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Statsionaarse faasi sigma faktori RpoS osalus *Pseudomonas putida* nälgivas populatsioonis toimuvates adaptatsiooniprotsessides

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Sisukord

Kasutatud lühendid

SOD - superoksiidi dismutaas

FeSOD - raua-seoseline superoksiidi dismutaas

MnSOD - mangaani-seoseline superoksiidi dismutaas

HPI - hüdroperoksidaas I

HPII - hüdroperoksidaas II

O_2^- - superoksiidradikaal

H_2O_2 - vesinikperoksiid

HO• - hüdroksüülradikaal

MMR - DNA valepaardumiste reparatsioon (*Mismatch Repair*)

(p)ppGpp - guanosiin (penta-) tetrafosfaat

RNAP - RNA polümeraas

GASP - statsionaarse faasi kasvueelis (*Growth Advantage In Stationary Phase*)

CFU – kolooniaid moodustavate rakkude arvukus (*Colony Forming Units*)

Sissejuhatus

Bakterirakud puutuvad oma elu jooksul kokku mitmete erinevate keskkonnatingimustega ning peavad sageli toime tulema toitainete puuduse ning erinevate stressifaktoritega. Stressitingimustes toimuvad bakterirakkudes laiaulatuslikud geeniekspressiooni muutused, mille tulemusena bakterirakud omandavad füsioloogilise seisundi, mis võimaldab neil ellu jääda ning adapteeruda uute keskkonnatingimustega. Paljude stressitingimustes vajalike geenide ekspressiooni reguleerib statsionaarse faasi sigma faktor RpoS, mille ekspressioon indutseeritakse vastusena rakkude kasvu aeglustumisele. RpoS tagab rakkudele laiaulatuliku kaitse mitmete stressitingimuste, sealhulgas pH ja temperatuuri kõikumiste, osmolaarsuse muutuste aga ka oksüdatiivse stressi eest. *E. coli* rakkudes on RpoS-i osalust näidatud ka statsionaarse faasi mutageneesil, mis suurendades bakteripopulatsiooni geneetilist mitmekesisust, annab samuti võimaluse bakteripopulatsiooni paremaks kohastumiseks erinevate keskkonnatingimustega.

Mehhanismidest, mille abil RpoS mõjutab *P. putida* nälgivas populatsioonis toimuvaid füsioloogilisi ja geneetilisi protsesse, on vähe teada.

Minu töö eesmärgiks oli uurida mehhanisme, mille kaudu statsionaarse faasi sigma faktor RpoS võiks mõjutada mullabakteri *P. putida* statsionaarse faasi nälgivates rakkudes toimuvaid adaptatsiooniprotsesse.

Kirjanduse ülevaade

Statsionaarse faasi sigma faktor RpoS on üldise stressivastuse regulaator. *E. coli* rakkudes on leitud ligikaudu 100 geeni, mille ekspressioon on reguleeritud RNA polümeraasi subühiku RpoS-i poolt (Loewen jt., 1998; Ishihama, 2000). Enamus nendest geenidest kodeerivad valke, mis aitavad rakkudel erinevates stressitingimustes (pH ja temperatuuri kõikumised, osmolaarsuse muutused, oksüdatiivne šokk) ellu jääda ja adapteeruda muutuvate keskkonnatingimustega (Lange ja Hengge-Aronis, 1991; Hengge-Aronis, 2002). Lisaks *E. coli´*le on RpoS olemasolu teada enamikes ?-perekonda kuuluvates proteobakterites, sealhulgas ka pseudomonaadides (Ramos-Gonzalez ja Molin, 1998; Suh jt., 1999), kus RpoS on oluline stressitingimustes toimetulekuks, kuid omab veelgi suuremat osatähtsust virulentsuse ja kolonisatsiooni regulatsioonis (Jorgensen jt., 1999; Suh jt., 1999). RpoS valgu taset rakkudes kontrollitakse nii transkriptsiooni, translatsiooni kui ka valgu stabiilsuse tasemel (Lange ja Hengge-Aronis, 1994; Loewen jt., 1998; Hengge-Aronis, 2002). Rakkude kasvu aeglustumisel ja üleminekul eksponentsiaalsest kasvufaasist statsionaarsesse faasi RpoS tase rakkudes suureneb, saavutades maksimumi juba varases statsionaarses faasis, kus RpoS valgu hulk moodustab ligikaudu 30 % vegetatiivse sigma faktori RpoD tasemest, võimaldades RpoS-il edukamalt konkureerida vabale RNA polümeraasile ja seeläbi aktiveerida stressitingimustes vajalike geenide transkriptsiooni (Jishage jt., 1996).

1. RpoS-i osalus statsionaarse faasi mutageneesis

Lisaks RpoS-i poolt mõjutatud füsioloogilistele ja morfoloogilistele muutustele, mis toimuvad rakkude sisenemisel statsionaarsesse faasi, on RpoS-i osalust näidatud ka statsionaarse faasi rakkude mutatsioonisageduse suurenemises, mis võimaldab rakkudel suurendada geneetilist mitmekesisust ning annab võimaluse kujuneda välja antud keskkonnaga paremini adapteerunud rakupopulatsioonil (Bjedov jt., 2003; Layton ja Foster, 2003; Lombardo jt., 2003; Matic ja Saint-Ruf, 2005).

RpoS-i osalust statsionaarse faasi rakkudes toimuvate mutatsioonide tekkel on peamiselt näidatud vigutegeva DNA polümeraas IV ehk DinB ekspressiooni positiivse

regulatsiooni (Layton ja Foster, 2003; Lombardo jt., 2003) ja peamise replikatsioonijärgselt DNA järjestust korrigeeriva DNA valepaardumiste reparatsioonisüsteemi (MMR-*Mismatch Repair*) funktsiooni vähendamise kaudu (Bjedov jt., 2003; Lombardo jt., 2003).

Adaptatiivsete ehk statsionaarse faasi nälgivates rakkudes toimuvate mutatsioonide tekkel on oluline osa vigutegeval DNA polümeraas IV ehk DinB valgul. DinB valgu ekspressioon *E. coli* rakkudes on reguleeritud SOS vastuse poolt (Kim jt., 1997; McKenzie jt, 2000). SOS vastus indutseeritakse rakkudes DNA kahjustuste korral ning on reguleeritud RecA ja LexA valgu poolt. Bakterirakule optimaalsetes keskkonnatingimustes on transkriptsioon SOS reguloni geenidelt LexA valgu poolt represseeritud (Little ja Mount, 1982; Walker, 1996). Kui bakteriraku DNA saab kahjustada, tekib rakus üheahelaline DNA (ssDNA), mis on märklauaks RecA valgule. ssDNA-ga seondunud RecA valk aktiveerub ja omandab koproteaasse aktiivsuse, põhjustades LexA repressorvalgu autokatalüütilise lagundamise (Little, 1991; Lou, 2001). Selle tulemusena suureneb rakus järk-järgult erinevate SOS geenide, sealhulgas *dinB* geeni ekspressioon (Friedberg jt., 1995; Courcelle jt., 2001).

Layton ja Foster on näidanud, et lisaks SOS induktsioonile on DinB ekspressioon *E. coli* rakkudes positiivselt reguleeritud ka statsionaarse faasi sigma faktori RpoS-i poolt (Layton ja Foster, 2003). *E. coli* DinB valgu hulk, mis eksponentsiaalselt kasvavates rakkudes püsib konstantsena, hakkab hilises statsionaarses faasis (24 tunni möödudes) tõusma. Samas väheneb *rpoS*-defektsetes bakterirakkudes DinB valgu hulk hilise nälja tingimustes 3-5 korda võrreldes eksponentsiaalse kasvufaasi rakkudega. Edasise nälgimisperioodi jooksul jääb DinB tase *E. coli* algse tüve rakkudes suhteliselt muutumatuna püsima, kusjuures *rpoS*-defektses tüves langeb DinB valgu hulk veelgi, moodustades 3-ndaks nälgimispäevaks vaid 2 % *E. coli* algse tüve DinB valgu tasemest. Isegi LexA repressorvalgu suhtes defektses tüves, kus DinB on konstitutiivselt ekspresseeritud, vajatakse maksimaalse DinB hulga saavutamiseks rakkudes RpoS-i olemasolu (Layton ja Foster, 2003).

DinB valgu hulga vähenemisest on tingitud ka 1-nukleotiidsete deletsioonide tekkesagaduse mitmekordne langus *E. coli rpoS*-defektsetes statsionaarse faasi rakkudes võrreldes algse *E. coli* tüve rakkudega. DinB-st sõltuvate 1-nukleotiidsete deletsioonide

arv *rpoS*-defektses tüves on 5-ndaks nälgimispäevaks ligikaudu 10 korda madalam kui algses tüves (Layton ja Foster, 2003).

DinB seost on näidatud ka *E. coli* statsionaarse faasi rakkudes toimuval amplifikatsioonilisel mutageneesil, mille tulemusena geeni koopiaarvu mitmekordistamise kaudu suureneb võimalus kasuliku mutatsiooni tekkimiseks vastavasse geeni (Anderssen jt., 1998; Hendrickson jt., 2002; Slechta jt., 2002). Amplifikatsioonilisi mutatsioone nagu ka 1-nukleotiidseid deletsioone on *E. coli* statsionaarse faasi nälgivates rakkudes uuritud peamiselt *lac* raaminihkesüsteemi abil. *E. coli* tüve FC40 rakkudes, kus on deleteeritud kromosomaalne *lacZ* geen ning F´ plasmiid sisaldab *lacZ* geeni, mille lugemisraami on viidud +1 raaminihe, on võimalik detekteerida statsionaarse faasi mutatsioonide toimumist, jälgides Lac^+ revertantide teket laktoosi sisaldaval minimaalsöötmel (Cairns ja Foster, 1991). *lacZ* geeni amplifikatsioone on võimalik detekteerida, kuna +1 raaminihe *lacZ* geenis ei pärsi *lacZ* geeni avaldumist, vaid võimaldab algse tüvega võrreldes 1-2 %-list ß-galaktosidaasi produktsiooni. Anderssoni ja Hendricksoni töögrupid on näidanud, et DNA amplifikatsioon on eelduseks 1-nukleotiidsete deletsioonide tekkele (Anderssen jt., 1998; Hendrickson jt., 2002). Spontaanselt toimuva *lac* operoni amlifikatsiooni käigus, mis võimaldab kasvu laktoosi minimaalsöötmel, tekib rakkudes üheahelaline DNA, mille tagajärjel indutseeritakse SOS-sõltuv DinB ekspressioon ning suureneb 1-nukleotiidsete deletsioonide tekkesagedus. Samuti võib 1-nukleotiidsete deletsioonide tekkesagedus amplifikatsioonilisel mutageneesil suureneda *dinB* geeni üleekpressiooni tõttu, mis on tingitud *lac* operoni läheduses paikneva *dinB* geeni koosamplifikatsioonist *lac* geeniga (Kofoid jt., 2002; Slechta jt., 2003). Rosenbergi töögrupp on vastupidiselt näidanud, et Lac^+ raaminihkemutatsioonide ja amplifikatsiooniliste mutatsioonide teke *E. coli* statsionaarse faasi rakkudes on teineteisest sõltumatud protsessid ning 1-nukleotiidsete deletsioonide teke ei vaja amplifikatsioonilise mutageneesi toimumist, vaid sõltub DinB poolt läbi viidavast vigaderohkest DNA replikatsioonist (Hastings jt., 2004). Samas on näidatud, et amplifikatsiooniliste mutatsioonide teke ei vaja funktsionaalset DinB valku, vaid sõltub Pol I olemasolust rakkudes (Hastings jt., 2004) ning on lisaks mõjutatud RpoS-i poolt. *rpoS*-defektsetes rakkudes on *lac*

amplifikatsioonide tekkesagedus 8-ndaks nälgimispäevaks võrreldes algse tüvega ligikaudu 6 korda vähenenud (Lombardo jt., 2003).

Lisaks *E. coli* statsionaarse faasi rakkudes toimuvatele 1- nukleotiidsetele deletsioonidele ja amplifikatsioonilistele mutatsioonidele on RpoS-i osalust näidatud ka *E. coli* vananevates kolooniates toimuvate asendusmutatsioonide tekkes (Bjedov jt., 2003). Asendusmutatsioonide, peamiselt G-A transitsioonide teke, tõuseb 7 päeva vanustes kolooniates ligikaudu 7 korda võrreldes esimesel päeval tekkinud kolooniatega. Mutatsioonisageduse tõus *E. coli* nädalavanustes kolooniates on sõltuv Pol II olemasolust ja MMR reparatsioonisüsteemi alatalitlusest. 73 % vananevates kolooniates tekkinud mutatsioonidest vajavad Pol II olemasolu, kuid on sõltumatud LexA valgust. See näitab, et kuigi Pol II kuulub SOS reguloni geenide hulka (Goodman, 2002), ei vajata asendusmutatsioonide tekkeks SOS vastuse induktsiooni ning Pol II basaalne ekspressioon rakkudes on piisav asendusmutatsioonide tekkesageduse suurenemiseks *E. coli* vananevates kolooniates (Bjedov jt., 2003). Kõrgenenud mutatsioonisageduse põhjuseks vananevates kolooniates on ka MMR-i võtmevalgu MutS-i vähenemine, mis on otseselt seotud RpoS valgu olemasoluga rakkudes (Bjedov jt., 2003). Nii *mutS* geeni mRNA kui ka MutS valgu hulk statsionaarse faasi rakkudes on kontrollitud RpoS-i ja Hfq valgu poolt (Tsui jt., 1997). *rpoS* ja *hfq* geeni suhtes üksikmutantse *E. coli* tüve nädalavanustes kolooniates mutatsioonisagedus ei suurene ja on võrreldav esimese päeva kolooniatega (Bjedov jt., 2003). Hfq kontrollib negatiivselt MutS valgu taset rakkudes nii RpoS valgust sõltuva kui sõltumatu mehhanismi kaudu. RpoS-st sõltumatu mehhanismi puhul arvatakse, et Hfq seondub kas otseselt *mutS* geeni mRNA-ga, blokeerides ribosoomi seostumissaidi ja mõjutades seeläbi translatsiooni initsiatsiooni või kontrollib MutS valgu hulka kaudselt, reguleerides näiteks *mutS* geeni mRNA degradatsiooni erinevate RNaaside taseme või aktiivsus kaudu rakus. RpoS valgust sõltuva mehhanismi puhul on teada, et Hfq reguleerib positiivselt *rpoS* geeni ekspressiooni (Muffler jt., 1996), seondudes *rpoS* geeni mRNA-ga ja eemaldades ribosoomi seondumissaidi lähedal paikneva mRNA sekundaarstruktuuri. Selle tulemusena väheneb *rpoS* geeni mRNA degradatsioon ning suureneb võimalus translatsiooni initsiatsiooniks *rpoS* geenilt. RpoS-i otsest mõju MutS valgu hulgale

senini näidatud ei ole, kuid arvatakse et see toimub RpoS-i kontrolli all olevate lisaregulaatorite, kas erinevate RNaaside või proteaaside osalusel (Tsui jt., 1997).

Lisaks *E. coli* statsionaarse faasi rakkudes toimuvatele mutatsiooniprotsessidele on RpoS-i osalust näidatud ka mullabakter *P. putida* nälgivas rakupopulatsioonis aset leidvate mutatsioonide ja DNA ümberkorralduste tekkeks. Saumaa jt. on näidanud, et RpoS-i olemasolu *P. putida* rakkudes on vajalik 2-3 nukleotiidsete deletsioonide ja insertsioonide tekkes, mille osakaal rakkudes tõuseb alates 6-ndast nälgimispäevast (Saumaa jt., 2002). RpoS-i osalust on näidatud ka *P. putida* nälgivates rakkudes toimuvate teisttüüpi DNA ümberkorralduste tekkes. On teada, et RpoS mõjutab negatiivset mobiilse DNA elemendi IS*1414* transpositsiooni aktivatsiooni, reguleerides ilmselt mõne faktori ekspressiooni, mis on vajalik IS*1414* transpositsiooni kontrolli all hoidmiseks (Saumaa jt., 2002). Samuti on näidatud, et transposooni Tn*4652* transpositsioon on positiivselt reguleeritud RpoS-i poolt. RpoS on otseselt vajalik transposooni Tn*4652* transposaasi geenilt *tnpA* lähtuva transkriptsiooni initsiatsiooniks (Ilves jt., 2001).

2. RpoS-i olulisus oksüdatiivse stressi tingimustes

RpoS-i vajalikkust oksüdatiivsete kahjustuste ärahoidmisel ja eemaldamisel on näidatud nii *E. coli* kui *P. putida* rakkudes (Almiron jt., 1992; Visick ja Clarke, 1997; Peters jt., 2001; Nunoshiba jt., 1996). Oksüdatiivsed kahjustused tekivad raku normaalse elutegevuse käigus moodustuvate rakulisi komponente kahjustavate reaktiivsete hapnikuühendite nagu näiteks superoksiidradikaalide (O_2^-), hüdroksüülradikaalide (HO•) või vesinikperoksiidi (H_2O_2) mõjul. Aeroobses keskkonnas kasvavavatel mikroorganismidel tekivad reaktiivsed hapnikuühendid peamiselt hingamisahela elektrontranspordi käigus (Gonzalez-Flecha ja Demple, 1995; Messner ja Imlay, 1999). Molekulaarne hapnik, mis on piisavalt väike, penetreerub hingamisahela elektrontransportahelas töötavate ensüümide aktiivtsentrisse, mille tagajärjel toimub elektroni juhuslik ülekanne hapnikule ning moodustuvad O_2^- ja H_2O_2 (Massey, 1994). Need aktiivsed hapnikuosakesed põhjustavad mitmete ensüümide inaktiveerumist, oksüdeerides nende aktiivtsentris paiknevat [4Fe-4S] klastrit ([4Fe-4S]

+ O_2^- + $2H^+$ → [3Fe-4S] + $Fe2^+$ + H_2O_2). Selline [4Fe-4S] klaster on oksüdeerituna ebastabiilne ja degradeerub, vabastades rakkudesse raua aatomi (Flint jt., 1993), mis võib osaleda HO• tekkes (Imlay, 2003). Valkude puhul on kirjeldatud ka teisi oksüdatiivsete hapnikuradikaalide poolt põhjustatud kahjustusi, nagu näiteks disulfiidsildade redutseerimine, valkudevaheline ristsidemete teke ja peptiidide fragmentatsioon, mis kõik viivad valgu funktsiooni kadumiseni (Fucci jt., 1983; Stadtman, 1990). Lisaks valkudele kahjustavad vabad hapnikuradikaalid ka membraanis paiknevaid lipiide, indutseerides nende peroksüdatsiooni ja muutes seeläbi membraani omadusi (Cabiscol jt., 2000).

