containing strain PC24 fluctuates differently, possibly through qualitative and quantitative changes in chemical composition of degrading pollutants. Fluctuations in catabolic activity studied using *pheA* and C23O genes as markers suggest that the activity of each strain in a mixed culture is regulated within a specific time scale. It is well known that *meta* and *ortho* ring fission of phenolic compounds are alternative pathways for a single strain and that pollutants are degraded faster under mixed culture conditions. In this case commensal interactions between different species support degradation.

Intermediates of a catabolic pathway of one strain (e.g., strain PC18) may be further degraded by another strain (e.g., strain PC24) possessing suitable catabolic pathway. Additionally, in strain PC18, catechol *meta* and protocatechuate *ortho* pathways are induced with phenol and *p*-cresol, respectively [25], and this strain can decompose dimethylphenols in the presence of phenol and *p*-cresol [4].

The planktonic growth form in aqueous suspensions represents just one possible survival strategy of microorganisms. A likely advantage of the alternative strategy, formation of biofilms [7], is higher availability of nutrients. Many pollutant-degrading bacteria are known to secrete biosurfactants that increase aqueous concentration of naphthalene, indicating that a microorganism can promote solubilisation of its substrate [40]. Despite this fact the majority of microorganisms live in biofilms, in both nutrient-rich and oligotrophic environments [41]. Among the strains used in this study, PC18 grows mostly in aggregated forms in oil-amended microcosm. The functional activity in oil-amended microcosms and flexibility of using different contaminants show that this strain may be effective in bioremediation. It is important to examine biodegradation of pollutants at their low concentrations, because due to their poor solubility in water, they may often not be degraded.

Acknowledgements

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ORIGINAL PAPER

Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol-degrading *Pseudomonas* species and biotypes

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Abstract Phenol- and *p*-cresol-degrading pseudomonads isolated from phenol-polluted water were analysed by the sequences of a large subunit of multicomponent phenol hydroxylase (LmPH) and catechol 2,3-dioxygenase (C23O), as well as according to the structure of the plasmid-borne *pheBA* operon encoding catechol 1, 2-dioxygenase and single component phenol hydoxylase. Comparison of the *carA* gene sequences (encodes the small subunit of carbamoylphosphate synthase) between the strains showed species- and biotype-specific phylogenetic grouping. LmPHs and C23Os clustered similarly in *P. fluorescens* biotype B, whereas in *P. mendocina* strains strong genetic heterogeneity became evident. *P. fluorescens* strains from biotypes C and F were shown to possess the *pheBA* operon, which was also detected in the majority of *P. putida* biotype B strains which use the *ortho* pathway for phenol degradation. Six strains forming a separate LmPH cluster were described as the first pseudomonads possessing the Mop type LmPHs. Two strains of this cluster possessed the genes for both single and multicomponent PHs, and two had genetic rearrangements in the *pheBA* operon leading to the deletion of the *pheA* gene. Our data suggest that few central routes for the degradation of phenolic compounds may emerge in bacteria as a result of the combination of genetically diverse catabolic genes.

Keywords Phenol and *p*-cresol-degrading bacteria · Carbamoylphosphate synthase gene (*carA*) · Phenol hydroxylase · Catechol 2,3-dioxygenase · *pheBA* operon

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Introduction

The metabolic capacity of indigenous microbial consortia present in a continuously polluted environment may reflect adaptation of various catabolic pathways in pollutant-degrading indigenous bacterial strains (El Fantroussi and Agathos 2005). Numerous studies on microbial catabolism of phenol and alkylphenols (e.g. cresols) have led to a deeper understanding of the degradation of aromatic compounds, in terms of enzymology, genetics, microbial diversity, and bioremediation. The microbial strategy for aerobic degradation of aromatic compounds involves two critical steps: first, the ring hydroxylation of adjacent carbon atoms and second, the ring cleavage of the resulting catecholic intermediates. In the case of phenol degradation, the aromatic ring is first monohydroxylated by phenol hydroxylase (PH, phenol 2-monooxygenase, EC 1.14.13.7) at *ortho* position to the pre-existing hydroxyl group. The next step is catalysed by either catechol 1,2-dioxygenase (C12O, initiating the *ortho* pathway leading to formation of succinyl-CoA and acetyl-CoA) or catechol 2,3-dioxygenase (C23O, initiating the *meta* pathway leading to formation of pyruvate and acetaldehyde) (Nozaki et al. 1970; Mason and Cammack 1992).

Two different types of PHs have been identified: single (sPH) and multicomponent (mPH) (Shingler et al. 1989; Kukor and Olsen 1990; Nurk et al. 1991). PHs from *Pseudomonas pickettii* PKO1 (encoded by *tbuD*) and *Pseudomonas* sp. EST1001 (encoded by *pheA*) belong to the first group. The *tbuD* gene is co-transcribed with the

C23O gene (*tbuE*) and the latter gene shares the operon with the *pheB* gene encoding C12O (Kukor and Olsen 1991; Kivisaar et al. 1991). mPHs are composed of six subunits and have a very similar protein structure; the catabolic site is located within the largest subunit (Watanabe et al. 1998). The genetic organisation of operons coding for mPHs is different: the genes that code for the *dmp* type (from *Pseudomonas* CF600) and the *mop* type (from *Acinetobacter calcoaceticus* NCIB8250) of mPHs are located upstream of the genes that are followed by the genes that code for C23O and C12O, respectively (Shingler et al. 1992; Ehrt et al. 1995). This may reflect the preference of different mPHs to catabolise differently substituted phenols.