Peamiseks nukleiinhappeid kahjustatvaks oksüdatiivseks hapnikuradikaaliks rakkudes on hüdroksüülradikaal (HO•) (Imlay, 2003), mis moodustub Fentoni reaktsioonis, kui vaba rauaioon kannab elektroni üle vesinikperoksiidile (H_2O_2+ Fe^{2+} → OH^- + FeO^{2+} + H^+ → Fe^{3+} + OH^- + HO•) (Keyer ja Imlay, 1996; Liochev ja Fridivich, 1994). HO• on ainus reaktiivne hapnikuühend, mis otseselt võib kahjustada enamikke biomolekule. Kuna nukleiinhapped seovad kergesti vabu rauaioone (Rai jt., 2001), siis on DNA peamine, mis Fentoni reaktsiooni käigus kahjustatud saab. HO• võib DNA-s kõrvaldada elektrone suhkrujäägilt ja lämmastikalustelt, aga ka lisada elektrone küllastumata lämmastikalustele, tekitades seeläbi laiaulatuslikke kahjustusi, milleks võivad olla katked DNA ahelas, lämmastikaluse ja suhkrujäägi vahelised ühendused või ristsidemed teiste molekulidega, mille tulemuseks on replikatsiooni peatumine rakkudes (Sies, 1993; Sies ja Menck, 1992).

Oksüdatiivsete kahjustuste kaitseks on bakterirakkudel välja kujunenud mitmesugused mehhanismid, milles osalevate valkude ekspressiooniks on vajalik RpoS-i olemasolu. Alljärgnevalt kirjeldan neid mehhanisme lähemalt.

Dps

Dps on DNA-d kaitsev valk, mille induktsioon *E. coli* statsionaarse faasi rakkudes on otseselt RpoS-st sõltuv (Almiron jt., 1992; Finkel ja Nair, 2004). Dps valgu kaitsefunktsioon seisneb selle mittespetsiifilises seostumises DNA-ga, mille tulemusena moodustub kõrgelt struktureeritud stabiilne nukleoproteiinkompleks (Wolf jt., 1999), kus kromosomaalne DNA on kaitstud lisaks oksüdatiivsele stressile ka temperatuuri

tõusust tingitud stressi, happe ja aluse šoki, erinevate kiirguste (UV-ja ?-kiirgus) ning samuti raua ja vase toksilisuse eest (Finkel ja Nair, 2004). DNA topoloogia muutmise kaudu reguleerib Dps ka mitmete pikaajalise nälgimise tingimustes vajalike geenide ekspressiooni (Almiron jt., 1992; Martinez ja Kolter, 1997). *E. coli* Dps valk, mis on homoloogne rauda siduva valgu ferritiiniga, omab ka ferroksidaasset aktiivsust, mille abil oksüdeeritakse rakkudes vabu metalliioone, peamiselt rauda (Ilari jt., 2002), mis osaleb Fentoni reaktsioonis elektroni doonori ja ülekandjana vesinikperoksiidile, põhjustades rakkudes reaktiivsete hüdroksüülradikaalide teket (Stadtman ja Berlett, 1991). Dps valgul on näidatud ka nõrka katalaasset aktiivsust, mis võimaldab sel ensüümil neutraliseerida vesinikperoksiidi, vähendadades seega veelgi DNA-d ja valke kahjustavate hüdroksüülradikaalide moodustumise võimalust Fentoni reaktsiooni käigus (Zhao jt., 2002).

Superoksiidi dismutaasid

Peamise rakulise kaitse superoksiidi stressi vastu tagavad superoksiidi dismutaasid (SOD-id), mis konverteerivad superoksiidradikaali vesinikperoksiidiks ja molekulaarseks hapnikuks (Fridovich, 1995). *E. coli* rakkudes on teada kaks rakusiseste superoksiidi radikaalide kahjutustamisel osalevat superoksiidi dismutaasi - *sodA* geeni poolt kodeeritav mangaani-seoseline MnSOD ja *sodB* geeni poolt kodeeritav raua-seoseline FeSOD (Beyer jt., 1991; Fridovich, 1995). Suuremat osatähtsust *E. coli* statsionaarse faasi rakkude kaitsel oksüdatiivsete kahjustuste eest aeroobses keskkonnas on näidatud *sodA* geeni poolt kodeeritud MnSOD-i puhul. *sodA* geeni suhtes defektsetes rakkudes suureneb märgatavalt O_2^- tundlikkus võrreldes *sodB*-defektsete või *E. coli* algse tüve rakkudega (Carlioz ja Touati, 1986).

sodA ja *sodB* geenide ekspressioon *E. coli* rakkudes on transkriptsiooni tasemel raua poolt kontrollitud. *sodA* geenilt lähtuv transkriptsioon on sõltuv mangaani olemasolust rakkudes ning negatiivselt reguleeritud raua poolt, mistõttu *sodA* geen ekspresseerub statsionaarse faasi rakkudes, kus raua kontsentratsioon on minimaalne (Moody ja Hassan, 1984; Katsuwon ja Anderson, 1990; Hassan ja Sun, 1992; Katsuwon jt., 1993). *sodA* geeni ekspressioon *E. coli* rakkudes on reguleeritud ka SoxR ja SoxS regulaatorvalkude poolt (Nunoshiba ja Demple, 1994). Kuna *soxS* geeni transkriptsioon

on indutseeritud RpoS-st sõltuvalt (Nunoshiba jt., 1996) on *sodA* geeni ekspressioon kaudselt mõjutatud RpoS-i olemasolust rakkudes. *sodB* geeni transkriptsioon on vastupidiselt *sodA* geenile indutseeritud logaritmiliselt paljunevates rakkudes ning väheneb, nagu juba öeldud, statsionaarse faasi rakkudes raua limitatsiooni tõttu (Pugh ja Fridivich, 1985; Katsuwon ja Anderson, 1990; Niederhoffer jt., 1990; Katsuwon jt., 1993).

*P. putida´*s on SOD valgu olemasolu näidatud nii tüve Corvallis rakkudes kui tüve KT2440 rakkudes (Kim jt., 1999; Kim jt., 2000; Heim jt., 2003). *P. putida* tüvi Corvallis omab sarnaselt *E. coli´*le kahte erinevat SOD valku: SodA-d ja SodB-d. Oksüdatiivsete kahjustuste eemaldamisel statsionaarse faasi rakkudes on suurem osatähtsus SodB valgul. *sodB* geeni suhtes defektne tüvi on palju tundlikum O_2^- kõrgenenud kontsentratsioonile ning märgatavalt aeglasema kasvuga võrreldes algse *P. putida* Corvallis tüvega. *sodA* ja *sodB* geenide suhtes defektne tüvi on veelgi tundlikum oksüdantidele ning selle kasv minimaalsöötmel on täielikult pärsitud. Selle põhjuseks arvatakse olevat O_2^- akumuleerumise tõttu inaktiveerunud Entner-Douderoffi raja ensüümid, mis on vajalikud glükoosi metabolismiks. Samas on näidatud, et *sodA* geeni suhtes defektse tüve fenotüüp sarnaneb nii O_2^- kõrgenenud kontsentratsiooni taluvuse kui ka kasvuvõime poolest algse *P. putida* Corvallis tüve fenotüübiga. Seega on FeSOD-il *P. putida* Corvallis tüve statsionaarse faasi rakkudes olulisem kaitsefunktsioon oksüdatiivse stressi tingimustes kui MnSOD-il. Kuigi sarnaselt *E. coli´*le, on *P. putida* Corvallis rakkudes FeSOD-i kodeeriva *sodB* geeni ekspressioon statsionaarses faasis alla reguleeritud, on SodB valk *P. putida´*s suhteliselt stabiilne ja olemas ka statsionaarse faasi rakkudes (Kim jt., 2000).

P. putida KT2440 tüve rakkudest on aga leitud täiesti uudne SOD valk. Seni teada olevad SOD valgud on kõik homodimeersed, kuid *P. putida* KT2440 rakkudes on kirjeldatud SodA ja SodB monomeeridest koosnevat heterodimeerset valku, mille ekspressioon on sõltumatu nii mangaani kui raua olemasolust kasvukeskkonnas (Heim jt., 2003).

Katalaasid

Teiseks ensümaatiliseks kaitsemehhanismiks oksüdatiivsete kahjustuste vastu on katalaasid, mis redutseerivad vesinikperosiidi (H_2O_2) veeks ja molekulaarseks hapnikuks (Loewen jt., 1985; Visick ja Clarke, 1997).

E. coli rakkudes on kaks kromosomaalset katalaasi: hüdroperoksidaas I (HPI), mida kodeerib *katG* geen ja hüdroperoksidaas II (HPII), mis on *katE* geeni poolt kodeeritud (Loewen jt., 1985). HPI ekspressioon on peamiselt indutseeritud logarimiliselt kasvavates rakkudes vastusena juba madalale H_2O_2 kontsentratsioonile (Visick ja Clarke, 1997). H_2O_2 olemasolu bakterite kasvukeskkonnas aitab tunnetada globaalne regulaatorvalk OxyR, mis oksüdatiivse stressi tingimustes omandab aktiivse konformatsiooni ning aktiveerib otseselt mitmete oksüdatiivsel stressil vajalike geenide, sealhulgas ka *katG* geeni, transkriptsiooni eksponentsiaalselt kasvavates *E. coli* rakkudes (Storz jt., 1990; Hidalgo ja Demple, 1996).

Rakkude üleminekul eksponentsiaalsest kasvufaasist statsionaarsesse faasi suureneb rakkudes märgatavalt (ligikaudu 7 korda) HPII valgu hulk (von Ossowski jt., 1991), mis ei ole indutseeritud H_2O_2 poolt aktiveeritud OxyR regulaatorvalgu kaudu, vaid on otseselt sõltuv RpoS-i olemasolust rakkudes. RpoS reguleerib positiivselt HPII kodeeriva geeni *katE* transkriptsiooni initsiatsiooni *E. coli* statsionaarse faasi rakkudes (Loewen ja Triggs, 1984; Mulvey jt., 1990; Schellhorn ja Hassan, 1988). RpoS-i puudumisel on HPII aktiivsus statsionaarse faasi rakkudes vaid vaevu detekteeritav (Visick ja Clarke, 1997).

Vähest aktiivsuse tõusu (kuni 2 korda) *E. coli* satsionaarse faasi rakkudes on märgatud ka HPI puhul, kuid see ei ole sõltuv funktsionaalse RpoS-i olemasolust (Visick ja Clarke, 1997).

RpoS-i positiivset mõju on näidatud ka *P. fluorescens´*i biodegradeerivast plasmiidist pAM10.6 leitud katalaasi geeni *katA* ekspressioonile (Peters jt., 2001). Nii *P. fluorescens´*i kui ka *P. putida* statsionaarse faasi rakkudes on plasmiidse KatA ekspressioon umbes 4 korda tõusnud võrreldes eksponentsiaalselt kasvavate rakkudega. Samas on *rpoS* geeni suhtes defektsetes *P. putida* statsionaarse faasi rakkudes plasmiidse KatA aktiivsus 3-4 korda langenud, mis viitab sellele, et KatA on RpoS-i poolt reguleeritud (Peters jt., 2001).

3. RpoS-i osalus bakterirakkude adapteerumisel stressitingimustes

Stressitingimustes toimuvad bakterirakkudes laiaulatuslikud geenieksрessiooni muutused, mille tulemusena bakterid omandavad füsioloogilise seisundi, mis võimaldab neil adapteeruda uute keskkonnatingimustega ja seeläbi ellu jääda (Ojha ja Chatterji, 2001). Üheks oluliseimaks globaalseks geenieksрessiooni regulaatoriks on guanosiin tetrafosfaat (p)ppGpp, mille süntees rakkudes suureneb peamiselt toitainete limitatsiooni (Spira jt., 1995) aga ka mitmete teiste stressitingimuste korral, mis viivad bakterirakkude kasvu aeglustumiseni (Teich jt., 1999; Magnusson jt., 2005). (p)ppGpp tase rakkudes on kontrollitud kahe ensüümi, RelA ja SpoT poolt (Cashel jt., 1996; Gentry ja Cashel, 1996). RelA on ribosoomiga seonduv valk, mis katalüüsib (p)ppGpp sünteesi peamiselt aminohapete nälja korral vastusena laadimata tRNA seondumisele ribosoomi A saiti, mis põhjustab translatsiooni elongatsiooni peatumise (Haseltine ja Bock, 1973; Egli ja Wick, 2004). SpoT omab nõrka (p)ppGpp süntetaasi aktiivsust, kuid on peamiselt hüdrolaas, mis degradeerib (p)ppGpp-d (Hernandez ja Bremer, 1991; Xiao jt., 1991) ning on oluline (p)ppGpp akumuleerumiseks erinevates stressitingimustes (Magnusson jt., 2005). SpoT hüdrolüüsi aktiivsuse inhibeerimine stressifaktorite poolt võimaldab neil reguleerida positiivselt (p)ppGpp taset rakkudes (Murray ja Bremer, 1996).

(p)ppGpp-sõltuv geenieksрessiooni regulatsioon toimub transkriptsiooni tasemel, (p)ppGpp otsese seostumise kaudu RNA polümeraasiga (Hernandez ja Cashel, 1995; Reddy jt., 1995). (p)ppGpp mõju geenieksрessioonile võib olla nii positiivne kui negatiivne. Raku kasvu ja jagunemisega seotud geenide (näiteks rRNA, tRNA, ribosomaalsete valkude, rasvhapete ja lipiidide biosünteesiga seotud ning DNA replikatsiooniks vajalike geenide) transkriptsioon on (p)ppGpp poolt negatiivselt kontrollitud (Magnusson jt., 2005). Näiteks rRNA geenide puhul on näidatud, et (p)ppGpp seondumine RNAP-ga destabiliseerib transkriptsiooni initsiatsiooni avatud kopleksi ning vähendab sellega transkriptsiooni toimumist rRNA geenidelt (Paul jt., 2004; Barker jt., 2001). rRNA sünteesi vähenemise tagajärjel suureneb rakkudes vaba RNAP hulk, mis võimaldab aktiveerida transkriptsiooni RpoD-st sõltuvate madala RNAP sidumisefektiivsusega geenide promootoritelt. Kuna nende geenide

transkriptsiooni initsiatsioonil moodustuv avatud kompleks on võrreldes rRNA geenide promootoritel moodustuva avatud kompleksiga püsivam, siis nende geenide promootoritelt lähtuvat transkriptsiooni (p)ppGpp ei vähenda (Egli ja Wick, 2004). Sellist kaudset (p)ppGpp positiivset mõju geenieksprессiooni regulatsioonile vaba RNAP hulga suurendamise kaudu rakkudes on näidatud aminohapete biosünteesi ja proteolüüsiga seotud geenide puhul (Egli ja Wick, 2004).

Positiivselt reguleerib (p)ppGpp ka *rpoS* geeni transkriptsiooni, suurendades seeläbi rakkudes RpoS-i hulka (Gentry jt., 1993; Lange jt., 1995; Zgurskaya jt., 1997). Lisaks on näidatud, et (p)ppGpp seondumine RNAP-ga muudab viimase konformatsiooni, võimaldades RpoS-il veelgi efektiivsemalt RNA polümeraasile seostuda ning aktiveerida transkriptsiooni mitmetelt *rpoS* reguloni kuuluvatelt geenidelt (Ojha ja Chatterji, 2001; Jishage jt., 2002). Arvatakse, et RpoS on üheks faktoriks, mille kaudu (p)ppGpp tagab vajalike geenide ekspressiooni rakkude kasvu aeglustumisel või kokkupuutel stressitingimustega (Magnusson jt., 2005).

rpoS reguloni kuuluvad geenid on lisaks stressitaluvuse suurendamisele seotud ka metabolismi regulatsiooniga, võimaldades rakkudel stressitingimustes üle minna anaeroobsele metabolismile, et vältida reaktiivsete hapnikuühenite tekkimist, mis võiksid kahjustada rakulisi komponente ja põhjustada oksüdatiivset stressi (Nyström, 2004). Samuti on RpoS oluline raku morfoloogilistes ümberkorraldustes (Lange ja Hengge-Aronis, 1991) ja virulentsusgeenide regulatsioonis (Hengge-Aronis, 2002). RpoS, mis tagab rakkudele laiaulatusliku kaitse, on vajalik rakkude samaaegsel kokkupuutel mitme erinava stressifaktoriga (Egli ja Wick, 2004). Samas, kui bakterirakud on eksponeeritud vaid ühele konkreetsele stressile, näiteks süsinikuallika limitatsioon, on RpoS-i olemasolu rakkudes pigem puuduseks, sest konkureerides RpoD-ga RNAP holoensüümile seondumises, vähendab RpoS glükoosi omastamiseks vajalike RpoD-st sõltuvate geenide transkriptsiooni ja limiteerib seeläbi ka bakterirakkude kasvu (Farewell jt., 1998; Notley-McRobb jt., 2002). Kuna näljatingimustes on toitainete efektiivne omastamine väga oluline, on sellega seletatav ka *rpoS* geeni suhtes mutantsete statsionaarses faasis kasvueelist (GASP–*Growth Advantage In Stationary Phase*) omavate bakterirakkude välja selekteerumine näljatingimustes (Tanaka jt., 1995).

GASP fenotüüpi on kirjeldatud väga paljudes bakteriliikides ning defineeritud kui pikka aega toitainetevaeses keskkonnas viibinud vananevate rakkude võimet välja konkureerida noorema kultuuri rakke (Zambrano jt., 1993; Finkel ja Kolter, 1999). *E. coli* puhul on GASP fenotüübiga rakke leitud 10 päeva vanustest populatsioonidest. On näidatud, et sellise „vana" kultuuri kokkusegamisel ja kooskultiveerimisel üleöö kasvanud, „värske" *E. coli* tüve rakkudega hakkab „värske" populatsiooni arvukus järjest vähenema ning 7-10 päeva möödudes sisaldab bakteripopulatsioon vaid „vana" kultuuri rakke (Zambrano jt., 1993; Finkel ja Kolter, 1999). GASP fenotüübi teke on geneetiline ega ole tingitud füsioloogilisest adaptatsioonist statsionaarse faasi keskkonnaga. Seda tõestab GASP fenotüübi säilimine rakkudes, mis on korduvalt läbinud logarimilise kasvufaasi (Zambrano jt., 1993).