C23O (catechol:oxygen 2,3-oxidoreductase; EC 1.3.11.2) is a key enzyme of many bacterial pathways for the degradation of aromatic compounds. The majority of C23Os are phylogenetically closely related, belonging to the subfamily 1.2.A of the 1.2 extradiol dioxygenase family, and are of particular importance in the degradation of monocyclic aromatic compounds (Eltis and Bolin 1996). Methyl-substituted aromatic compounds are usually degraded through the *meta* pathway catalysed by C23O or via o*rtho* ring cleavage of protocatechuate (*ortho*^{prot}) by using *p*-cresol methylhydroxylase as a key enzyme of this route (Dagley and Patel 1957).

We have previously shown that phenol/*p*-cresol-degraders isolated from river water continuously polluted with phenolic compounds of oil shale ash leachate group into three different catabolic types: (a) phenol and *p*-cresol are both degraded via the catechol *meta* pathway (*meta–meta* type), (b) phenol is degraded via the catechol *ortho* and *p*-cresol, via the protocatechuate *ortho* pathway (*ortho–ortho*^{prot}), or (c) phenol is degraded via the catechol *meta* and *p*-cresol via the protocatechuate *ortho* pathway (*meta–ortho*^{prot}) (Heinaru et al. 2000). The main aim of the present study was to compare the gene sequences of PHs and C23Os, responsible for the efficient degradation of phenols in these strains, in order to elucidate their species- and biotype-specific clustering, and to prove the assumption that different combinations of genetically diverse catabolic genes may lead to functionally similar central routes of degradation of phenolic compounds.

Materials and methods

Bacterial strains and growth conditions

A total of 38 phenol- and *p*-cresol-degrading strains from the genus *Pseudomonas* isolated from phenol-polluted river water were studied (Table 1). The strains and the culture media have been described in our previous work (Heinaru et al. 2000).

DNA extraction, plasmid characterisation and Southern hybridisation

Genomic DNA of bacterial strains was extracted from 2 ml of Luria-Bertani (LB) overnight culture using the UltraClean microbial DNA isolation kit (MO BIO Laboratories), according to the manufacturer's instructions. The extracted DNA was stored at −20°C. Plasmid DNA was detected according to the procedures of Connors and Barnsley (1982). To specify the location of phenol degradation genes in studied strains, a Southern blot analysis of plasmid DNA was performed as described by Sambrook et al. (1989). Digestion of DNA with a restriction enzyme was performed according to the manufacturer's (Fermentas) guidelines. For Southern hybridisation of the isolated plasmid DNA, *pheA* probes were generated via PCR from the strain *Pseudomonas putida* EST1412 using *pheA* primers (Table 2) as previously described (Heinaru et al. 2001). LmPH gene probes for hybridisation were generated from the strains PC16, PC17, PC30 and P69 using primers described in Table 2. The purified DNA fragments were labelled with [α^{32}P]dCTP (Amersham Pharmacia Biotech, Inc.) using a DNA labelling kit (DecaLabel™, Fermentas). Autoradiograms were analysed using PhosphorImager (Molecular Dynamics).

Primers and PCR conditions

The extracted DNA was quantified spectrophotometrically and subjected to PCR amplification. The primers used for the amplification of the studied genes are listed in Table 2. Amplification was performed by using a 25 µl mixture containing about 15 ng of template DNA, 1 × PCR buffer consisting of $(NH_4)_2SO_4$ as provided in the Fermentas *Taq* DNA polymerase kit, 200 µM concentration of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 20 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Fermentas). The amplification and sequence determination of 16S rRNA genes of the strains were performed as described in our previous work (Heinaru et al. 2001). Amplification of the *carA* gene used as a species- and biotype-specific marker in pseudomonads was performed as described by Hilario et al. (2004).

The PCR programs for the amplification of 580 bp LmPH gene fragments, as well as for the 924 bp C23O and the 947 bp *pheA* gene fragments have been described previously (Futamata et al. 2001; Junca and Pieper 2003; Heinaru et al. 2005). Step cycles of the

Table 1 Bacterial strains used and their GenBank accession numbers for *carA* and 16S rDNA

Strain	Identified by *carA*	Strain abbreviation	Accession no. for *carA*	Accession no. for 16S rDNA
PC1	*P. mendocina*	Pmen PC1	DQ178181	AF232713
PC2	*P. mendocina*	Pmen PC2	DQ178182	DQ178219
PC3	*P. mendocina*	Pmen PC3	DQ178183	ND
PC4	*P. mendocina*	Pmen PC4	DQ178184	DQ178220
PC5	*P. mendocina*	Pmen PC5	DQ178185	DQ178221
PC6	*P. mendocina*	Pmen PC6	DQ178186	DQ178222
PC7	*P. mendocina*	Pmen PC7	DQ178187	DQ178223
PC8	*P. mendocina*	Pmen PC8	DQ178188	ND
PC9	*P. mendocina*	Pmen PC9	DQ178189	ND
PC10	*P. mendocina*	Pmen PC10	DQ178190	DQ178224
PC11	*P. mendocina*	Pmen PC11	DQ178191	ND
PC12	*P. mendocina*	Pmen PC12	DQ178192	DQ178225
PC13	*P. putida* B	Ppu PC13	DQ178193	ND
PC14	*P. putida* B	Ppu PC14	DQ178194	AY973266
PC15	*P. putida* B	Ppu PC15	DQ178195	AY973267
PC16	*P. putida* B	Ppu PC16	DQ178196	AY918067
PC17	*P. fluorescens* F	PfF PC17	DQ178197	AY538263
PC18	*P. fluorescens* B	PfB PC18	DQ178198	AF228366
PC19	*P. mendocina*	Pmen PC19	DQ178199	DQ178226
PC20	*P. fluorescens* F	PfF PC20	DQ178200	AY538264
PC21	*P. fluorescens* B	PfB PC21	DQ178201	DQ178227
PC22	*P. fluorescens* B	PfB PC22	DQ178202	DQ178228
PC23	*P. fluorescens* B	PfB PC23	DQ178203	DQ178229
PC24	*P. fluorescens* C	PfC PC24	DQ178204	AF228367
PC25	*P. fluorescens* C	PfC PC25	DQ178205	ND
PC26	*P. fluorescens* C	PfC PC26	DQ178206	ND
PC28	*P. fluorescens* C	PfC PC28	DQ178207	ND
PC30	*P. putida* B	Ppu PC30	DQ178208	AY918068
PC31	*P. fluorescens* C	PfC PC31	DQ178209	ND
PC32	*P. fluorescens* B	PfB PC32	DQ178210	DQ178230
PC33	*P. fluorescens* B	PfB PC33	DQ178211	DQ178231
PC34	*P. fluorescens* B	PfB PC34	DQ178212	DQ178232
PC35	*P. putida* B	Ppu PC35	DQ178213	ND
PC36	*P. putida* B	Ppu PC36	DQ178214	DQ178233
PC37	*P. fluorescens* B	PfB PC37	DQ178215	DQ178234
PC38	*P. fluorescens* B	PfB PC38	DQ178216	DQ178235
PC39	*P. putida* B	Ppu PC39	DQ178217	ND
P69	*P. fluorescens* F	PfF P69	DQ178218	AY973265

ND not determined

probe reactions for the *pheBA* operon were as follows: the *pheB* gene fragment (847 bp) and IS*1472* (330 bp) were amplified at 94°C for 1 min, 52°C for 45 s, 7°C for 1 min; IS*1411* (996 bp) and IRL (1112 bp) were amplified at 94°C for 1 min, 61°C for 45 s, 72°C for 1 min. All amplifications were performed for 32 cycles, and an additional extension step of 10 min at 72°C was employed in the end. To evaluate PCR product sizes, 5 µl of the PCR reaction was analysed using agarose gel electrophoresis (1.5–2.0%, 1× TAE running buffer, at 100 V). DNA bands were visualised by ethidium bromide (final concentration, 0.5 µg l^{-1}) staining.

PCR products were purified from 2% agarose gels using the QIAquick Gel Extraction Kit (Qiagen) or UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories) according to the instructions provided. The PCR products were ligated into pTZ57R using the InsT/Aclone™ PCR Product Cloning Kit (MBI Fermentas) and cloned into *E. coli* DH5α competent cells (Inoue et al. 1990). The *E. coli* cells were cultured on LB medium at 37°C, containing ampicillin 15 µg ml^{-1}, isopropyl β-D-thiogalactopyranoside 48 µg ml^{-1} and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside 80 µg ml^{-1}. For the detection of LmPH and C23O genes, *Pseudomonas* sp. CF600 was used as a reference strain (Shingler et al. 1989).

Analysis of the *pheBA* operon

The genetic organisation of the *pheBA* operon was determined by PCR using the primer pairs shown in

Table 2 PCR primers used for amplification of DNA probes

Probe/Primer	Nucleotide sequence (5′→3′)	References
LmPH/pheUf	CCAGGSBGARAARGAGARGAARCT	Futamata et al. (2001)
pheMHr	GATBGGCACRTTGTCTTC	
pheA/pheA1	CAGGATCGAATATCGGTGGCCTCG	Heinaru et al. (2000)
pheA2	CTTCACGCTGGCGTAACCAATCGC	
pheB/pheB1	TCGTTGCTGGTCTCGACC	This study
pheB2	TTGCAGCTCCTCTTCTGC	
IS*1472*/IS1	GTTGTTCCTGATCGAGATG	Peters et al. (2004)
IS2	GCCGCGGTCGCCCAGATAGC	
IS*1411*/pheA3	GCGATTGGTTACGCCAGCGTGAAG	Peters et al. (2004)
IS3	GCTTGAGGCAGGGCTTCTTGCG	This study
IRL/pheA1	CAGGATCGAATATCGGTGGCCTCG	Heinaru et al. (2000)
IRL	CGGTCAGATTTTTTCTACACCC	This study
C23O/ORF-F	AGGTGWCGTSATGAAMAAAGG	Junca and Pieper (2003)
ORF-R	TYAGGTSAKMACGTTCAKGAA	
carA/carA-F	TTCAACACCGCCATGACCGG	Hilario et al. (2004)
carA-R	TGATGRCCSAGGCAGATRCC	

Table 2. The sequences of amplified products were compared with those of the *pheBA* operon in the GenBank. The cells for enzyme assay were harvested in the late exponential growth phase. Crude extracts were prepared and enzyme activity was assayed as described previously (Heinaru et al. 2000). The activity of sPH was monitored spectrophotometrically by examining oxidation of NADPH at 340 nm (Hegeman 1966). Protein concentration was measured according to the Bradford method (1976). Enzyme activities were determined from at least three separate independent experiments. *P. putida* EST1412 was used as a reference strain for the measurement of the sPH activity (Kivisaar et al. 1990).

Northern hybridisation

For northern analysis, bacterial strains were grown overnight in a minimal medium containing 0.2% (w/v) casamino acids (CAA) and inoculated into fresh CAA medium supplemented with 2.5 mM phenol as inducer at initial optical density of ca. 0.1 at 580 nm. Total RNA was extracted from exponential-phase cultures using the Nucleospin® RNA II extraction kit (Macherey-Nagel) according to the manufacturers' instructions. About 20 μg of total RNA per lane was used for northern blot analysis. Agarose-formaldehyde gel electrophoresis was performed as described in Sambrook et al. (1989). The RNA was transferred onto nylon membrane (Hybond-N$^+$; Amersham Bioscience) using the capillary method and fixed by UV cross-linking. Gene-specific DNA probes were generated by PCR (Table 2) and hybridisation was performed according to standard protocols (Sambrook et al. 1989).