Senini on GASP fenotüüpi omavates rakkudes leitud mutatsioone kolmes geenis. Lisaks juba mainitud *rpoS* geenile, tagavad kasvueelise ka mutatsioonid *lrp* (*leucine responsive protein*) geenis ja *ybeJ-gltJKL* geeniklastris, mis kodeerivad vastavalt aspartaadi ja glutamaadi transportereid (Zinser ja Kolter, 1999). Juhul, kui katkestada kasvõi üks nendest geenidest, on GASP fenotüüpi võimalik tekitada ka mittevananevates rakkudes (Zambrano jt., 1993). Kuigi need geenid on seotud erinevate protsessidega rakus ning GASP mutatsioonid nendes on molekulaarsel tasemel erinevad, viivad need siiski sarnase fenotüübi tekkele, mis suurendab rakkude võimet kataboliseerida ühte või mitut aminohapet süsiniku- ja energiaallikana (Zinser ja Kolter, 1999). Kasvueelis on näljatingimustes äärmiselt oluline, võimaldades surnud rakkudest vabanenud aminohappeid paremini omandada ja nende arvelt ellu jääda (Finkel, 2006). Kuigi GASP fenotüübi tekkeks ei ole hädavajalik mutatsiooni toimumine *rpoS* geenis, on enamuste rakkude kasvueelis põjustatud siiski *rpoS* geeni muteerumisest (Finkel ja Kolter, 1999; Farrell ja Finkel, 2003). Mutatsioon *rpoS* geenis ei põhjusta selle inaktiveerumist, vaid vähendab RpoS valgu aktiivsust (Bohannon, 1991). Seega võib GASP fenotüübi teke vähemalt osaliselt olla seotud RpoS reguloni kuuluvate geenide ekspressiooni muutusega (Vijayakumar jt., 2004; Patten jt., 2004). Arvatakse, et nõrgenenud RpoS aktiivsus võimaldab rakkude kiiremat kasvu, kuid on samas piisav, et aktiveerida oksüdatiivse stressiga toimetulekuks vajalike geenide ekspressioon (Farrell ja Finkel, 2006).

Materjal ja metoodika

1. Söötmed, bakteritüved ja plasmiidid

Söötmetena kasutasime LB-söödet (Miller, 1972) ja minimaalsöödet M9 baasil (Adams, 1959), millele lisasime mikroelemente (2,5 ml/l; Bauchop ja Elsden, 1960), aminohapete hüdrolüsaati (CAA, 10 ml 20 % lahust 1 l söötme kohta) ning süsinikuallikana glükoosi (Glc) lõppkontsentratsiooniga 10 mM. *E. coli* kasvatamisel minimaalsöötmel lisasime veel vitamiini P1. Tardsöötme saamiseks lisasime vedelsöötmele agarit (15 g/l). Plasmiidi selektsiooniks kasutasime antibiootikume: karbenitsilliini (Cb; 1-3 mg/ml) ja ampitsilliini (Amp; 0,1 mg/ml) *P. putida* rakke kasvatasime temperatuuril 30 ºC, *E. coli* rakke temperatuuril 37 ºC. Vedelsöötmes kasvatamisel aereerisime kultuure loksutil.

Töös kasutatud bakteritüved ja plasmiidid on toodud tabelis 1.

Tabel 1. Töös kasutatud bakteritüved ja plasmiidid.

Bakteritüvi	Genotüüp või iseloomustus	Allikas
Escherichia coli		
DH5α	*supE44*? *lacU169 (f80 lacZ* ? M15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
HB101	*subE44 subF58 hsdS3* ($r_B^-m_B^-$) *recA13pro*? *2 lacY1 galK2 rsp20 xyl-5 mt1* -1	Boyer jt., 1969
CC118?pir	*galE galK phoA20 thi*-1 *rpsE rpoB argE* (Am) *recA*1 ?pir phage lysogen	Herrero jt., 1990
Pseudomonas putida		
PaW85	algne tüvi, identne tüvega KT2440	Bayley jt., 1997
PaW85tet	*tnpAC*::tet	Tark jt., 2005
PaWdinB	*dinB*::tet	Tegova jt., 2004
PaWrpoS	*rpoS*::Km	Ojangu jt., 2000
PaWrpoSsm	*rpoS*::Km, Sm resistentsusgeen plasmiidist pBK-miniTn7-? Sm1	käesolev töö
PaWrpoStet	*rpoS*::Km, *tnpAC*::tet	käesolev töö
PaWrpoSlacItacrpoS	*rpoS*::Km, *rpoS* geen P*tac* promootori kontrolli all. Konstrueeritud analoogselt PaWRpoS tüvele (Ojangu jt., 2000)	käesolev töö
PaWrpoSlacItacsodAB	*rpoS*::Km, *sodA* ja *sodB* geenid P*tac* promootori kontrolli all	käesolev töö

Plasmiid		
pBluescript, KS(+)	kloneerimisvektor (Amp^r)	Stratagene
pKTlacZ	reporterplasmiid (Amp^r)	Hõrak ja Kivisaar, 1998
pUC18Not	kloneerimisvektor (Amp^r)	Herrero jt., 1990
pUTmini-Tn5Tel	Tn5 Tel minitransposooni sisaldav pUT plasmiid (Amp^r,Tel^r)	Sanchez-Romero jt., 1998
pBRlacItac	P*tac* promootor ja *lacI*^{q} repressor plasmiidis pBR322	Ojangu jt., 2000
pBK-miniTn7-? Sm1	Tn7 Sm minitransposooni sisaldav pUC19 plasmiid (Amp^r,Sm^r)	Koch jt., 2001
pKTpheA56+A	plasmiidis pKT240 sisalduva *pheA* geeni 56-ndas positsioonis asuvasse ACC (Thr) koodonisse on inserteeritud A nukleotiid	Tegova jt., 2004
pKTpheA22TAG	plasmiidis pKT240 sisalduva *pheA* geeni 22. koodon CTG (Leu) on asendatud translatsiooni stoppkoodoniga TAG	Tegova jt., 2004
pKSdinB	*dinB* promootorregioon kloneeritud vektorisse pBluescript, KS(+)	käesolev töö
pKTPdinBlacZ	*dinB* promootorregioon plasmiidist pKSdinB kloneeritud vektorisse pKTlacZ	käesolev töö
pKSsodB	*P. putida sodB* geen kloneeritud vektorisse pBluescript, KS(+)	käesolev töö
pKSsodAB	*sodA* geen kloneeritud plasmiidi pKSsodB	käesolev töö
pBRlacItacsodAB	*sodA* ja *sodB* geenid plasmiidist pKSsodAB kloneeritud plasmiidi pBRlacItac	käesolev töö
pUC18NotlacItacsodAB	*lacI*^{q}-P*tac-sodAB* ekspressioonikassett plasmiidist pBRlacItacsodAB kloneeritud plasmiidi pUC18Not	käesolev töö
pUTtellacItacsodAB	*lacI*^{q}-P*tac-sodAB* ekspressioonikassett plasmiidist pUC18NotlacItacsodAB kloneeritud plasmiidi pUTmini-Tn5Tel	käesolev töö
pUTkmlacItacrpoS	*lacI*^{q}-P*tac-rpoS* ekspressioonikassett plasmiidis pUTmini-Tn5Km	Ojangu jt., 2000
pKTPdinBluxAB	*dinB* promootorregioon kloneeritud vektorisse pKTluxAB	Tegova jt., 2004

2. mRNA alguspunkti määramine pöördtranskriptaasi reaktsiooni meetodil

mRNA alguspunkti määrasime pöördtranskriptaasi reaktsiooni abil, kasutades radioaktiivselt märgitud praimerit dinBXho, mis oli komplementaarne *P. putida dinB* geeni 5´ otsa DNA-ga positsioonides 40 kuni 58 *dinB* geeni ATG koodoni suhtes. Praimeri märkisime radioaktiivselt, võttes kinaasireaktsiooni 10 pmol praimerit, 10 kordset kinaasi puhvrit (100 mM $MgCl_2$; 50 mM DTT; 1mM spermidiin; 1 mM EDTA; 100 mM Tris-HCl, pH7,6) 1/10 reaktsiooni mahust, 10 ühikut kinaasi („Fermentas") ning 0,5 µl ?^{32}P ATP-d („Amersham"). Reaktsioon toimus 1 tund temperatuuril 37 ºC,

seejärel sadestasime praimerit temperatuuril -20 ºC 1/10 mahu 5M NaCl-i ja 3,5 kordse mahu 96 % etanooliga, pesime 75 % etanooliga ning lahustasime 6 µl-s vees. Totaalse RNA eraldasime *P. putida* rakkudest RNA Rneasy MiniKit („QIAGEN") protokolli järgi. Reaktsioonisegusse, mis sisaldas dNTP-d (0,5 mM), reaktsioonipuhvrit (75 mM KCl; 10 mM $MgCl_2$; 0,5 mM spermidiin; 50 mM Tris, pH 8,3), 4 ühikut pöördtranskriptaasi („GibcoBRL") ning 2 ühikut RNasiini („Fermentas"), võtsime võrdse koguse (~5 µg) RNA-d. Segasime kokku totaalse RNA ja praimeri, denatureerisime mRNA kuumas vesivannis (96 ºC), lisasime 10 kordset reaktsioonipuhvrit 1/10 reaktsiooni mahust ning langetasime aegalaselt temperatuuri 54 ºC-ni. Seejärel lisasime RNasiini ja pöördtranskriptaasi ning nukleotiidide segu. Reaktsioon toimus temperatuuril 42 ºC 30 minutit, mille järel peatasime reaktsiooni 1M ammooniumatsetaadiga 96 % etanoolis, sadestasime 15 minutit temperatuuril -20 ºC, pesime sadet 75 % etanooliga ning lahustasime 5 µl-s „Amersham" DNA sekveneerimiskiti stopplahuses (95 % spermidiin; 20 mM EDTA; 0,05% broomfenoolsinine; 0,05 % ksüleen tsüanool FF). Enne geelile kandmist denatureerisime proove 5 minutit temperatuuril 96 ºC.

Geelelektroforeesi viisime läbi 6,5 %-ses akrüülamiidgeelis TBE puhvris (45 mM boorhape; 0,9 mM EDTA; 45 mM TrisHCl, pH 8,3) temperatuuril 55 ºC, pingel 1800V. seejärel kuivatasime geeli ning eksponeerisime fosfoimageri ekraanile.

3. *P. putida dinB* geenilt lähtuva transkriptsiooni uurimine

Selleks, et uurida *P. putida dinB* geeni promootorilt lähtuva transkriptsiooni taseme sõltuvust statsionaarse faasi sigma faktorist RpoS, amplifitseerisime *P. putida* tüve PaW85 genoomist 410 bp suuruse ala, mis sisaldas *dinB* geeni promootorala ning sellest allapoole paiknevat *dinB* geeni transkriptsiooni alguspunkti koos 58 nt pikkuse järjestusega *dinB* geeni algusest. *dinB* geeni eelset ala sisaldava PCR-i produkti kloneerisime restriktaasiga *EcoR*V avatud vektorisse pBluescriptKS. Saadud plasmiidist pKSdinB lõikasime *P. putida dinB* promootorregiooni restriktaaside *BamH*I ja *Xho*I fragmendina välja ning kloneerisime samade ensüümidega avatud vektorisse pKTlacZ. Saadud plasmiidi nimetasime pKTPdinBlacZ.

dinB promootorilt lähtuva transkriptsiooni taset hindasime ß-galaktosidaasi hulka määrates. Selleks kasvatasime plasmiidi pKTPdinBlacZ sisaldavaid *P. putida* rakke minimaalsöötmes. Reaktsioonisegu sisaldas 1,6 ml Z-puhvrit (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 50 mM ß-merkaptoetanool; 0,001 % SDS, pH 7), 0,4 ml ONGP-lahust (o-nitrofenüül- ß-D-galaktopüranosiid, 4 mg/ml), 100 µl kloroformi ning 10-200 µl rakukultuuri. Mõõtsime lisatud rakukultuuri optilise tiheduse (OD_{580}) ja fikseerisime aja reaktsiooni käivitamisest kuni reaktsiooni peatamiseni 1 ml 1M Na_2CO_3 lahusega. ß-galaktosidaasi hulga määrasime spektofotomeetriliselt, lähtudes lainepikkusel 420 nm valgust neelava produkti o-nitrofenooli tekkimisest ajaühikus rakutiheduse kohta.

4. Superoksiidi dismutaaside SodA ja SodB üleekspressioonikonstrukti konstrueerimine

Selleks, et uurida SodAB valgu hulga mõju mutatsioonide tekkesagedusele nälgivates bakterites amplifitseerisime *P. putida* tüve PaW85 genoomist 662 bp pikkuse *sodA* geeni ja 639 bp pikkuse *sodB* geeni. *sodB* geeni sisaldava PCR-i produkti kloneerisime restriktaasiga *EcoR*V avatud vektorisse pBluescriptKS. Saadud plasmiidi pKSsodB kloneerisime *sodA* geeni sisaldava PCR-i produkti, kasutades selleks restriktaasi *Sma*I. *sodA* ja *sodB* geene sisaldavale plasmiidi nimetasime pKSsodAB. Edasi lõikasime plasmiidist pKSsodAB välja *sodA* ja *sodB* geene sisaldava ala, kasutades selleks restriktaase *Xba*I ja *Sal*I ning sisestasime samade restriktaasidega avatud vektorisse pBRlacItac. Saadud plasmiidist pBRlacItacsodAB lõikasime *sodA ja sodB* geenid koos *tac* promootori ja repressorgeeniga *lacI BamH*I fragmendina välja ning kloneerisime sama ensüümiga avatud vektorisse pUC18Not, saades plasmiidi pUC18NotlacItacsodAB. Viimase etapina lõikasime plasmiidist pUC18NotlacItacsodAB välja *sodA ja sodB* geenid koos *tac* promootori ja *lacI* geeniga, kasutades selleks restriktaasi *Not*I ning sisestasime sama restriktaasiga avatud vektorisse pUTmini-Tn5Tel. Saadud plasmiidi nimetasime pUTtellacItacsodAB.

5. *P. putida* nälgivates rakkudes toimuvate mutatsioonide tekkesageduse määramine

P. putida rakke, mis sisaldasid 1-nukleotiidsete deletsioonide detekteerimiseks vajalikku pKTpheA56+A või asendusmutatsioonide detekteerimiseks vajalikku pKTpheA22TAG plasmiidset testsüsteemi kasvatasime üleöö 2,3 ml-s minimaalsöötmes (vaata punkt 1). 18-20 tundi kasvanud rakukultuurist võtsime rakususpesnsiooni, tsentrifuugisime rakud söötmest välja ja suspendeerisime 1 x M9 lahuses. Seejärel plaatisime ligikaudu 5 x 10^8 bakterirakku fenooli minimaaltassidele, mis sisaldasid plasmiidi selektsiooniks vajalikku antibiootikumi karbenitsilliin (Cb). Tüvede PaWrpoSlacItacrpoS ja PaW85rpoSlacItacsodAB puhul oli lisatud ka IPTG lõppkontsentratsiooniga 0,5 mM. Phe^+ revertantide akumuleerumist selektiivtassidel jälgisime 15 päeva jooksul peale plaatimist. Teisel päeval tassidele ilmunud Phe^+ kolooniad sisaldasid mutatsiooni, mis oli tekkinud kasvavas bakterikultuuris enne rakkude plaatimist fenooli minimaalsöötmele. Kolmandal päeval ja hiljem tassidele ilmunud Phe^+ kolooniate puhul olid mutatsioonid tekkinud bakterite fenooli minimaaltassil nälgimise käigus ning seega olid need Phe^+ revertandid statsionaarse faasi mutandid. Phe^+ revertantide tekkesageduse arvutasime nälgivas populatsioonis olevate elusrakkude kohta.

6. *P. putida* nälgiva populatsiooni elumuse määramine

Bakterirakkude elumust määrasime kogu nälgimisperioodi jooksul kolooniaid moodustavate rakkude (CFU-*Colony Forming Units)* arvukuse alusel samadelt fenooli minimaaltassidelt, millel jälgisime Phe^+ revertantide teket. 1-ml pipetiotsikut kasutades lõikasime tassidelt ilma Phe^+ kolooniateta agaritükikesed, suspendeerisime nendel olevad rakud 1 x M9 lahuses, tegime vastavad lahjendused ja plaatisime LB tassidele. Lähtudes tassidele tekkinud kolooniate arvust ja tehtud lahjendustest arvutasime agaritükil olevate elusrakkude arvu. Saadud arvust tuletasime tassi pindala ja agaritüki pindala suhte alusel kogu elusrakkude arvu fenooli minimaaltassidel.

7. Süsinikunäljas adapteerunud *P. putida rpoS*-defektse populatsiooni konkurentsikatsed

Selleks, et uurida kas süsinikunälja tingimustega adapteerunud *P. putida rpoS* geeni suhtes defektne rakupopulatsioon on näljatingimustes elujõulisem, kasutasime *P. putida* tüvesid, mis olid märgitud erinevate antibiootikumide resistentsusgeenidega. Tüvedes PaW85tet ja PaWrpoStet olid bakteri kromosoomis asuva transposooni Tn*4652 tnpA* ja *tnpC* geenid katkestatud tetratsükliini resistentsusgeeniga. Tüve PaWrpoSsm korral oli viidud kromosoomi streptomütsiini resistentsust tagav geen, kasutades plasmiidi pBK-miniTn7-? Sm1. See võimaldas neid tüvesid antibiootikumi resistentsuse alusel segakultuuris eristada. Kõigepealt, selleks et tekitada süsinikunäljas adapteerunud *P. putida rpoS*-defektne rakupopulatsioon, plaatisime glükoosi sisaldavas minimaalsöötmes üleöö kasvanud *P. putida* PawrpoSsm tüve rakukultuurist ligikaudu 5 x 10^8 bakterirakku fenooli minimaalsöötmele ning näljutasime neid 10 päeva temperatuuril 30 ºC. *P. putida rpoS*-defektsed rakud, mis olid 10 päeva fenooli minimaaltassidel adapteerunud, pesime tassidelt 1 x M9 puhvriga maha ning plaatisime seguna suhtes 1:100, kas üleöö minimaalsöötmes kasvanud PaW85tet või PaWrpoStet tüve rakkudega fenooli minimaalsöötmele. Adapteerunud *P. putida rpoS*-defektse rakupopulatsiooni konkurentsivõimet hindasime mõõtes rakkude elumust eelpool kirjeldatud meetodil, määrates 7 päeva jooksul iga päev fenooli minimaaltassidelt tetratsükliini ja streptomütsiini resistentsete bakterite arvukuse.