DNA sequencing and phylogenetic analyses

Nucleotide sequencing of the cloned inserts was carried out on an ABI Prism™ 377 DNA sequencer (Perkin-Elmer) using the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) and the protocols provided by the manufacturer. The GenBank database search was conducted using BLAST programs. CLUSTAL W version 1.83 was used for the sequence alignments (Thompson et al. 1994). Phylogenetic trees were constructed from the evolutionary distance matrix by the neighbour-joining method (Saitaou and Nei 1987). The sequence data reported in this study has been deposited in GenBank under accession numbers AY875721–AY875748, and DQ387868 for LmPH, and AY887949–AY887972 for C23O genes. The *carA* and 16S rRNA gene sequences obtained in this study are available in GenBank under the accession numbers shown in Table 1.

Results and discussion

Species- and biotype-specific grouping of pseudomonads based on sequences of the *carA* gene

Although sequencing of 16S rRNA genes has proven to be a valuable tool in taxonomic analysis of bacteria (Woese 1987), its application in the case of the genus *Pseudomonas* is limited, especially within the *fluorescens* lineage of pseudomonads (Hilario et al. 2004). Quite expectedly, we were unable to construct a phylogenetic tree precisely distinguishing the 27 *Pseudomonas* strains studied in this work (Table 1) according to the determined 16S rRNA gene sequences. The high identity

level of this gene within *Pseudomonas* strains, especially among *P. fluorescens* biotypes (about 99%) hampers its use in phylogenetic analysis. Therefore, other genes including the *carA* gene encoding the small subunit of carbamoylphosphate synthase (Lawson et al. 1996) have been used for more precise phylogenetic analysis (Eisen 1995; Yamamoto et al. 2000). Carbamoylphosphate synthase genes are essentially immune to horizontal gene transfer (Cammarano et al. 2002) and evolve much faster than ribosomal rRNA genes, thus providing higher resolution power to discriminate between closely related species (Yamamoto et al. 2000). In this study, the phylogenetic tree of 38 strains of phenol/*p*-cresol-degraders and the reference strains was constructed on the basis of the partial sequences (617 bp) of the *carA* gene. As expected, it revealed distinct groupings of the *Pseudomonas fluorescens* biotypes B, C and F (Fig. 1, groups I, IV and IIIb, respectively), *P. mendocina* (Fig. 1, group II) and *P. putida* species (Fig. 1, group IIIa). It also became obvious that *P. putida* biotype B is closely related to *P. fluorescens* biotype F, groups IIIa and IIIb, respectively. The latter result is also supported by the fact that no metabolic differences between these strains were detected by Hilario et al. (2004).

Phylogeny of LmPHs and C23Os

In order to study diversity between the catabolic genes, the partial sequences of LmPH (580 bp) and C23O (924 bp) genes of the phenol/*p*-cresol-degraders were analysed. The respective PCR amplifications resulted in 29 LmPH and 24 C23O positive strains. The deduced amino acid sequences of these PCR products were aligned against sequences of reference strains. The phylogenetic tree of the proteins deduced from the sequences