Selleks, et kindlaks teha, kas *P. putida rpoS*-defektse rakupopulatsiooni adapteerumine näljatingimustes on füsioloogiline või geneetiline, proovisime *rpoS*-defektsest *P. putida* nälgivast populatsioonist isoleerida kloone, kus võiks olla toimunud süsinikunäljas paremat adapteerumist võimaldav mutatsioon. Selleks tegime katse, kus alustuseks sarnaselt konkurentsikatsele plaatisime minimaalsöötmes üleöö kasvanud *rpoS*-defektse tüve PaWrpoSsm rakud fenooli minimaaltassile ja jätsime nad 10 päevaks süsinikunälja tingimustega adapteeruma. Seejärel lõikasime adapteerunud *rpoS*-defektsete rakkudega fenooli minimaaltassilt 1-ml pipetiotsikuga agaritükikese, pesime bakterirakud sellelt M9 puhvriga maha ning plaatisime üksikkolooniate saamiseks sobiva lahjendusega LB täissöötmele. Tekkinud üksikkolooniatest pärit rakud panime rikkasse vedelsöötmesse kasvama ning plaatisime 24 tunni möödudes fenooli

minimaalsöötmele nii puhaskultuurina, ligikaudu 10^9 *P. putida* PawrpoSsm tüve rakku, kui ka segatuna adapteerumata üleöö minimaalsöötmes kasvanud PaWrpoStet tüve rakkudega suhtes 1:1 ja 1:100. *P. putida rpoS*-defektse rakupopulatsiooni eluvõimet peale logaritmilise kasvufaasi läbimist hindasime samuti eelpool toodud CFU arvukuse meetodil, määrates 7 päeva jooksul iga päev fenooli minimaaltassidelt tetratsükliini ja streptomütsiini resistentsete bakterite arvukuse.

8. *P. putida* nälgiva rakupopulatsiooni iseloomustamine agarsöötmel rakkude LIVE/DEAD kitiga värvimisel

P. putida süsinikunäljas viibiva populatsiooni värvimiseks kasutasime LIVE/DEAD *Bac*Light (Molecular Probes, Inc., Eugene, Oregon, USA) kitti, mis võimaldab eristada elusaid rakke surnutest rakkude tsütoplasma membraani erineval läbilaskvusel nukleiinhappe spetsiifilistele fluorestseeruvatele värvidele. Roheline fluorokroom SYTO 9 on väike molekul, mis on võimeline rakkudesse sisenema läbi intaktse plasmamembraani, samas kui suurema molekulmassiga punase fluorokroomi propiidiumjodiidi sisenemine on võimalik ainult kahjustatud membraaniga rakkudesse.

Kõigepealt lõikasime *P. putida* nälgivate rakkudega fenooli minimaaltassilt umbes $1cm^2$ suuruse agaritüki ja tõstsime selle skalpelliga alusklaasile. Seejärel pipeteerisime agaritükile 10 µl lahjendatud LIVE/DEAD *Bac*Light kiti värvide segu (1,5 µl SYTO 9 värvi ja 1,5 µl propiidiumjodiidi 1-ml-s destilleertud vees) ja katsime agaritüki katteklaasiga. Bakterirakkude värvumiseks asetasime agaritüki 10 minutiks pimedasse kohta. Värvunud bakterirakke vaatasime fluorestsentsmikroskoobis 1000 kordse kogusuurendusega, kasutades filtrit, mis võimaldas üheaegselt näha nii punaselt kui ka roheliselt fluorestseeruvaid bakterirakke.

9. PCR

PCR-i (polümeraasi ahelreaktsioon) kasutasime *P. putida dinB* geeni eelse ala ning *sodA* ja *sodB* geenide amplifitseerimiseks genoomist ning meie poolt tehtud konstruktide õigsuse kontrollimiseks. Reatsiooni maht oli 20 µl. Reaktsioon toimus PCR-i puhvris, mis sisaldas: 75 mM Tris-HCl (pH 8,8), 20 mM $(NH_4)_2SO_4$, 0,01 %

Triton X 100, 0,5 % Ficoll 40, 1 mM tartrasiini. Eelnevale lisasime veel 2,5 mM $MgCl_2$, 0,2 mM dNTP, 0,5 u firma „Fermentas" Taq polümeraasi, 10 pmol praimereid (tabel 2) ja ~10 ng isoleeritud DNA-d või rakke.

PCR-i teostasime firmade „Biometra" või „Techne" PCR-i masinatega. Reaktsiooni tingimused: denaturatsioon 96 ºC 1 minut; praimeri seondumine 54 ºC 1 minut; DNA süntees temperatuuril 72 ºC 1 minut.

Tabel 2. Töös kasutatud praimerid

nimetus	**järjestus**	**seondumiskoht**
dinBBamHI	5´AAGCTTTTTAACGGGCAAAGAAA3´	komplementaarne *dinB* geeni eelneva alaga positsioonides -328 kuni -351
dinBXhoI	5´CGCATCTCGATCGCAGCGT3´	komplementaarne *dinB* geeniga positsioonides +40 kuni +58
sodA1	5´CGCTGCCAAGCCGGATGT3´	komplementaarne *sodA* geeni eelneva alaga positsioonides -15 kuni -32
sodA2	5´TTACTTCAGGGCTTCAAGGTA3´	komplementaarne *sodA* geeniga positsioonides +610 kuni +627
sodB1	5´CGGCCTTGCGCAAACCGC3´	komplementaarne *sodB* geeni eelneva alaga positsioonides -28 kuni -45
sodB2	5´TTAGGCCTTGAAGGTCTTGCC3´	komplementaarne *sodA* geeniga positsioonides +574 kuni +591

10. Plasmiidse DNA eraldamine „*boiling*" meetodil

Üleöö 4 ml-s LB vedelsöötmes kasvanud plasmiidi sisaldavaid *E. coli* DH5α rakke tsentrifuugisime firma "Eppendorf„ lauatsentrifuugiga "MiniSpin„ täispööretel (12 100 x g) 30 sekundit ja suspendeerisime 300 µl-s SET puhvris (10 % sahharoos; 50 mM EDTA; 50 mM Tris-HCl, pH 8,0). Rakukestade lagundamiseks lisasime 50 µl lüsotsüümi lahust (20 mg/ml), segasime ja hoidsime toatemperatuuril. 5 minuti möödudes lisasime 300 µl TET puhvrit (1 % Triton X 100; 50 mM EDTA; 50 mM Tris-HCl, pH 8,0), segasime õrnalt ja kuumutasime 3 minutit temperatuuril 95 ℃. Pärast kuumutamist tsentrifuugisime 20 minutit täispööretel ning eemaldasime steriilse tikuga

ependorfi põhja sadenenud membraanivalkude-kromosoomi kompleksi. Seejärel lisasime DNA sadestamiseks 800 µl isopropanooli ja tsentrifuugisime täispööretel 6 minutit. Eemaldasime supernatandi, lahustades sademe 100 µl-s H_2O-s, millele oli RNA lagundamiseks lisatud 2 µg/ml RNaasi ning hoidsime 30 minutit temperatuuril 37 ℃. Pärast seda töötlesime DNA lahust fenooli ja kloroformiga ning sadestasime DNA 1/10 mahu 5 M NaCl-i ja 2,5 mahu 96° etanooliga. Seejärel tsentrifuugisime 8 minutit täispööretel, eemaldasime supernatandi ja pesime sadet 150 µl 75° etanooliga. Tsentrifuugisime 1 minuti täispööretel, eemaldasime supernatandi ja kuivatasime sadet ~ 20 minutit temperatuuril 37 ℃. DNA sademe lahustasime 50 µl-s H_2O-s.

Geelelektroforeesiks lisasime DNA proovile 0,04 %-list broomfenoolsinise lahust 50 %-ses glütseroolis, 20 µl proovi kohta 4 µl. Proovid kandsime horisontaalsele 1 %-sele agaroosgeelile TAE puhvris (1 mM EDTA; 50 mM Tris-atsetaat, pH 8,2). Geel sisaldas etiidiumbromiidi 0,1 µg/ml. Elektroforeesi viisime läbi toatemperatuuril, pingel 100-150 volti. Geeli pildistasime ultraviolettvalguses.

DNA restriktsiooniks kasutasime firma „Fermentas" ensüüme. Reaktsioonid viisime läbi tingimustel, mis olid ette nähtud firma kataloogis. Peale restriktsiooni lisasime proovile broomfenoolsinise lahust glütseroolis ja kandsime proovid agaroosgeelile.

11. Transformatsioon

Kompetentsete rakkude valmistamiseks kasvatasime *E. coli* DH5α rakke üleöö 4 ml-s LB puljongis temperatuuril 37 ℃. Seejärel lahjendasime rakukultuuri ~20 korda ja kasvatasime rakke samadel tingimustel tiheduseni A_{580} = 0,2-0,5. Tsentrifuugisime rakud söötmest põhja ja eemaldasime supernatandi. Suspendeerisime sadenenud rakud 0,5 ml-s RF1 lahuses (10 mM NaOAc; 50 mM $MnCl_2$; 5 mM NaCl, pH 5,6-6) ja asetasime 10 minutiks jääle. Pärast seda tsentrifuugisime rakud põhja, suspendeerisime 60 µl-s RF2 lahuses (10 mM NaOAc; 70 mM $CaCl_2$; 5 mM $MnCl_2$; 5 % glütserool, pH 5,6-6) ja hoidsime 30 minutit jääl.

Transformatsiooniks lisasime kompetentsetele rakkudele ligaasisegu või ~100 ng plasmiidset DNA-d ja hoidsime 20 minutit jääl. Seejärel viisime rakud 5 minutiks

temperatuurile 37 ℃ ning pärast seda 5 minutiks jääle. Edasi lisasime rakkudele 1 ml LB-d ja kasvatasime rakke temperatuuril 37 ℃. 1 tunni möödudes tsentrifuugisime rakud põhja, suspendeerisime ~50 µl-s LB-söötmes ning plaatisime LB tardagarile, mis sisaldas 100 mg/ml ampitsilliini (Amp).

12. Bakterite konjugatsioon

Minitransposooni koosseisus olevaid geene sisaldavate plasmiidide viimiseks *P. putida* rakkudesse kasutasime bakterite konjugatsiooni. Selleks kasvatasime üleöö *E. coli* tüve CC118λpir, mis sisaldas ülekantavat plasmiidi, retsipienttüve ja plasmiidide ülekandeks vajalikku helperplasmiidi RK2013 sisaldavat *E. coli* tüve HB101. Edasi lahjendasime üleöö LB-vedelsöötmes plasmiidide selektsiooniks vajalike antibiootikumide juuresolekul kasvanud kultuurid tiheduseni A_{580} = 0,1 värskesse LB vedelsöötmesse, mis antibiootikume ei sisaldanud ja kasvatasime kultuure loksutil ~2-3 tundi. Seejärel segasime kokku võrdse koguse doonor-, retsipient- ja helpertüve kultuurist ja pipeteerisime 100 µl segu LB-tassile. Rakke kasvatasime üleöö temperatuuril 30 °C. Järgnevalt suspendeerisime rakukultuuri 5 ml-s 1 x M9 lahuses ja plaatisime sellest 100 µl bakterikultuuri selektiivsöötmele.

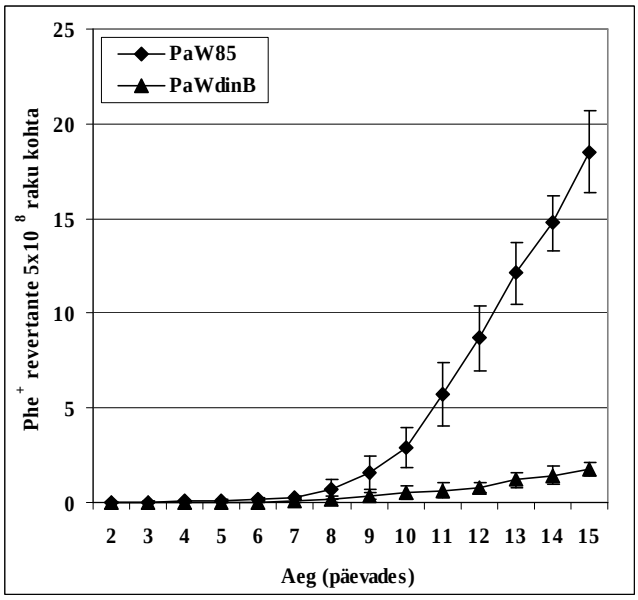
Mutatsiooniprotsesside uurimiseks vajalike testplasmiidide ülekandeks *P. putida* rakkudesse segasime 100 µl LB-s kokku võrdses mahus plasmiidi sisaldavat doonortüve DH5α, retsipienttüve ja plasmiidi ülekandeks vajaliku helperplasmiidi sisaldava tüve HB101[pRK2013]. 15 minuti möödudes plaatisime ristamissegust ~2 µl rakke karbenitsilliini (Cb) sisaldavale minimaalsöötmele.

Tulemused

Statsionaarse faasi sigma faktor RpoS reguleerib bakterirakkudes paljude stressitingimustes ellu jäämiseks vajalike geenide ekspressiooni (Lange ja Hengge-Aronis, 1991). *E. coli* puhul on näidatud RpoS-i osalust ka statsionaarse faasi mutageneesil, kus RpoS reguleerib positiivselt vigaderohke DNA polümeraas IV ehk DinB ekspressiooni (Layton ja Foster, 2003). DinB on vajalik *E. coli* statsionaarse faasi rakkudes 1-nukleotiidsete raaminihete tekkeks, mis suurendades rakupopulatsioonis geneetilist mitmekesisust, võimaldavad rakkudel nii stressitingimustes kohaneda ja ellu jääda (Foster jt., 1999; Rosenberg jt., 2001). Lisaks raaminihkemutatsioonidele on RpoS-i osalust näidatud ka *E. coli* vananevates kolooniates toimuvate asendusmutatsioonide tekkes, kus RpoS mõjutab negatiivselt peamise reparatsioonisüsteemi võtmevalgu MutS-i hulka (Bjedov jt., 2003).

Käesoleva töö eesmärgiks on uurida mehhanisme, mille kaudu statsionaarse faasi sigma faktor RpoS võiks mõjutada mullabakteri *P. putida* statsionaarse faasi rakkudes toimuvaid adaptatsiooniprotsesse.

DNA polümeraas IV ehk DinB mõjutab *P. putida* statsionaarse faasi rakkudes 1-nukleotiidsete deletsioonide teket. Esimese nälgimisnädala jooksul on 1-nukleotiidsete deletsioonide teke *P. putida* algses tüves ja *dinB* suhtes defektses tüves võrreldav. Mutatsioonide tekkesagedus tõuseb drastiliselt alles alates 9. nälgimispäevast ja on otseselt sõltuv funktsionaalse *dinB* olemasolust rakkudes (joonis 1) (Tegova jt., 2004).

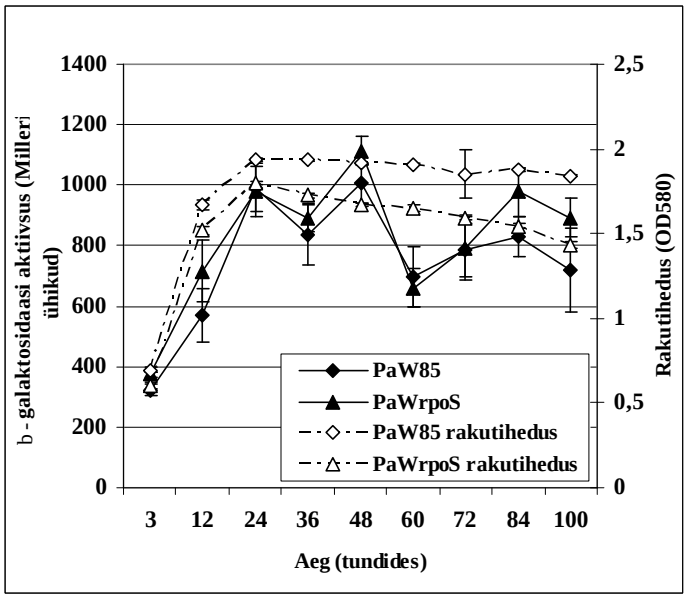


Joonis 1. Phe$^+$ revertantide akumulatsioon fenooli minimaaltassidel *P. putida* algse tüve PaW85 ja *dinB* geeni suhtes defektse tüve rakkudes, mis sisaldavad 1-nukleotiidsete deletsioonide detekteerimiseks vajalikku testsüsteemi pKTpheA56+A (Tegova jt., 2004).

Kuna DinB-st sõltuvate mutatsioonide tase suureneb hilise nälja tingimustes (Tegova jt., 2004), mil RpoS tase rakkudes peaks olema maksimaalne, otsustasime kindlaks teha, kas *P. putida dinB* geeni transkriptsioon on sarnaselt *E. coli´*le RpoS valgu poolt positiivselt kontrollitud.

1. *P. putida dinB* geeni transkriptsioon ei sõltu statsionaarse faasi sigma faktorist RpoS

Selleks, et uurida *P. putida dinB* geeni promootorilt lähtuva transkriptsiooni taseme sõltuvust statsionaarse faasi sigma faktorist RpoS, konstrueerisime plasmiidi pKT*lacZ* baasil testsüsteemi, kus *dinB* promootorregioon oli kloneeritud *lacZ* geeni ette. Mõõtes ß-galaktosidaasi hulka, jälgisime *dinB* promootorilt lähtuva transkriptsiooni taset nii *P. putida* algse tüve PaW85 kui ka *rpoS*-defektse tüve rakkudes. Tulemused, mis on esitatud joonisel 2, näitavad, et ß-galaktosidaasi hulk *P. putida* rakkudes tõusis hilises näljas maksimaalselt 3 korda võrreldes eksponentsiaalse faasi rakkudes toimuva transkriptsiooniga, kuid seda nii algse kui ka *rpoS*-defektse *P. putida* tüve rakkudes.