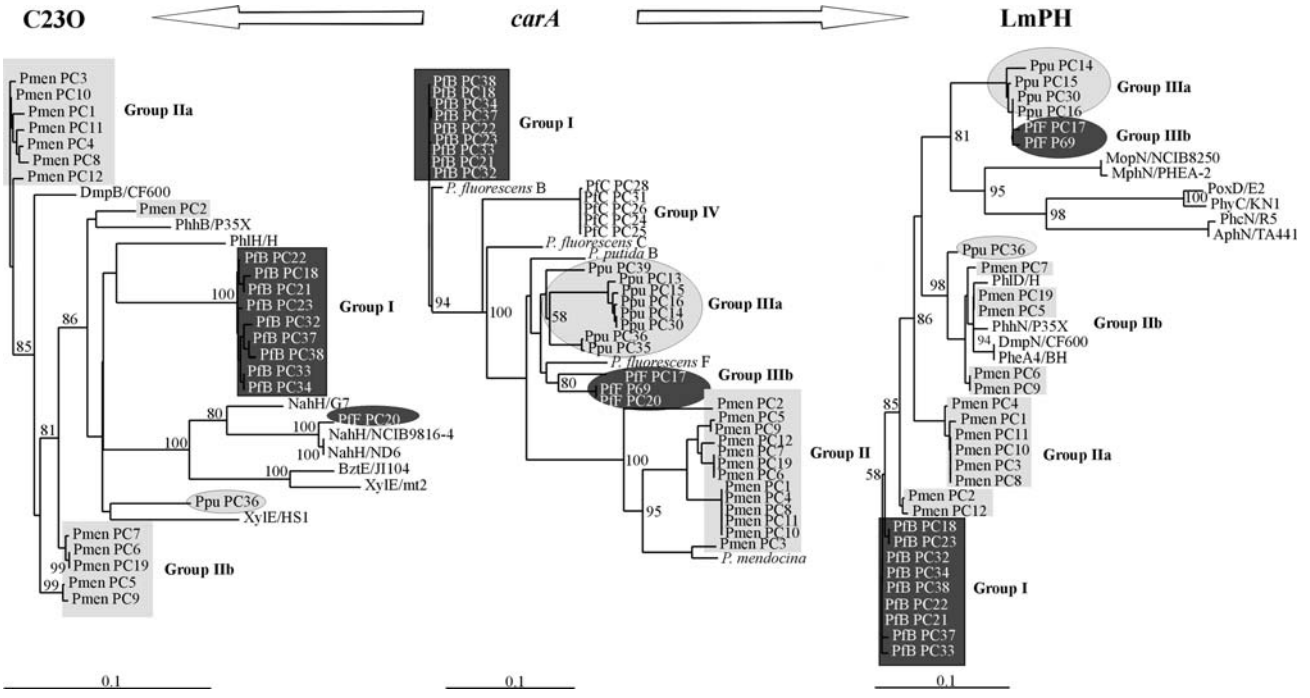

Fig. 1 Neighbour-joining trees based on the 617 bp of *carA* gene sequences, on the deduced amino acid sequences of the LmPHs (580 bp) and C23Os (924 bp) of the phenol/*p*-cresol degraders and reference strains. The *carA* gene sequences obtained from the GenBank database belong to the following type strains: *P. mendocina* ICMP 13540 (AJ414222), *P. putida* biotype B ICMP 13630 (AJ414224), *P. fluorescens* biotype B ICMP 13619 (AJ414216), biotype C ICMP (AJ414217) and biotype F ICMP 13616 (AJ414218). The sequences obtained in this study were aligned with the known LmPHs: MopN from *Acinetobacter calcoaceticus* NCIB8250 (Z36909), MphN from *A. calcoaceticus* PHEA-2 (AJ564846), PoxD from *Ralstonia* sp. E2 (AF026065), PhyC from *Ralstonia* sp. KN1 (AB031996), PhcN from *Comamonas testosteroni* R5 (AB024741), AphN from *C. testsseroni* TA441 (AB006479), PhlD from *P. putida* H (X80765), PhhN from *P. putida* P35X (X79063), DmpN from *Pseudomonas* sp. CF600 (M60276), PheA4 from *P. putida* BH (D28864), and C23Os: DmpB from *P. putida* CF600 (M33263), PhlH from *P. putida* H (X80765), NahH *P. putida* G7 (P08127), NahH from *P. putida* NCIB9816-4 (AA064305), NahH from *Pseudomonas* sp. ND6 (NP-943120), BztE from *P. aeruginosa* JI104 (X60740), XylE from *P. putida* mt2 (V01161), XylE from *P. putida* HS1 (M65205), PhhB from *P. putida* P35X (X77856). Bootstrap values (per 1000 trials) higher than 50% are indicated at the nodes. The scale bars represent 0.1 substitutions per nucleotide (*carA*) or amino acid site (LmPH, C23O). The groups of different species and biotypes revealed according to the *carA* gene sequence analysis are designated using different grades of *grey* shading and shapes, which are used to track the position of these strains in phylogenetic trees of C23O and LmPH

revealed four main groups (I, IIa, IIb, IIIa + IIIb) of LmPH genes (Fig. 1). Only in the case of two strains, Pmen PC2 and Pmen PC12, the LmPH genes grouped outside those four clusters. Six strains (Ppu PC14-PC16, Ppu PC30, PfF PC17, PfF P69) harbouring group IIIa + IIIb LmPHs did not contain C23O genes. The phylogenetic tree for the C23O genes indicated the presence of three distinct groups (I, IIa, IIb) with the exception of strains Pmen PC2, Ppu PC36 and PfF PC20. Notably, the LmPH gene was absent in the PfF PC20 strain.

Comparison of the clustering data **(**Fig. 1**)** of ten strains belonging to the *meta–ortho*^{prot} degradation type of phenol and *p*-cresol (Table 3) shows that all nine *P. fluorescens* biotype B strains analysed (PC18, PC21-PC23, PC32-PC34, PC37, PC38) form a unique set within the group I LmPH and the group I C23O genes. We suppose that it may reflect selective pressure of phenolic pollutants in the environment on *P. fluorescens* biotype B bacteria. Pmen PC12 strain from the *meta–ortho*^{prot} type is exceptional, because it clusters according to the LmPH analysis with *P. mendocina* strain PC2, while according to C23O analysis, with the group IIa (Fig. 1; Table 3).

P. mendocina strains (12 out of 13) degrade phenol and *p*-cresol through the *meta* pathway. The deduced amino acid sequences of LmPH and C23O are not congruent with *carA* (Fig. 1). Two main clusters of strains were revealed: six strains (PC1, PC3, PC4, PC8, PC10, PC11) form group IIa and five strains (PC5-PC7, PC9, PC19) form group IIb, according to sequences of LmPH and C23O. LmPHs from group IIb are closely related to those of the reference strains and belong to the Dmp family (DmpN, PhlD, PhhN and PheA4) (Fig. 1). The C23O genes of group IIb are similar to DmpB from *Pseudomonas* sp. CF600. LmPH gene from the strain Pmen PC2 groups together with that of the strain Pmen PC12, and C23Os of Pmen PC2 and PhhB are similar (Table 3). The *P. putida* strain PC36 from biotype B possessing the *meta–meta* catabolic type for degradation of phenol and *p*-cresol is exceptional: it is the only strain in this phylogenetic group with the C23O gene (Table 3). LmPH of the strain Ppu PC36 groups close to LmPHs of the Dmp family, and C23O close to *xylE* of *P. putida* HS1 (Fig. 1). Thus, our analysis concerning catabolic genes showed genetic heterogeneity of *P. mendocina* strains that may reflect adaptation of these bacteria to presence of phenolic pollutants in the environment.