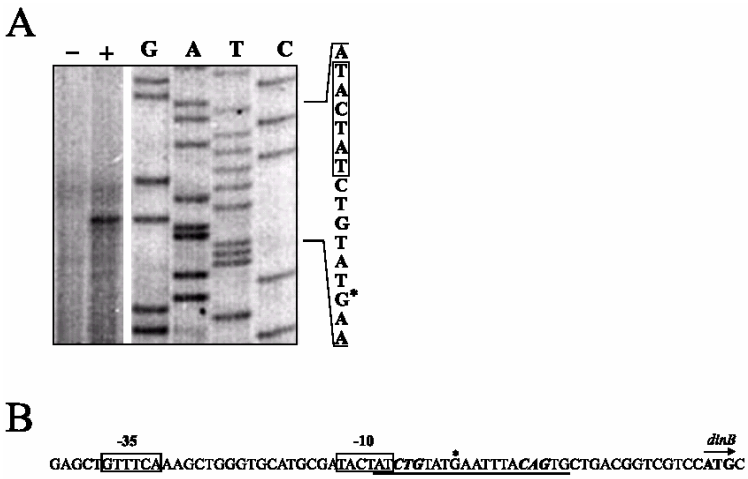


Joonis 2. *P. putida dinB* geeni transkriptsiooni sõltuvus bakterikultuuri kasvufaasist mõõdetuna ß-galaktosidaasi Milleri ühikutes (MÜ) nii *P. putida* algses tüves PaW85 kui ka *rpoS*-defektses tüves. Katkendlike joontega on näidatud tüvede PaW85 ja PaWrpoS kasvukõver.

Järelikult on *P. putida dinB* geeni transkriptsioon vähesel määral mõjutatud bakterite kasvufaasi poolt, kuid ei ole sõltuv statsionaarse faasi sigma faktori RpoS olemasolust.

Saadud tulemust kinnitab ka see, et *P. putida dinB* geeni promootorala ei lange kokku RpoS-i poolt äratuntavate promootorite -10 heksameerse konsensusjärjestusega G/TCTATACT. Nagu on näha jooniselt 3, algab *dinB* geeni mRNA süntees G nukleotiidilt, millest 6 nukleotiidi ülesvoolu paikneb promootori -10 ja -35 heksameerne järjestus (GATACTAT-N_{17}-GTTTCA). Kui võrrelda *P. putida dinB* geeni promootorala järjestust mõnede tüüpiliste RpoS-i poolt kontrollitavate geenide promootorjärjestuste konsensusega, näeme, et *dinB* geeni promootorjärjestuse -10 heksameeris puudub RpoS-i äratundmiseks ja promootorregiooniga interakteerumiseks vajalik -13 positsioonis paiknev C nukleotiid (Hengge-Aronis ja Becker, 2001; Hengge-Aronis, 2002). *P. putida dinB* geeni promootorilt lähtuva transkriptsiooni alguspunkti määramine ja tulemused, mis me saime statsionaarse faasi sigma faktori RpoS mõju

uurimisel *dinB* geeni transkriptsioonile, on avaldatud artiklis Tegova jt., 2004 (vaata lisa 1).



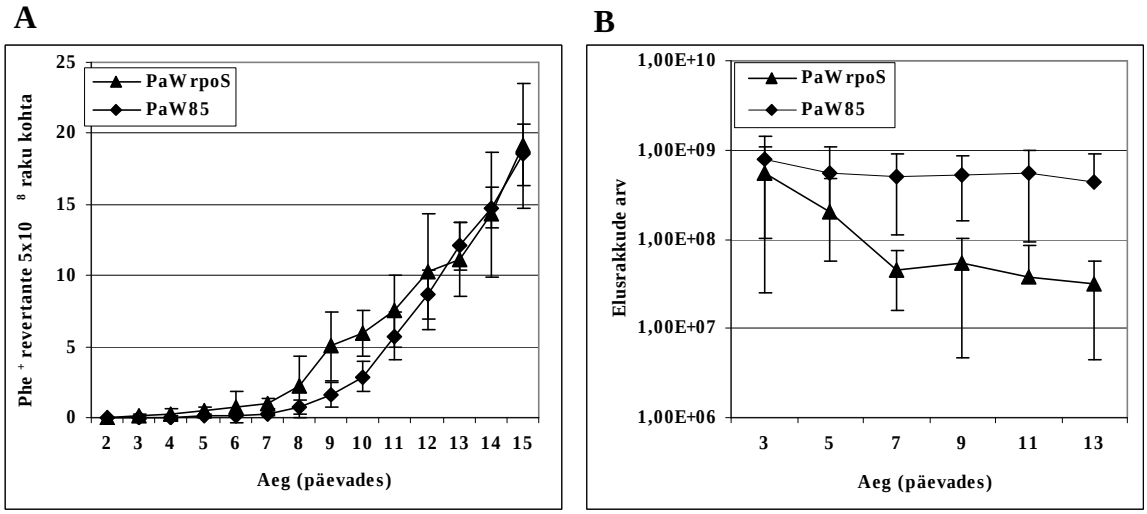
Joonis 3. *P. putida dinB* geeni transkriptsiooni alguspunkti määramine. (A) Radadel G, A, T, C on *P. putida dinB* geeni promootorregiooni DNA sekveneerimise reaktsioonid. – ja + tähistatud radadel on näha pöördtranskriptaasi reaktsiooni tulemus ilma plasmiidita (-) ning plasmiidi pKTPdinBluxAB sisaldavatest (+) *P. putida* PaW85 rakkudest eraldatud RNA-ga. (B) Joonisel tärniga tähistatud G nukleotiid näitab *dinB* mRNA alguspunkti ning kastikesega on ümbritsetud *dinB* promootori -10 ja -35 heksameersed järjestused.

Kuigi RpoS ei reguleeri otseselt *P. putida dinB* geenilt lähtuvat transkriptsiooni, jääb võimalus, et RpoS võib mõjutada DinB taset kaudselt, näiteks reguleerides geene, mis on seotud DinB ekspressiooniga või DinB valgu stabiilsuse tagamisega nälgivates *P. putida* rakkudes. Seega tekkis meil järgmisena küsimus, kas RpoS võib kaudselt osaleda DinB-sõltuval mutageneesil.

2. RpoS-i olemasolu ei mõjuta 1-nukleotiidsete deletsioonide teket *P. putida* nälgivas rakupopulatsioonis

Raaminihkemutatsioonide detekteerimiseks kasutasime testsüsteemi, kus plasmiidis asuva fenooli monooksügenaasi geeni *pheA* kodeerivat järjestust oli muudetud, inserteerides *pheA* geeni algusesse A nukleotiidi ning tekitades sellega +1 raaminihke *pheA* geeni kodeerivas alas. Kuna *pheA* geeni olemasolu võimaldab *P. putida* rakkudel kasutada fenooli ainsa süsinikuallikana, saavad fenooli sisaldaval

minimaalsöötmel hakata kasvama vaid need *P. putida* rakud, milles on *pheA* geenis toimunud 1-nukleotiidne deletsioon ja taastunud *pheA* geeni esialgne järjestus (Tegova jt., 2004). Phe^+ revertantide tekkimist fenooli minimaaltassidele jälgisime 15 päeva jooksul nii *rpoS* geeni suhtes defektses *P. putida* tüves kui ka *P. putida* algses tüves. Jooniselt 4A on näha, et 1-nukleotiidsetest deletsioonidest põhjustatud Phe^+ revertantide akumulatsioon oli *rpoS*-defektses tüves kogu nälgimisperioodi jooksul võrreldav algse tüvega. Kuna nälgimise käigus langes *rpoS*-defektse tüve elumus ligikaudu 1,5 suurusjärku (joonis 4B), on joonisel 4A toodud akumulatsioonitulemuste esitamisel arvestatud ka seda.

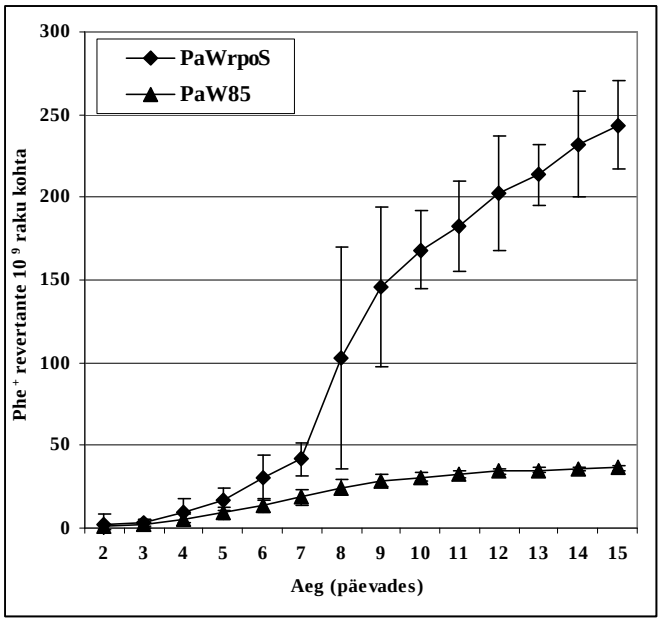


Joonis 4 (A) Phe^+ revertantide akumulatsioon fenooli minimaaltassidel *P. putida* algse tüve PaW85 ja *rpoS* geeni suhtes defektse tüve rakkudes, mis sisaldavad 1-nukleotiidsete deletsioonide detekteerimiseks vajalikku testsüsteemi pKTpheA56+A. **(B)** *P. putida* algse tüve PaW85 ja *rpoS* suhtes defektse tüve elumus fenooli minimaaltassidel.

Need tulemused näitavad, et erinevalt *E. coli´*st, kus RpoS-i olemasolu on vajalik DinB-st sõltuvate raaminihkemutatsioonide tekkeks (Layton ja Foster, 2003; Lombardo jt., 2003), ei ole *P. putida* statsionaarse faasi nälgivates rakkudes 1-nukleotiidsete deletsioonide teke RpoS-i poolt mõjutatud.

3. RpoS-i olemasolu *P. putida* nälgivates rakkudes mõjutab negatiivselt asendusmutatsioonide teket

Kuna RpoS mõjutab nälgivas rakupopulatsioonis paljusid erinevaid protsesse (näiteks valkude süntees, aminohapete transport ja metabolism) (Nystöm, 2004), otsustasime uurida, kas RpoS-il võiks olla mõju teisttüüpi punktmutatsioonide tekkele *P. putida* nälgivates rakkudes. Asendusmutatsioonide detekteerimiseks kasutasime testsüsteemi, kus plasmiidis asuva fenooli lagundamiseks vajaliku *pheA* geeni kodeerivasse järjestusse oli sisse viidud translatsiooni stoppkoodon TAG. Fenooli sisaldavatel minimaaltassidel said hakata kasvama rakud, kus asendusmutatsioonide tulemusena oli *pheA* geeni stoppkoodoni asemel tekkinud aminohapet kodeeriv koodon (Tegova jt., 2004). Võrreldes Phe^+ revertantide tekkesagedust plasmiidset testsüsteemi pKTpheA22TAG sisaldavates *P. putida* algse tüve ja *rpoS*-defektse tüve rakkudes, nägime, et asendusmutatsioonide tekkesagedus nälgimise algperioodil (3-7 päev) oli nii *P. putida* algses tüves kui ka *rpoS* geeni suhtes defektses tüves võrreldav (joonis 5). Alates 7-ndast nälgimispäevast suurenes *rpoS*-defektses tüves asendusmutatsioonide tekkesagedus ja püsis edasise nälgimisperioodi jooksul ligikaudu 5-7 korda kõrgemal tasemel võrreldes *P. putida* algse tüvega (joonis 5).

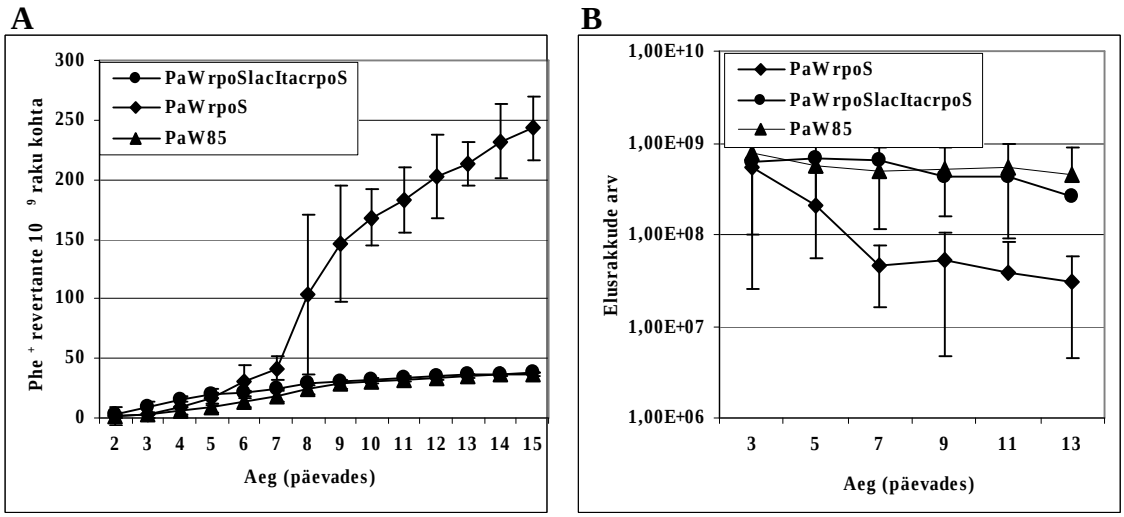


Joonis 5. Phe$^+$ revertantide akumulatsioon fenooli minimaaltassidel *P. putida* algse tüve PaW85 ja *rpoS* geeni suhtes defektse tüve rakkudes, mis sisaldavad asendusmutatsioonide detekteerimiseks vajalikku testsüsteemi pKTpheA22TAG.

Vastupidiselt *E. coli´*st teadaolevatele andmetele, kus on näidatud, et RpoS on vajalik nii raaminihkemutatsioonide (Layton ja Foster, 2003; Lombardo jt., 2003), kui ka vananevates kolooniates toimuvate asendusmutatsioonide tekkeks (Bjedov jt., 2003), näitavad meie tulemused, et *P. putida* statsionaarse faasi rakkudes mõjub RpoS-i olemasolu asendusmutatsioonide tekkele negatiivselt ning võib seega olla seotud mõne asendusmutatsioonide teket vähendava mehhanismiga.

Selleks, et teada saada, kas *rpoS*-defektses *P. putida* tüves suurenenud asendusmutatsioonide tekkesagedus on põhjustatud vaid RpoS valgu puudumisest või on selles tüves toimunud veel lisaks geneetilised muutused, mis põhjustavad asendusmutatsioonide tekkesageduse suurenemist bakterite pikaajalisel nälgimisel, viisime *P. putida rpoS*-defektse tüve kromosoomi IPTG-ga indutseeritava *tac* promootori kontrolli all oleva *rpoS* geeni (tüvi PaWrpoSlacItacrpoS). Jooniselt 6 on näha, et *tac* promootori kontrolli all oleva *rpoS* geeni viimine *rpoS*-defektse *P. putida*

tüve kromosoomi, taastas nii tüve elumuse, kui ka alandas mutatsioonisagedust *P. putida* algse tüve tasemele.



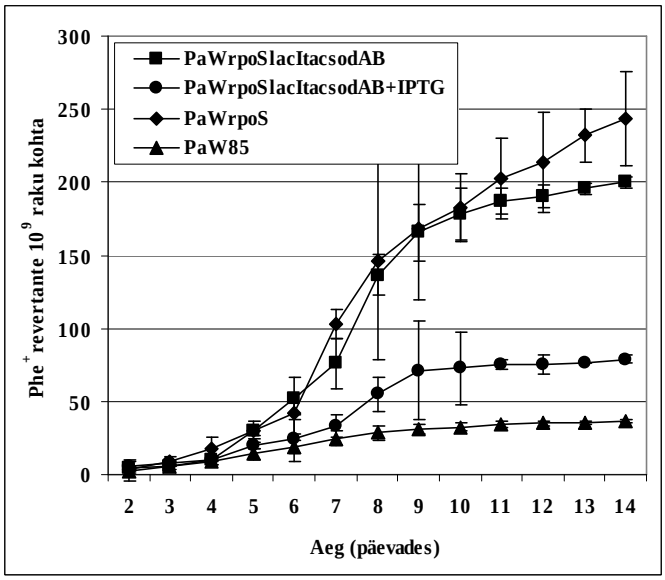
Joonis 6. (A) Phe$^+$ revertantide akumulatsioon fenooli minimaaltassidel testsüsteemi pKTpheA22TAG sisaldavates *P. putida* algse tüve PaW85, *rpoS* geeni suhtes defektse tüve ning PaWrpoSlacItacrpoS tüve rakkudes. **(B)** *P. putida* algse tüve PaW85, *rpoS* geeni suhtes defektse tüve ning PaWrpoSlacitacrpoS tüve elumus fenooli minimaaltassidel.

Millest võiks olla tingitud mutatsioonisageduse suurenemine *P. putida rpoS*-defektses tüves?

Kõikides õhuhapnikule eksponeeritud organismides tekib normaalse elutegevuse käigus reaktiivseid hapnikuradikaale, mis võivad kahjustada nii valke kui ka nukleiinhappeid (Imlay, 2003). *E. coli* põhjal on teada, et nii otseselt DNA-d oksüdatiivsete kahjustuste eest kaitsvat valku kodeeriva *dps* geeni (Almiron jt., 1992; Finkel ja Nair, 2004), kui ka reaktiivseid hapnikuühendeid detoksifitseerivaid ensüüme kodeerivate *katE* ja *sodA* geenide ekspressioon on statsionaarse faasi sigma faktori RpoS poolt kontrollitud (Loewen jt., 1984; Mulvey jt., 1990; Schellhorn ja Hassan, 1998; Nunoshiba jt., 1996). Seda, kuidas mõjutavad oksüdatiivsed kahjustused mutatsioonide tekkeprotsesse bakterites *in vivo*, on vähe uuritud.