Strains harbouring sPH

The *pheBA* operon determines the synthesis of sPH (encoded by the *pheA*) and C12O (encoded by the *pheB*), it is plasmid-borne and there is strong evidence of horizontal transfer of this operon in nature (Peters et al. 1997). The *pheBA* operon is flanked by two IS elements, IS*1472* and IS*1411*. The promoter of the *pheBA* operon is located upstream of IS*1472* (Kasak et al. 1993). IS*1411* was discovered as a consequence of insertional activation of the promoterless *pheBA* genes in *P. putida* due to the presence of outward-directed promoters at the left end of IS*1411* (Kallastu et al. 1998). In this study we analysed the presence of the

Table 3 Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among catabolic types and *Pseudomonas* species

Species and biotypes identified by *carA*	Strain designation (PC)	Catabolic type of phenol–*p*-cresol degradation	LmPH group[a]	C23O group[a]	*pheBA* operon[a,b]
P. fluorescens B	18, 21 –23, 32–34, 37, 38	*meta–ortho*^{prot}	I	I	–
P. mendocina	1, 3, 4, 8, 10, 11	*meta–meta*	IIa	IIa	–
	5–7, 9, 19	*meta–meta*	IIb	IIb	–
	2	*meta–meta*	Similar to 12	Similar to PhhB	–
	12	*meta–ortho*^{prot}	Similar to 2	Similar to IIa	–
P. putida B	14, 15	*ortho–ortho*^{prot}	IIIa	–	–
	16	*ortho–ortho*^{prot}	IIIa	–	+
	30	*ortho–ortho*^{prot}	IIIa	–	d
	13, 35, 39	*ortho–ortho*^{prot}	–	–	+
	36	*meta–meta*	IIb	Similar to XylE	–
P. fluorescens F	17	*ortho–ortho*^{prot}	IIIb	–	d
	P69	*ortho*^{c}	IIIb	–	+
	20	*ortho–meta*	–	Similar to NahH	+
P. fluorescens C	24–26, 28, 31	*ortho–ortho*^{prot}	–	–	+

ortho^{c}, the strain does not degrade *p*-cresol; *d, pheA* gene from *pheBA* operon absent

[a] –, *pheBA* operon, genes for LmPH or C23O are absent

[b] +, *pheBA* operon is present

pheBA operon in our strains by using PCR analysis with the *pheBA*-specific primers described in Table 2 (data not shown). We found this operon in 13 strains belonging to the *ortho–ortho*^{prot} type of degradation of phenol and *p*-cresol (Table 3). However, these strains belong to different *Pseudomonas* species and biotypes: *P. fluorescens* biotype C (PC strains 24–26, 28, 31), *P. putida* biotype B (PC strains 13, 16, 35, 39, 30) and *P. fluorescens* biotype F (PC strains 20, 17 and P69) (Table 3). The strain PfF PC20 is particularly interesting, as it is the only strain with the *ortho–meta* type of degradation for phenol and *p*-cresol possessing the C23O gene related to NahH gene of *P. putida* strain NCIB 9816-4. Therefore, it is reasonable to assume that the *pheBA* operon was transferred horizontally into this strain.

Co-presence of mPH and sPH genes

LmPH group IIIa + IIIb is the most distant cluster revealed by phylogenetic analyses. It contains four strains of *P. putida* B (PC14-16, PC30) and two strains