4. Superoksiidi dismutaaside SodA ja SodB üleekspressioon *P. putida rpoS*-defektsetes rakkudes vähendab asendusmutatsioonide tekkesagedust.

P. putida KT2440 genoomis on kaks superoksiidi dismutaasi geeni: *sodA* ja *sodB*, mille poolt kodeeritud SodA ja SodB monomeeridest moodustub heterodimeerne superoksiidi dismutaas, mis on vajalik rakulisi komponente kahjustavate superoksiidi radikaalide (O_2^-) eemaldamiseks bakterirakust (Heim jt., 2003). Selleks, et välja selgitada, kas asendusmutatsioonide suurenenud tekkesagedus *P. putida rpoS*-defektse tüve nälgivates rakkudes võiks olla põhjustatud superoksiidi radikaalide kuhjumisest rakus, otsustasime konstrueerida *P. putida rpoS*-defektse tüve (PaWrpoSlacItacsodAB), mille kromosoomis oleksid *sodA* ja *sodB* geenid IPTG-ga indutseeritava *tac* promootori kontrolli all, et suurendada SodAB valgu hulka rakkudes. Jooniselt 7A on näha, et *sodAB* geenide üleekspressioonil IPTG juuresolekul vähenes asendusmutatsioonide tekkesagedus *P. putida rpoS*-defektses tüves ligikaudu 2,5 korda. Võrreldes *P. putida* algse tüvega jäi asendusmutatsioonide tekkesagedus siiski ligikaudu 2 korda kõrgemale tasemele. Üllatav on see, et kuigi *sodAB* geenide üleekspressioon vähendas *rpoS*-defektses *P. putida* tüves mutatsioonisagedust, ei mõjutanud SodAB valgu hulga suurendamine rakkude elumust, mis langes võrreldavalt *P. putida rpoS*-defektse tüvega.



Joonis 7. Phe$^+$ revertantide akumulatsioon fenooli minimaaltassidel testsüsteemi pKTpheA22TAG sisaldavates *P. putida* algse tüve PaW85, *rpoS* geeni suhtes defektse tüve ning PaWrpoSlacItacsodAB tüve rakkudes nii IPTG juuresolekul kui ilma.

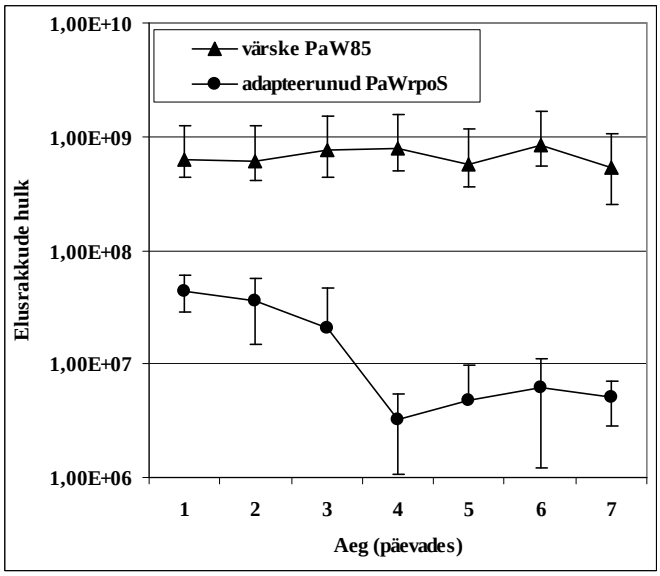
Seega võib öelda, et üheks suurenenud asendusmutatsioonide tekkesageduse põhjuseks *P. putida rpoS*-defektses tüves võib olla superoksiidi radikaalide hulga suurenemine nälgivas populatsioonis.

Reaktiivsete hapnikuühendite hulga suurenemine rakus, mis põhjustab nii oksüdatiivselt kahjustatud DNA kui ka valkude hulga tõusu (Imlay jt., 2003), võib olla põhjuseks, miks *P. putida rpoS*-defektse tüve elumus langeb süsinikunäljas ligikaudu 1,5 suurusjärku (joonis 4B). Samas peale 7-ndat nälgimispäeva *rpoS*-defektse tüve elumus enam ei muutunud ja püsis stabiilsena vähemalt 40 päeva jooksul. Selle nähtuse seletuseks on vähemalt kaks võimalust. Esiteks võivad nälgivas *rpoS*-defektse tüve populatsioonis tekkida mutatsioonid, mis muudavad selle populatsiooni kohasemaks antud keskkonnatingimustega. Teiseks võib bakteripopulatsiooni kohastumine süsinikunäljaga toimuda ka füsioloogiliselt, raku eluprotsesside ümberkorralduse arvelt.

5. Süsinikunäljas adapteerunud *rpoS*-defektne *P. putida* rakupopulatsioon on võimeline konkureerima *rpoS*-defektse adapteerumata populatsiooniga, kuid ei suuda konkureerida *P. putida* algse populatsiooniga

Selleks, et uurida, kas süsinikunälja tingimustega adapteerunud *P. putida rpoS* geeni suhtes defektne populatsioon on näljatingimustes elujõulisem kui adapteerumata populatsioon, tegime erinevate bakteripopulatsioonidega konkurentsikatseid. Selleks, et eristada adapteerunud ja adapteerumata bakteritüvesid, märkisime need erinevate antibiootikumide resistentsusmarkeritega. *P. putida rpoS*-defektsed rakud, mis olid 10 päeva fenooli sisaldavatel minimaaltassidel adapteerunud, pesime agarsöötmelt maha ja plaatisime seguna, kas „värske" üleöö kasvanud *P. putida* algse tüve või *rpoS*-defektse tüve rakkudega fenooli sisaldavale minimaalsöötmele.

Joonisel 8. on näha, et kui süsinikunäljas adapteerunud *rpoS*-defektsest populatsioonist pärit rakud olid segatud adapteerumata algse tüve populatsiooniga, langes nende elumus 4-ndaks päevaks ligikaudu 1 suurusjärgu ning püsis seejärel edasise nälgimisperioodi jooksul muutumatuna. Samas *P. putida* algse tüve rakkude elumus ei langenud ja jäi kogu katse vältel samale tasemele.

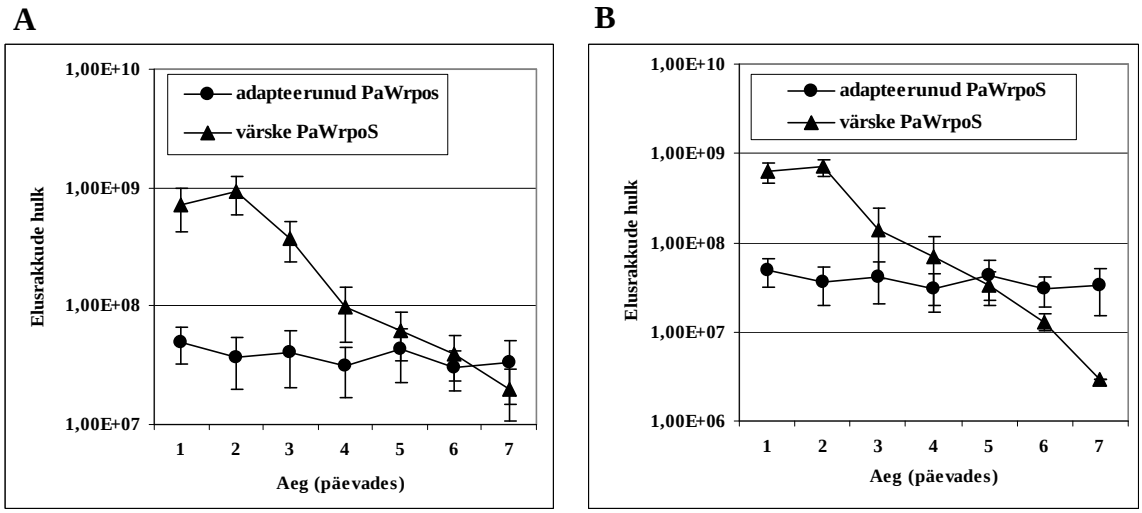


Joonis 8. Süsinikunäljas 10 päeva adapteerunud *rpoS*-defektse rakupopulatsiooni ja „värske" üleöö kasvanud *P. putida* algse rakupopulatsiooni 1:100 suhtes kokkusegamisel ja fenooli minimaalsöötmel kooskultiveerimisel toimuv kahe tüve konkurents.

Seega võime öelda, et adpteerunud *rpoS*-defektne populatsioon, segatuna „värske" *P. putida* algse rakukultuuriga, ei suutnud nälgimise käigus oma arvukust säilitada ega segapopulatsioonis *P. putida* algse tüve rakkudega konkureerida. Samuti ei suutnud süsinikunäljas adapteerunud *rpoS*-defektne populatsioon konkureerida samades tingimustes adapteerunud *P. putida* algse populatsiooniga (andmed esitamata).

Sarnase konkurentsikatse viisime läbi ka süsinikunälja tingimustega adapteerunud ja adapteerumata *rpoS*-defektsete *P. putida* populatsioonidega. Jooniselt 9 on näha, et adapteerunud *rpoS*-defektse populatsiooni elumus ei langenud, juhul kui sellest populatsioonist pärit rakud olid plaaditud segatuna üleöö kasvanud *rpoS*-defektse rakukultuuriga, mille elumus hakkas alates teisest nälgimispäevast langema. Kuni 4-nda nälgimispäevani langes „värske" *rpoS*-defektse tüve rakkude osakaal kooskultiveerimisel adapteerunud *rpoS*-defektse tüve rakkudega erinevates katsetes võrreldavalt, kuid seejärel kujunes välja kaks konkurentsitüüpi. Ühel juhul (joonis 9A) langes „värske" *rpoS*-defektse tüve rakkude arvukus segakultuuris 7-ndaks päevaks ligikaudu 1,5 suurusjärku, võrreldes nende esialgse arvukusega. Teisel juhul langes „värske" *rpoS*-defektse tüve rakkude arvukus ühe nädala möödudes ligikaudu 2,5

suurusjärku võrreldes nende esialgse arvukusega (joonis 9B). Samas, kui plaatisime fenooli minimaaltassidele ainult adapteerunud *rpoS*-defektse populatsiooni, siis langes selle elumus näljatingimustes sarnaselt adapteerumata *rpoS*-defektsele populatsioonile ligikaudu 1,5 suurusjärku (andmed esitamata).



Joonis 9. (A), (B) Süsinikunäljas 10 päeva adapteerunud ja „värske" üleöö kasvanud *rpoS*-defektse rakupopulatsiooni 1:100 suhtes kokkusegamisel ja fenooli minimaalsöötmel kooskultiveerimisel toimuv konkurents, mis on jälgitav elumuse muutusena.

Need tulemused viitavad sellele, et süsinikunäljas adapteerunud *rpoS*-defektses *P. putida* populatsioonis toimunud muutused võimaldavad rakkudel samades keskkonnatingimustes konkureerida „värske" *rpoS*-defektse populatsiooni rakkudega, kusjuures adapteerunud *rpoS*-defektse populatsiooni rakkude arvukuse püsima jäämine antud tingimustes sai toimuda vaid vähem kohastunud, „värske" *rpoS*-defektse rakupopulatsiooni arvelt. Samas ei anna need tulemused vastust küsimusele, kas *rpoS*-defektse *P. putida* populatsiooni rakud olid adapteerunud füsioloogiliselt või olid nendes toimunud geneetilised muutuded.

6. *rpoS*-defektse *P. putida* populatsiooni adapteerumine ei toimu ilmselt geneetilise mehhanismi abil

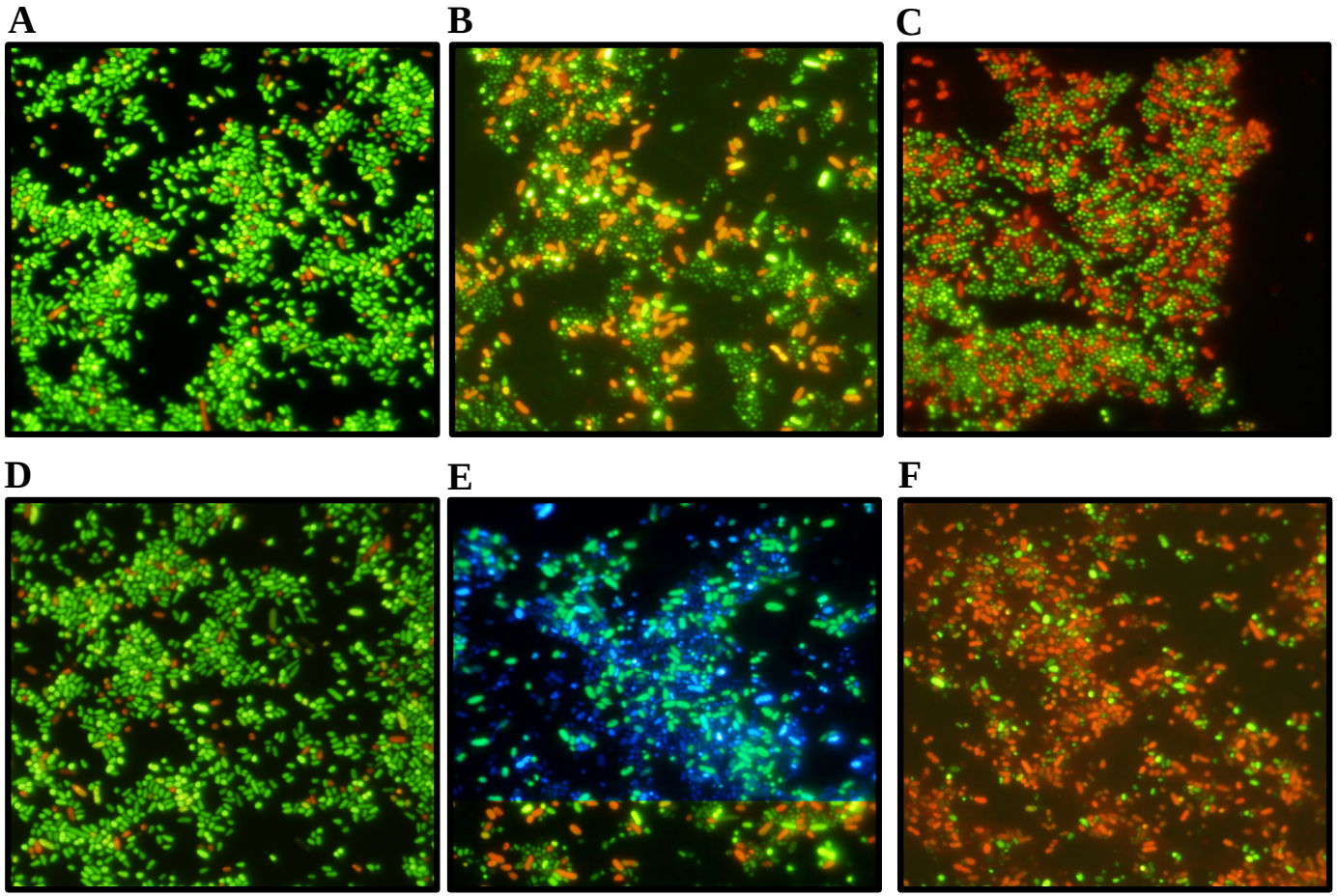
Selleks, et välja selgitada, kas *rpoS*-defektses *P. putida* nälgivas rakupopulatsioonis adapteerumisel toimuvad muutused on füsioloogilised või geneetilised, proovisime näljatingimustega adapteerunud *P. putida rpoS*-defektse tüve populatsioonist isoleerida kloone, kus võiksid olla toimunud mutatsioonid, mis võimaldaksid rakkudel paremini süsinikunälja tingimustega adapteeruda. Selleks tegime katse, kus plaatisime *P. putida rpoS*-defektse tüve 10 päeva süsinikunälja tingimustes adapteerunud rakud lahjendusega täissöötmele, et tekitada üksikkolooniad. Üksikkolooniatest pärit rakke kasvatasime üleöö rikkas vedelsöötmes ja plaatisime fenooli minimaaltassile segatuna adapteerumata üleöö kasvanud *rpoS*-defektse *P. putida* tüve rakkudega. Jälgides adapteerunud populatsioonist üksikkolooniateks viidud rakkude ja adapteerumata rakukultuuri elumusi segapopulatsioonis 7 nälgimispäeva jooksul, võime öelda, et eelpool ilmnenud adapteerunud *rpoS*-defektse populatsiooni võime konkureerida üleöö kasvanud *rpoS*-defektsete rakkudega, oli kõigi sellest populatsioonist puhastatud kloonide puhul kadunud. Erinevalt *E. coli´*st, kus statsionaarses faasis kasvueelist omavates rakkudes toimuvad muutused on geneetilised ning GASP (*Growth Advantage In Stationary Phase*) fenotüüp ilmneb ka rakkudes, mis on korduvalt läbinud logaritmilise kasvufaasi (Finkel, 2006), ei saa meie katse tulemuste põhjal väita, et *rpoS*-defektse *P. putida* tüve adapteerumine süsinikunälja tingimustes oleks geneetiline. Seega arvame, et pigem võivad osades *P. putida rpoS*-defektse tüve rakkudes toimuda muutused nende füsioloogias, mis võimaldavad nendel rakkudel näljatingimustes ellu jääda ja edukalt konkureerida.

7. *P. putida rpoS*-defektne rakupopulatsioon on heterogeensem ja rakkude suremus süsinikunäljas on suurem võrreldes *P. putida* algse tüve rakkudega.

Süsinikunäljas viibiv *P. putida* rakupopulatsioon on pidevas muutumises ning äärmiselt heterogeene. Kuigi nälgiv populatsioon püsib näiliselt muutumatuna (*P. putida* algse tüve elumus kolooniaid moodustavate rakkude arvukuse määramise alusel ei lange), toimub nälgivas populatsioonis siiski pidev suremise protsess ning surnud

rakkudest vabanenud toitainete arvelt ka rakkude juurdekasv. *P. putida* nälgiva populatsiooni dünaamilisust oleme kirjeldanud artiklis Saumaa jt., 2006 (vaata lisa 2). Selleks, et iseloomustada nälgivat *P. putida rpoS*-defektset populatsiooni, värvisime nii *P. putida* algse tüve kui *rpoS*-defektse tüve rakke agarsöötmel *in situ*, kasutades bakteri elusrakkude arvukuse määramise LIVE/DEAD kitti, mis võimaldas meil fluorestsentsmikroskoobis eristada punaseks värvunud surnud rakke ja rohelisi elusaid rakke. Vahetult peale plaatimist nägime mõlema tüve rakupopulatsioonides valdavalt elusaid rakke (joonis 10A ja 10D). Teiseks päevaks oli nii *P. putida* algse tüve kui *rpoS*-defektse tüve nälgiv rakupopulatsioon muutunud heterogeenseks. Surnud rakkude arvukus tõusis mõlemas rakupopulatsioonis võrreldavalt, kusjuures elus ja surnud rakkude arvukus rakukultuuris oli sama tardsöötme eri piirkondades märkimisväärselt erinev (joonis 10B ja 10E). 5-ndal nälgimispäeval oli näha, et *rpoS*-defektne rakupopulatsioon sisaldas võrreldes algse tüvega palju rohkem surnud rakke ning mitmes agarsöötmele plaaditud rakukultuuri piirkonnas oli näha vaid mõni üksik elus rakk valdava enamuse surnud rakkude seas (joonis 10F). Kuigi ka *P. putida* algse tüve populatsioonis suurenes oluliselt surnud rakkude arvukus, ei täheldanud me 5-ndal nälgimispäeval sellist drastilist surnud rakkude osakaalu suurenemist *P. putida* algse tüve populatsioonis (joonis 10C). Kuna *P. putida* algse tüve elumus süsinikunälja tingimustes kolooniaid moodustavate rakkude (CFU-*Colony Forming Units)* arvukuse määramise alusel ei vähenenud, kuid visuaalselt hinnates surnud rakkude arvukus populatsioonis siiski suurenes, siis järelikult pidi osa *P. putida* rakupopulatsioonist suutma surnud rakkudest vabanevate toitainete arvelt kasvada.

rpoS-defektse *P. putida* populatsiooni adapteerumine süsinikunäljas võib olla samuti seotud osade rakkude suremisega nälgivas rakupopulatsioonis ning teiste rakkude juurdekasvuga. *rpoS*-defektse populatsiooni rakkude arvukuse langus nälgimise esimesel nädalal võib olla põhjustatud sellest, et *rpoS*-defektse tüve rakud on tundlikumad reaktiivsete hapnikuühendite poolt põhjustatud kahjustustele. Hilisema nälja tingimustes elusrakkude arvukus enam ei vähene ja toimub adapteerumine. On võimalik, et surnud rakkudest vabanenud toitained aitavad mingil rakupopulatsiooni alaosal kasvada ja annavad seeläbi võimaluse välja kujuneda populatsioonil, mis on süsinikunälja tingimustega paremini adapteerunud.



Joonis 10. *P. putida* nälgiva algse tüve PaW85 **(A, B, C)** ja tüve PaWrpoS **(D, E, F)** populatsioonide iseloomustamine fenooli minimaaltassidel. Bakterirakud on värvitud LIVE/DEAD kitiga ja visualiseeritud fluorestsentsmikroskoobis 1000 kordse suurendusega. Ülemine rida kajastab algse tüve PaW85 nälgivas populatsioonis toimuvaid muutuseid ja alumine rida kajastab tüve PaWrpoS nälgivas populatsioonis tomuvaid muutuseid. Nälgivat populatsiooni on kirjeldatud **(A,D)** vahetult peale plaatimist, **(B,E)** teisel nälgimispäeval, **(C,F)** viiendal nälgimispäeval.

Arutelu

Bakteris *E. coli* on RpoS-i osalust näidatud nii statsionaarse faasi rakkudes toimuvates füsioloogilistes protsessides kui ka geneetilistes ümberkorraldusetes, mis võimaldavad bakterirakkudel, kas geenieksрessiooni muutuste või geneetilise mitmekesisuse suurendamise tulemusena adapteeruda uute keskkonnatingimustega ning tagavad seeläbi populatsiooni ellujäämise stressitingimustes (Lange ja Hengge-Aronis, 1991; Hengge-Aronis, 2002). RpoS-i olemasolu on vajalik nälgivates *E. coli* rakkudes nii 1-nukleotiidsete deletsioonide, amplifikatsioonide kui ka asendusmutatsioonide tekkeks (Bjedov jt., 2003; Layton ja Foster, 2003; Lombardo jt., 2003; Matic ja Saint-Ruf, 2005).

Meie katsete tulemusena selgus, et *P. putida* statsionaarse faasi nälgivates rakkudes toimuvate 1-nukleotiidsete deletsioonide teke on erinevalt *E. coli´*st RpoS-i olemasolust sõltumatu ning enamgi veel, asendusmutatsioonide tekkesagedus on vastupidiselt *E. coli´*le RpoS-i poolt negatiivselt mõjutatud. Seega on ilmne, et *P. putida* nälgivas rakupopulatsioonis toimuvate mutatsiooniliste protsesside regulatsioon on kontrollid teistsuguste mehhanismide kaudu kui *E. coli* rakkudes. Erinevus *E. coli* ja *P. putida* mutatsiooniprotsesside toimumises võib tuleneda sellest, et *E. coli* kasvukeskkond on stabiilsem, võrreldes mullabakter *P. putida´*ga, kes elab pidevalt muutuvates keskkonnatingimustes ning on sagedamini eksponeeritud mitmetele stressifaktoritele. Sellistes sagedasti vahelduvates kasvutingimustes ellu jäämiseks peavad *P. putida* rakud olema võimelised muutunud keskkonnatingimustega kiiresti adapteeruma. Kui võrrelda mehhanisme, mis viivad geneetilise adaptatsioonini *E. coli* ja *P. putida* rakkudes, siis *P. putida* genoom sisaldab rohkem vigutegevaid DNA polümeraase kodeerivaid geene. *E. coli* rakkudes on teada kaks vigutegevat DNA polümeraasi: Pol IV (DinB) ja Pol V (UmuD´C), mille olemasolu rakkudes võimaldab bakteritel kiiremini kohaneda muutunud keskkonnatingimustega (Yeiser jt., 2002). *P. putida* tüve PaW85 rakkudes on vigutegevale DNA polümeraas IV-le leitud aga koguni kaks homoloogi (Abella jt., 2004). Samuti on *P. putida* genoomis Pol III katalüütilist subühikut kodeeriva *dnaE* geeni homoloog *dnaE2* (Saumaa jt., 2006), mille osalust mutageneesil on näidatud nii *Mycobacterium tuberculosis´*e (Boshoff jt., 2003) kui

*Caulobacter crescentus´*e rakkudes (Galhardo jt., 2005). DNA Pol V homoloogi *P. putida* tüve PaW85 rakkude genoomist küll leitud ei ole, kuid selle olemasolu on kirjeldatud erinevatest looduslikult kasvavatest pseudomonaadidest isoleeritud suurtes plasmiidides (McBeth, 1990; Sundin, 1996). Kuna looduslikes bakteripopulatsioonides toimub pidev geneetilise materjali vahetus, on Pol V homoloogi mitteomavatel bakterirakkudel võimalus see plasmiidi ülekandumise teel omandada ja saada seeläbi eelis paremaks kohastumiseks. Pol V homoologi kodeerivaid *rulAB* geene plasmiidis TOL pWW0 omava *P. putida* populatsiooni võimet konkureerida stressitingimustes plasmiidi mitte sisaldava *P. putida* populatsiooniga, on kirjeldatud meie töögrupi poolt avaldatud artiklis Tark jt., 2005.

P. putida kiiremat adapteerumist muutunud kasvutingimustega võimaldab ilmselt ka see, et erinevalt *E. coli´*st, kus nii vigutegevate DNA polümeraaside kui ka paljude DNA reparatsioonisüsteemide ekspressioon on SOS vastuse poolt kontrollitud (Friedberg jt., 1995; Goodman, 2002), ei ole *P. putida* rakkudes ei *dinB* geeni ega ka *rulAB* geenide transkriptsioon DNA kahjustuste poolt indutseeritud, vaid toimub pidevalt kõrgel tasemel (Tegova jt., 2004). Lisaks näitavad meie katse tulemused, et erinevalt *E. coli´*st, kus DinB valgu ekspressioon on lisaks SOS-induktsioonile ka RpoS valgu poolt kontrollitud (Layton ja Foster, 2003), ei ole *P. putida dinB* geeni transkriptsioon ka RpoS-i olemasolust sõltuv.

Samas on meie grupi tulemuste põhjal näidatud, et DinB-st sõltuvad mutatsiooniprotsessid *P. putida* nälgivates rakkudes peavad olema mingi *E. coli´*st erineva mehhanismi kaudu kontrollitud. Kui *E. coli* rakkudes toimub DinB-sõltuvate mutatsioonide teke kogu nälgimisperioodi jooksul ühtlasel tasemel (McKenzie jt., 2000), siis *P. putida* rakkudes on DinB olemasolu oluline alles väga pikka aega nälginud rakkudes toimuvate mutatsioonide tekkeks (Tegova jt., 2004). Seega on võimalik, et *P. putida* DinB valgu tase on reguleeritud seni veel teadmata posttranskriptsiooniliste või posttranslatsiooniliste mehhanismide poolt.

E. coli puhul on näidatud, et statsionaarses faasis toimuvate mutatsioonide teke on vähemalt osaliselt sõltuv MMR süsteemi alatalitlusest (Feng jt., 1996; Harris jt., 1997). Selle üheks põhjuseks on MutS valgu hulga vähenemine, mis on otseselt seotud RpoS valgu olemasoluga rakkudes (Bjedov jt., 2003). Erinevalt *E. coli´*st, on meie

laboris näidatud, et *P. putida* pikka aega nälgivates rakkudes toimuvate mutatsioonide teke ei ole põhjustatud MMR süsteemi alatalitlusest (Saumaa jt., 2006). Samuti on teada, et *P. pudida´*s ei ole *mutS* mRNA tase olulisel määral RpoS-i poolt alla reguleeritud (van den Broek, 2004). Kuna RpoS-i olemasolu *P. putida* nälgivates rakkudes ei põhjusta mutatsioonisageduse tõusu ning veelgi enam, mõjub hoopis asendusmutatsioonide tekkele negatiivselt, siis arvame, et vastupidiselt *E. coli´*le on RpoS *P. putida* nälgivates rakkudes vajalik mutatsiooniprotsesside kontrolli all hoidmiseks.

Püüdes leida põhjusi, miks *P. putida rpoS*-defektsetes nälgivates rakkudes suureneb asendusmutatsioonide tekkesagedus, leidsime mitmeid viiteid sellele, et *E. coli rpoS*-defektne tüvi on märgatavalt tundlikum reaktiivsetele hapnikuühenditele (Lange ja Hengge-Aronis, 1991; Nyström ja Dukan, 1998; Nyström ja Dukan, 1999). Reaktiivsed hapnikuühendid, mis põhjustavad nii nukleiinhapete, valkude kui ka lipiidide kahjustusi (Imlay, 2003), võivad mõjutada ka mutatsioonide tekkeprotsesse. Kuna *E. coli* põhjal on teada, et reaktiivseid hapnikuühendeid eemaldavate ensüümide SodA ja KatE ekspressioon vajab RpoS-i olemasolu, on võimalik, et ka *rpoS*-defektses *P. putida* nälgivas populatsioonis suurenenud asendusmutatsioonide tekkesagedus on tingitud reaktiivsete hapnikuühendite kuhjumisest. Tõepoolest, superoksiidi radikaale detoksifitseeriva ensüümi SodAB hulga suurendamine rakkudes võimaldas vähendada asendusmutatsioonide tekkesagedust nälgivas *P. putida rpoS*-defektses tüves. Seega on võimalik, et üheks mehhanismiks, mille kaudu RpoS võib alandada mutatsioonisagedust *P. putida* nälgivas populatsioonis, on oksüdatiivseid kahjustusi põhjustavate reaktiivsete hapnikuühendite eemaldamine neid detoksifitsserivate ensüümide ekspressiooni otsese või kaudse positiivse regulatsiooni kaudu.

Võimalik, et aktiivsete hapnikuosakeste hulga suurenemisest *rpoS*-defektsetes rakkudes on põhjustatud ka nälgiva *rpoS*-defektse *P. putida* populatsiooni elumuse langus. Meie tulemustest on näha, et *rpoS*-defektse tüve elumus langeb nälgimise esimesel nädalal ligikaudu 1,5 suurusjärku ning jääb seejärel stabiilsena püsima. Kuna *rpoS*-defektse *P. putida* populatsiooni elumus teisel nälgimisnädalal enam ei lange, võib järeldada, et *rpoS*-defektse tüve rakkudes peavad toimuma mingisugused muutused, mis aitavad *rpoS*-defektset rakupopulatsiooni alal hoida. Kuna meil ei ole õnnestunud *P.*

putida rpoS-defektsest adapteerunud populatsioonist isoleerida „mutanti”, mille võime adapteeruda süsinikunälja tingimustega säiliks ka peale logaritmilise kasvufaasi läbimist, siis arvame, et *rpoS*-defektse tüve ellu jäämine ja adapteerumine on tagatud rakuprotsesside füsioloogilise ümerkorralduse tulemusena ja ei ole geneetiline. Nii nälgivatest rakukultuuridest kolooniat moodustavate rakkude arvukuse määramisel kui ka visuaalselt rakkude värvimisel LIVE/DEAD kitiga, mis peaks võimaladama eristada surnud rakke elusatest, oleme näidanud, et süsinikunäljas viibiv *rpoS*-defektne populatsioon sureb esimesel nälgimisnädalal võrreldes algse tüvega palju kiiremini. Seega on võimalik, et *rpoS*-defektse tüve massilise suremise tulemusena nälgimise algperioodil vabaneb bakterirakkude kasvukeskkonda hulgaliselt toitaineid. Surnud rakkudest vabanenud toitained võivad aidata antud keskkonnas ellujäänud *P. putida rpoS*-defektsel alampopulatsioonil kasvada ja aktiveerida rakkudes füsioloogilisi protsesse, mis on vajalikud süsinikunälja tingimustega paremini adapteerunud populatsiooni väljakujunemiseks.

Kokkuvõte

Ebasoodsad kasvutingimused kutsuvad bakterirakkudes esile stressi, mille tagajärjel suureneb rakkudes statsionaarse faasi sigma faktori RpoS hulk, mis aitab bakterirakkudel stressitingimustes ellu jääda ja adapteeruda. *E. coli´*s on näidatud RpoS-i osalust nii statsionaarse faasi rakkudes toimuvates füsioloogilistes muutustes kui ka geneetilistes ümberkorraldustes, mille tulemusena suureneb rakkude adaptatsioonivõime ja stressitaluvus.

Käesoleva töö eesmärgiks oli uurida mehhanisme, mille kaudu statsionaarse faasi sigma faktor RpoS võiks mõjutada mullabakteri *P. putida* statsionaarse faasi nälgivates rakkudes toimuvaid adaptatsiooniprotsesse.

Töö tulemused võib kokku võtta järgnevalt:

1. RpoS on *P. putida* nälgivates rakkudes vajalik mutatsiooniprotsesside kontrolli all hoidmiseks.
    a) RpoS ei mõjuta vigutegevat DNA polümeraas IV ehk DinB-d kodeeriva *dinB* geeni transkriptsiooni ega DinB-sõltuvate 1-nukleotiidsete deletsioonide teket.
    b) RpoS-i olemasolu *P. putida* rakkudes vähendab asendusmutatsioonide tekkesagedust.
    c) Asendusmutatsioonide tekke suurenemine nälgivas *P. putida rpoS*-defektses populatsioonis on põhjustatud reaktiivsete hapnikuühendite hulga suurenemisest rakkudes.
2. RpoS mõjutab *P. putida* nälgiva populatsiooni adapteerumist süsinikunälja tingimustega.
    a) *P. putida rpoS*-defektse populatsiooni elumus süsinikunälja tingimustes esimesel nälgimisnädalal väheneb, seejärel toimuvad rakkudes ilmselt füsioloogilised muutused, mis võimaldavad *P. putida rpoS*-defektsel populatsioonil süsinikunälja tingimustes kohastuda ja ellu jääda.
    b) Süsinikunälja tingimustes püsima jäänud alampopulatsiooni välja kujunemine *rpoS*-defektses populatsioonis, võib toimuda toitainete arvelt, mis on vabanenud suurest hulgast surnud rakkudest.

Summary

In bacterial cells, growth limiting conditions cause stress and trigger the induction of stationary-phase sigma factor RpoS, which provides the ability to survive and adapt to different stress conditions. In *E. coli* RpoS has been shown to be involved in physiological changes and genetic alterations in stationary-phase cells, increasing fitness and stress tolerance.

The aim of this study was to investigate the mechanisms by which stationary-phase sigma factor RpoS may affect adaption processes in starving *P. putida* population.

The results of this study are summarized as following:

1. RpoS control negatively mutational processes in *P. putida* starving cells
    a) RpoS is not required for transcription of *dinB* gene which encodes error-prone DNA polymerase IV, neither does its absence affect occurrence of Pol IV-dependent 1-bp frameshift mutations.
    b) The presence of RpoS reduces the frequency of base substitution mutations.
    c) Increase in the frequency of occurrence of base substitution mutations in *rpoS*-defective *P. putida* starving population is probably caused by the increase in the amount of reactive oxygen species in starving cells.
2. RpoS affects the adaptation of *P. putida* population under conditions of carbon starvation.
    a) The viability of *P. putida rpoS*-defective population reduces during the first week of starvation. After that the physiological changes obviously take place, allowing survival of the remainder cells under carbon starvation conditions.
    b) Survival and maintenance of *rpoS*-defective *P. putida* subpopulation on agar plates under carbon starvation conditions could be achieved by availability of pool of nutrients released from dying cells.

Kasutatud kirjandus

Abella, M., Erill, I., Jara, M., Mazon, G., Campoy, S., Barbe, J. 2004. Widespread distribution of a *lexA*-regulated DNA damage-inducible multiple gene cassette in the *Proteobacteria phylum.* Mol Microbiol. 54(1):212-22.