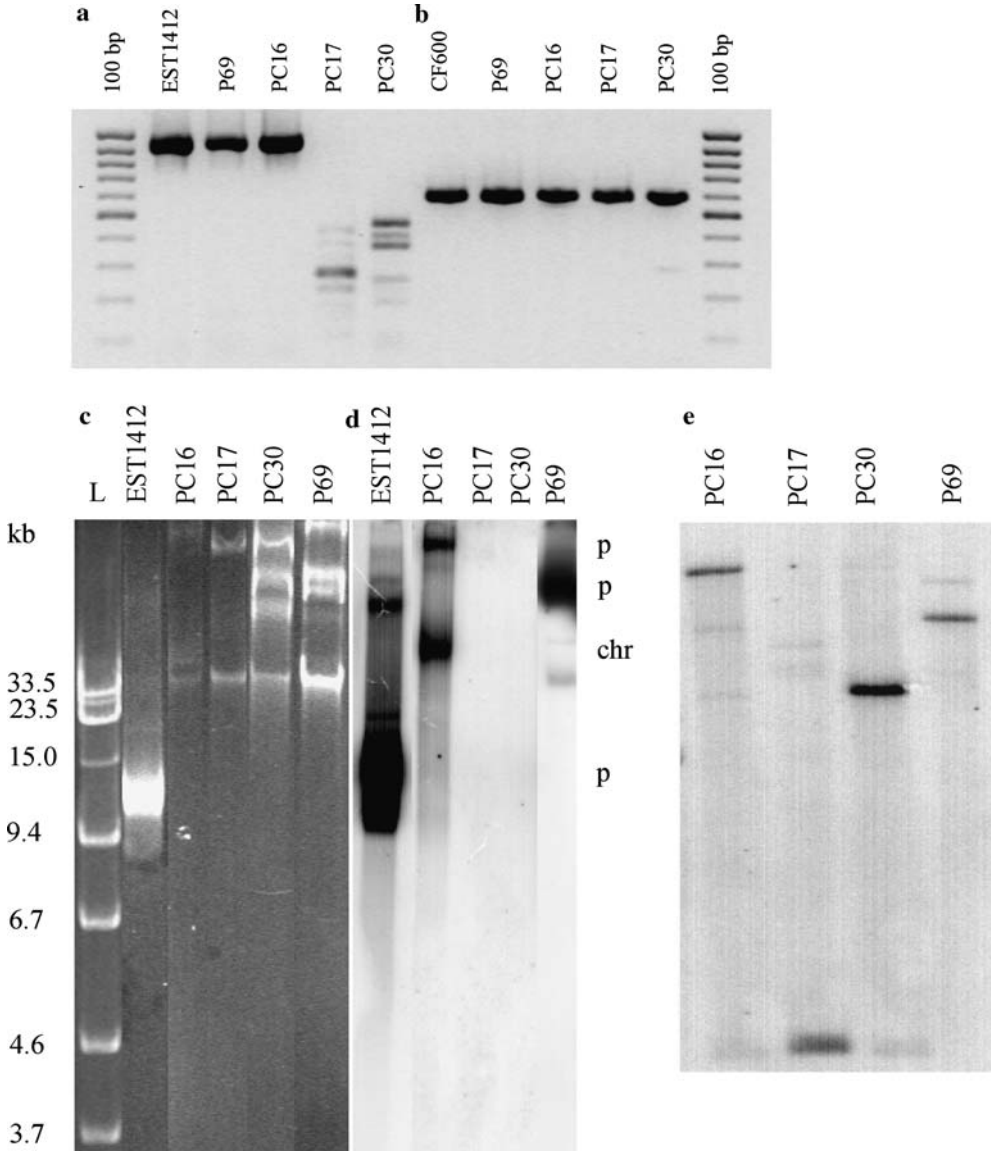

Fig. 2 Agarose gel electrophoresis of *pheA* (**a**) and LmPH (**b**) gene fragments amplified from the *pheA*$^{+}$ reference strain *P. putida* EST1412, the LmPH^{+} strain *Pseudomonas* sp. CF600 and the strains P69, PC16, PC17 and PC30. Plasmids retrieved from the *pheBA* operon possessing strains P69, PC16, PC17, PC30 and the positive control EST1412 (**c**) and Southern hybridisation of plasmids with the *pheA* gene probe (**d**). L, Bsp681/XhoI restriction fragments of λDNA were used as the DNA size standards; *chr* chromosomal DNA; *p* plasmid DNA. Autoradiograph of Southern analysis of total EcoRI-digested DNA of the studied strains using the LmPH gene probe (**e**). The strain designations are indicated above the *lanes*

of *P. fluorescens* F (PC17 and P69). Figure 1 indicates that MopN and MphN genes of reference strains are the nearest neighbours of group IIIa + IIIb. These six strains are the first examples of pseudomonads possessing the Mop type LmPHs coupled with the C12O gene. As shown in the previous paragraph, the *pheBA* operon was detected in four strains out of them: Ppu PC16, Ppu PC30, PfF PC17, PfF P69 (Table 3). The amplified products of sPHs and LmPHs from these strains were analysed together with those from reference strains using agarose gel electrophoresis. The co-presence of the two different genes of phenol hydroxylases, sPH (encoded by *pheA*) and mPH was found in strains Ppu PC16 and PfF P69**,** but not in PfF PC17 and Ppu PC30 (Fig. 2a, b). To examine the localisation of the gene coding for PHs, the plasmids of the four strains and the reference strain EST1412 were separated by electrophoresis and analysed by Southern hybridisation (Fig. 2c, d). We found that a *pheA* gene-specific probe yielded a strong hybridisation signal with the plasmid DNA of the strains Ppu PC16 and PfF P69, whereas no signal was detected in strains PfF PC17 and Ppu PC30. Applying an LmPH gene-specific probe, no hybridisation was detected in the case of the plasmid DNA from these strains, whereas the digested genomic DNA (EcoRI) showed positive hybridisation signal in all four lanes (Fig. 2e). These results verify the presence of the *pheA* gene in plasmids of two strains and indicate that the LmPH gene is chromosomally encoded in all four strains.

We analysed the structure of the *pheBA* operon of these four strains and in PfC PC24 by sequencing the amplified PCR products generated by PCR primers shown in Table 2. We revealed genetic changes in the *pheBA* operon in some strains compared to the original *pheBA* operon of pAT1140 (Kasak et al. 1993). The promoterless *pheBA* operon of pEST1412 was used as a control for the detection of the outward-directed promoter on the left side of IS*1411* (Kallastu et al. 1998). According to the analysis, the *pheBA* operon from strains PfC PC24 and PfF P69 is similar to that of pAT1140, whereas in Ppu PC16 it lacks IS*1411* (Fig. 3). The strains PfF PC17 and Ppu PC30 carry an incomplete *pheBA* operon without the *pheA*. It is known that some bacteria employ more than one pathway to degrade hydrocarbons, which allows formation of novel mixed metabolic pathways and may explain why bacterial strains capable of growing on contaminants emerge so quickly (Notomista et al. 2003). DNA

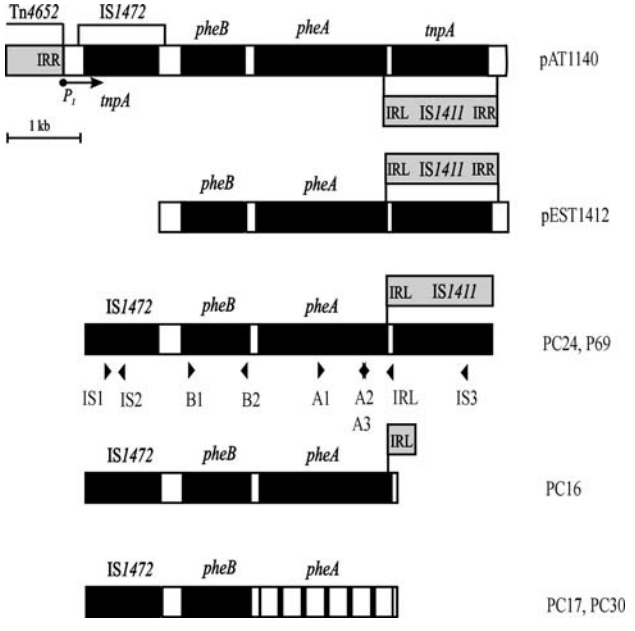

Fig. 3 Schematic organisation of the *pheBA* operon in strains possessing different level of the expression of sPH. Kasak and Kallastu determined the original structure of the pheBA operon in pAT1140 with co-workers (Kasak et al. 1993; Kallastu et al. 1998) (GenBank accession No. M57500). The *black* boxes show the locations of the *pheBA* genes and the transposase genes of IS*1472* and IS*1411*. The *open* boxes represent the intergenic regions, the *fragmented pheA* box indicates the eliminated region, and the *grey* box indicates the presence of the left end of inverted repeats (IRL) of IS*1411*

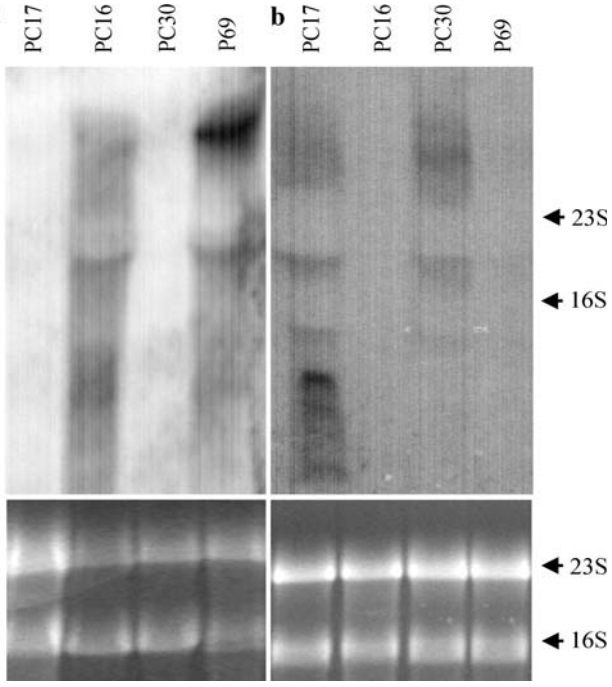

Fig. 4 Northern hybridisation analysis of *pheA* (**a**) and LmPH genes (**b**) from strains PC16, PC17, PC30 and P69. Hybridisation signals (*top*) and ethidium-bromide-stained total RNA in denatured agarose gel (*bottom*) is shown as a loading control

Table 4 PMO activity in *pheA*-expressing and -nonexpressing strains

Strain	Type of PH gene[a]	sPH[b] (nmol min^{-1} mg^{-1})
P. putida EST1412	*pheA*	1,008 ± 9
P. fluorescens C PC24	*pheA*	536 ± 73
P. fluorescens F PC17	mPH –	< 1
P. putida B PC30	mPH –	< 4
P. putida B PC16	mPH *pheA*	50 ± 6
P. fluorescens F P69	mPH *pheA*	469 ± 50
Pseudomonas sp. CF600	mPH	< 1

[a] mPH—contains a multicomponent phenol hydroxylase gene; *pheA*—contains a single component phenol hydroxylase gene;–, the *pheA* gene absent

[b] Specific activity values of sPH are means of three independent experiments ± standard errors of the means

fragments can move into new hosts to be incorporated or recombined, creating new mosaic genetic structures (van der Meer and Sentchilo 2003).

To evaluate the expression of the *pheBA* operon and the LmPH gene in the strains Ppu PC16, Ppu PC30, PfF PC17, PfF P69, a northern blot analysis and enzymatic study of sPH was performed. A hybridisation signal was detected with the *pheA* gene probe in case of RNA samples isolated from phenol-induced cells of Ppu PC16 and PfF P69 but not in PC17 and PC30, as expected from the absence of *pheA* in those strains (Fig. 4a). Interestingly, using a LmPH gene probe, RNAs from strains PC17 and PC30 hybridised, but no hybridisation signal was detected with RNA from Ppu PC16 and PfF P69 (Fig. 4b), although the respective gene was present in the genome of these strains (Fig. 2e). Specific activities of sPH of different *pheBA* positive strains and of the reference strain *P. putida* EST1412 showed that no sPH activity was present in cell extracts of PfF PC17 and Ppu PC30, and only low activity was detected in Ppu PC16. In contrast, the strains PfC PC24 and PfF P69 had a high sPH activity (Table 4). It has been previously reported that the mPH activity cannot be measured in crude cell extracts of pseudomonads (Powlowski and Shingler 1990). Consequently, the activity revealed in our assay should belong to sPH. This is further supported by the fact that mPH activity was not detectable using this assay in the reference strain *Pseudomonas* sp. CF600 that uses mPH for the growth on phenol (Table 4).

Thus, our results indicate elimination of the gene coding for sPH from the *pheBA* operon in strains PfF PC17 and Ppu PC30. However, these strains are still able to grow on phenol because they contain the LmPH gene. We assume that these strains have acquired the full-length *pheBA* operon through horizontal gene transfer, and further genetic rearrangements have led to the loss of the *pheA* gene. In strains Ppu PC16 and PfF P69 possessing genes for both sPH and mPH, the plasmid-encoded *pheA* gene is functional and expresses sPH activity but LmPH gene is probably not expressed as not detected in northern analysis. Next, we will attempt to find out how the LmPH gene has been inactivated in these strains and why some of the studied strains have selected sPH and others mPH for phenol degradation. It may be that the ability of bacterial cells to choose between different catabolic operons with similar function can be an effective and a flexible strategy to survive and function in a natural consortium under the conditions of heavy phenolic pollution.

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