Adams, M.H. 1959. Bacteriophages. Interscience Publishers Inc. N Y. 445-447

Almirón, M., Link, A.J., Furlong, D., Kolter, R. 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev. 6:2646-54

Andersson, D.I., Slechta, E.S., Roth, J.R. 1998. Evidence that gene amplification underlies adaptive mutability of the bacterial *lac* operon. Science 282: 1133-1135

Barker, M.M., Gaal, T, Josaitis, C.A., Gourse, R.L. 2001. Mechanism of regulation of transcription initiation by ppGpp. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J Mol Biol. 305(4):673-88.

Bauchop, T., Elsden, S.R. 1960. The growth of microorganisms in relation their energy supply. J. of Gen Microbiol. 23: 469-475

Bayley, S.A. Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G., Broda, P., 1997. Two modes of loss of the TOL function from *P. putida* mt-2. Mol. Gen. Genet. 154: 203-204

Becker, G., Hengge-Aronis, R. 2001. What makes an *Escherichia coli* promoter sigma(S) dependent? Role of the -13/-14 nucleotide promoter positions and region 2.5 of sigma(S). Mol Microbiol. 39(5):1153-65.

Beyer, W., Imlay, J., Fridovich, I. 1991. Superoxide dismutases. Prog Nucleic Acid Res Mol Biol. 40:221-53.

Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., Matic, I. 2003. Stress-induced mutagenesis in bacteria. Science 300: 1404-1409

Bohannon, D.E. 1991. Stationary phase-inducible "gearbox" promoters: differential effects of *katF* mutations and role of s^{70}. J. Bacteriol. 173: 4482-4492

Boshoff, H.I., Reed, M.B., Barry, C.E ., Mizrahi, V. 2003. DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. Cell. 113(2):183-93.

Boyer, H.W., Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* J. Mol. Biol. 41: 459-472

Cabiscol, E., Tamarit, J., Ros, J. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Internatl Microbiol. 3:3-8

Cairns, J., Foster, P.L. 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics 128: 695-701

Carlioz, A., Touati, D. 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J. 5(3):623-30.

Cashel, M., Gentry, D.R., Hernandez, V.J., Vinella, D. 1996. The stringent response. Cell Mol. Biol., Washington, DC: ASM Press, 1458-1496

Chatterji, D., Ojha, A.K. 2001. Revisiting the stringent response, ppGpp and starvation signaling. Curr Opin Microbiol. 4(2):160-5.

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O., Hanawalt, P.C. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. Genetics 158: 41-64

Dukan, S., Nystrom, T. 1998. Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Dev. 12(21):3431-41.

Dukan, S., Nystrom, T. 1999. Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. J Biol Chem. 274(37):26027-32.

Farell, M.J., Finkel, S.E. 2003. The growth advantage in stationary phase phenotype conferred by *rpoS* mutations is dependent of the pH and nutrient environment. J. Bacteriol. 185: 7044-7052

Farewell, A., Kvint, K., Nystrom, T. 1998. Negative regulation by RpoS: a case of sigma factor competition. Mol Microbiol. 29(4):1039-51.

Farrell, M.J., Finkel, S.E. 2003. The growth advantage in stationary-phase phenotype conferred by *rpoS* mutations is dependent on the pH and nutrient environment. J Bacteriol. 185(24):7044-52.

Feng, G., Tsui, H.C., Winkler, M.E. 1996. Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells. J Bacteriol. 178(8):2388-96.

Finkel, S.E. 2006. Long-term survival during stationary phase: evolution and the GASP phenotype.Nat Rev Microbiol. 4(2):113-20.

Finkel, S.E., Kolter, R. 1999. Evolution of microbial diversity during prolonged starvation. Proc Natl Acad Sci U S A. 96(7):4023-7.

Flint, D.H., Tuminello, J.F., Emptage, M.H. 1993. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J Biol Chem. 268:22369-76

Foster, P.L. 1999. Mechanism of stationary phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. 33: 57-88

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64:97-112

Friedberg, E.C., Walker, G.C., Siede, W. 1995. SOS responses and DNA damage tolerance in prokaryotes. In DNA Repair and Mutagenesis. American Society for Microbiology, Washington, DC, 407-464

Fucci, L., Oliver, C.N., Coon, M., Stadtman, E.R. 1983. Inactivation of key metabolic enzymes by mixed function oxidation reactions: possible implication in protein turnover and ageing. Proc Natl Acad Sci USA. 80:1521-5

Galhardo, R.S., Rocha, R.P., Marques, M.V., Menck, C.F. 2005. An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*. Nucleic Acids Res. 33(8):2603-14.

Gentry, D.R., Cashel, M. 1996. Mutational analysis of the *Escherichia coli spoT* gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. Mol Microbiol. 19(6):1373-84.

Gentry, D.R., Hernandez, V.J., Nguyen, L.H., Jensen, D.B., Cashel, M. 1993. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. J Bacteriol. 175(24):7982-9.

Gonzàlez-Flecha, B., Demple, B. 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J Biol Chem. 270:13681-7

Goodman, M.F. 2002. Error-prone repair DNA polymerases in prokaryotes and eukaryotes. Annu. Rev. Biochem. 71: 17-50

Harris, R.S., Feng, G., Ross, K.J., Sidhu, R., Thulin, C., Longerich, S., Szigety, S.K., Haseltine, W.A., Bock, R. 1973. Synthesisof guanosine tetra and pentaphosphate requires the presence of acodon-specific uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proc. Natl. Acad. Sci. USA 70: 1564-1568

Hassan, H.M., Sun, H.C. 1992. Regulatory roles of Fnr, Fur, and Arc in expression of manganese-containing superoxide dismutase in *Escherichia coli*. Proc Natl Acad Sci U S A. 89(8):3217-21.

Hastings, P.J., Slack, A., Petrosino, J.F., Rosenberg, S.M. 2004. Adaptive amplification and point mutation are independent mechanisms: evidence for various stress-inducible mutation mechanisms. PLoS Biol. (2)12: e399

Heim, S., Ferrer, M., Heuer, H., Regenhardt, D., Nimtz, M., Timmis, K.N. 2003. Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase. Environ Microbiol. 5(12):1257-69.

Hendrickson, H., Slechta, E.S., Bergthorsson, U., Andersson, D.I., Roth, J.R. 2002. Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. Proc Natl Acad Sci U S A. 99(4):2164-9.

Hengge-Aronis, R. 2002. Recent insights into the general stress response regulatory network in *Escherichia coli*.J Mol Microbiol Biotechnol. 4 :341-6.

Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev. 66(3):373-95.

Hernandez, V.J., Bremer, H. 1991. Escherichia coli ppGpp synthetase II activity requires *spoT*. J Biol Chem. 266(9):5991-9.

Hernandez, V.J., Cashel, M. 1995. Changes in conserved region 3 of *Escherichia coli* sigma 70 mediate ppGpp-dependent functions *in vivo*. J Mol Biol. 252(5):536-49.

Herrero, M., de Lorenzo, V., Timmis, K.M. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion on foreign genes in gram.negative bacteria. J. Bacteriol. 172: 6557-6567

Hersh, M.N., Ponder, R.G., Hastings, P.J., Rosenberg, S.M. 2004. Adaptive mutation and amplification in *Escherichia coli*: two pathways of genome adaptation under stress. Res Microbiol. 155(5):352-9.

Hidalgo, E., Demple, B. 1996. Activation of SoxR-dependent transcription in vitro by noncatalytic or NifS-mediated assembly of [2Fe-2S] clusters into apo-SoxR. J Biol Chem. 271(13):7269-72.

Hõrak, R., Kivisaar, M. 1998. Expression of the transposase gene *tnpA* of Tn4652 is positively affected by integration host factor. J. Bacteriol. 180: 2822-2829

Ilari, A., Ceci, P., Ferrari, D., Rossi, G.L., Chiancone, E. 2002. Iron incorporation into *Escherichia coli* Dps gives rise to a ferritin-like microcrystalline core. J Biol Chem. 277(40):37619-23.

Ilves, H., Hõrak, R., Kivisaar, M. 2001. Involvement of sigma(S) in starvation-induced transposition of *Pseudomonas putida* transposon Tn4652. J Bacteriol. 183(18):5445-8.

Imlay, J.A. 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. 57:395-418

Ishihama, A. 2000. Functional modulation of *Escherichia coli* RNA polymerase. Annu. Rev. Microbiol. 54: 499-518

Jishage, M., Iwata, A., Ueda, S., Ishihama, A. 1996. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of four species of sigma subunit under various growth conditions. J Bacteriol. 178(18):5447-51.

Jishage, M., Kvint, K., Shingler, V., Nystrom, T. 2002. Regulation of sigma factor competition by the alarmone ppGpp. Genes Dev. 16(10):1260-70.

Jorgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P., Stewart, G.S. 1999. RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. Microbiology. 145 (Pt 4):835-44.

Katsuwon, J., Anderson, A.J. 1990. Catalase and Superoxide Dismutase of Root-Colonizing Saprophytic Fluorescent Pseudomonads. Appl Environ Microbiol. 56(11):3576-3582.

Katsuwon, J., Zdor, R., Anderson, A.J. 1993. Superoxide dismutase activity in root-colonizing pseudomonads. Can J Microbiol. 39(4):420-9.

Keyer, K., Imlay, J.A. 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc. Natl. Acad. Sci. USA. 93:13635-40

Kim, S.-R., Maenhaut-Michel, G., Yamada, M., Yamamoto, Y., Matsui, K., Sofuni, T., Nohmi, T., Ohmori, H. 1997. Multiple pathways for SOS-induced mutagenesis in *Escherichia coli:* an overexpression of *dinP* / *dinB* results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. Proc. Natl. Acad. Sci. USA 94: 13792-13797

Kim, Y.C., Miller, C.D., Anderson, A.J. 1999. Transcriptional regulation by iron of genes encoding iron- and manganese-superoxide dismutases from *Pseudomonas putida.* 239(1):129-35.

Kim, Y.C., Miller, C.D., Anderson, A.J. 2000. Superoxide dismutase activity in *Pseudomonas putida* affects utilization of sugars and growth on root surfaces. Appl Environ Microbiol. 66(4):1460-7.

Koch, B., Jensen, L.E., Nybroe, O. 2001. A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomalsite. J Microbiol Methods. 45(3):187-95.

Kofoid, E., Bergthorsson, U., Slechta, E.S., Roth, J.R. 2003. Formation of an F' plasmid by recombination between imperfectly repeated chromosomal Rep sequences: a closer look at an old friend (F'(128) pro *lac*). J Bacteriol. 185(2):660-3.

Lange, R., Fischer, D., Hengge-Aronis, R. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*. J Bacteriol. 177(16):4676-80.

Lange, R., Hengge-Aronis, R. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol. 5(1):49-59.

Lange, R., Hengge-Aronis, R. 1994. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev. 8(13):1600-12.

Layton, J.C., Foster, P.L. 2003. Error-Prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*. Mol. Microbiol. 50: 549-561

Liochev, S.I., Fridovich, I. 1994. The role of O2- in the production of OH• *in vitro* and *in vivo*. Free Rad Biol Chem. 16:29-33

Little, J.W. 1991. Mecanism of specific LexA cleavage: autodigestion and the role of RecA coprotease. Biochim. 73: 411-421

Little, J.W., Mount, D.W. 1982. The SOS regulatory system of *Escherichia coli.* Cell 29: 11-22

Loewen, P.C., Hu, B., Strutinsky, J., Sparling, R. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. 44: 707-717

Loewen, P.C., Switala, J., Triggs-Raine, B.L. 1985. Catalases HPI and HPII in Escherichia coli are induced independently. Arch Biochem Biophys. 243(1):144-9.

Loewen, P.C., Triggs, B.L. 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia col*i. J Bacteriol. 160(2):668-75.

Lombardo, M.J., Aponyi, I., Rosenberg, S.M. 2004. General stress response regulator RpoS in adaptive mutation and amplification in Escherichia coli. Genetics. 166(2):669-80.

Luo, Y., Pfuetzner, R.A., Mosimann, S., Paetzel, M., Frey, E.A., Cherney, M., Kim, B., Little, J.W., Strynadka, C.J. 2001. Crystal structure of LexA: a conformational switch for regulation of self-cleavage. Cell 106: 585-594

Magnusson, L.U., Farewell, A., Nystrom, T. 2005. ppGpp: a global regulator in *Escherichia coli*. Trends Microbiol. 13(5):236-42.

Martinez, A., Kolter, R. 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J Bacteriol. 179:5188-94

Massey, V. 1994. Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem. 269(36):22459-62.

McBeth, D.L. 1990. The CAM-OCT plasmid enhances UV responses of *Pseudomonas aeruginosa recA* mutants. J Bacteriol.172(3):1340-4.

McKenzie, G.J., Harris, R.S., Lee, P.L., Rosenberg, S.M. 2000. The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. USA 97: 6646-6651

Messner, K.R., Imlay, J.A. 1999. The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. J Biol Chem. 274(15):10119-28.

Moody, C.S., Hassan, H.M. 1984. Anaerobic biosynthesis of the manganese-containing superoxide dismutase in *Escherichia coli*. J Biol Chem. 259(20):12821-5.

Muffler, A., Fischer, D., Hengge-Aronis, R. 1996. The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for *rpoS* translation in *Escherichia coli*. Genes Dev. 10(9):1143-51.

Mulvey, M.R., Switala, J., Borys, A., Loewen, P.C. 1990. Regulation of transcription of *katE* and *katF* in *Escherichia coli.* J Bacteriol. 172(12):6713-20.

Murray, K.D., Bremer, H. 1996. Control of *spoT*-dependent ppGpp synthesis and degradation in *Escherichia coli*. J Mol Biol. 259(1):41-57.

Nair, S., Finkel, S.E. 2004. Dps protects cells against multiple stresses during stationary phase. J Bacteriol. 186:4192-98

Niederhoffer, E.C., Naranjo, C.M., Bradley, K.L., Fee, J.A. 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. J Bacteriol. 172(4):1930-8.

Notley-McRobb, L., King, T., Ferenci, T. 2002. *rpoS* mutations and loss of general stress resistance in *Escherichia coli* populations as a consequence of conflict between competing stress responses. J Bacteriol. 184(3):806-11.

Nunoshiba, T. 1996. Two-stage gene regulation of the superoxide stress response soxRS system in *Escherichia coli*. Crit Rev Eukaryot Gene Expr. 6(4):377-89.

Nyström, T. 2004. Stationary-phase physiology. Annu. Rev. Microbiol. 58:161-81

Ojangu, E.L., Tover, A., Teras, R., Kivisaar, M. 2000. Effects of combination of different -10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. J Bacteriol. 182(23):6707-13.

Patten, C.L., Kirchhof, M.G., Schertzberg, M.R., Morton, R.A., Schellhorn, H.E. 2004. Microarry analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. Mol. Gen. Genomics 272: 580-591

Paul, B.J., Ross, W., Gaal, T., Gourse, R.L. 2004. rRNA transcription *in Escherichia coli*. Annu Rev Genet. 38:749-70.

Peters, M., Heinaru, A., Nurk, A. 2001. Plasmid-encoded catalase KatA, the main catalase of *Pseudomonas fluorescens* strain Cb36. FEMS Microbiol Lett. 200(2):235-40.

Pugh, S.Y., Fridovich, I.J. 1985. Induction of superoxide dismutases in *Escherichia coli* by metal chelators. Bacteriol. 162(1):196-202.

Rai, P., Cole, T.D., Wemmer, D.E., Linn, S. 2001. Localization of Fe(2+) at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe(2+) and H2O2. J Mol Biol. 312(5):1089-101.

Ramos-Gonzales, M.I., Molin, S. 1998. Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. J. Bacteriol. 180: 3421-3431

Reddy, P.S., Raghavan,A., Chatterji, D. 1995. Evidence for a ppGpp-binding site on *Escherichia coli* RNA polymerase: proximity relationship with the rifampicin-binding domain. Mol Microbiol. 15(2):255-65.

Rosenberg, S.M. 2001. Evolvingresponsively: adaptive mutation. Nat. Rev. Genet. 2: 504-515

Saint-Ruf, C., Taddei, F., Matic, I. 2004. Stress and survival of aging Escherichia coli rpoS colonies. Genetics. 168(1):541-6.

Sanchez-Romero, J.M., Diaz-Orejas, R., De Lorenzo, V. 1998. Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. Appl Environ Microbiol. 64(10):4040-6.

Saumaa, S., Kasak, L., Kivisaar, M. 2002. Different spectrum of stationary phase mutations in early-arising versus late-arasing mutants of *Pseudomonas putida*: involment of DNA repair enzyme MutY and stationary-phase sigma factor RpoS. J. Bacteriol. 184:6957-65

Saumaa, S., Tarassova, K., Tark, M., Tover, A., Tegova, R., Kivisaar, M. 2006. Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida.* DNA Repair (Amst). 5(4):505-14.

Schellhorn, H.E., Hassan, H.M. 1988. Transcriptional regulation of *katE* in *Escherichia coli* K-12. J Bacteriol. 170(9):4286-92.

Sies, H. 1993. Damage to plasmid DNA by singlet oxygen and its protection. Mutat Res. 299:183-91

Sies, H., Menck, C.F. 1992. Singlet oxygen induced DNA damage. Mutat Res. 275:367-75

Slechta, E.S., Bunny, K.L., Kugelberg, E., Kofoid, E., Andersson, D.I., Roth, J.R. 2003. Adaptive mutation: general mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*. Proc Natl Acad Sci U S A. 100(22):12847-52.

Slechta, E.S., Liu, J., Andersson, D.I., Roth, J.R. 2002. Evidence that selected amplification of a bacterial lac frameshift allele stimulates Lac(+) reversion (adaptive mutation) with or without general hypermutability. Genetics. 161(3):945-56.

Spira, B., Silberstein, N., Yagil, E. 1995. Guanosine 3',5'-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for Pi. J Bacteriol. 177(14):4053-8.

Stadtman, E.R, Berlett, B.S. 1991. Fenton chemistry. Amino acid oxidation. J. Biol. Chem. 266:17201-11

Stadtman, E.R. 1990. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med. 9:315-25

Storz, G., Tartaglia, L.A., Ames, B.N. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science. 248:189-248

Suh, S.J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E., Ohman, D.E. 1999. Effect of rpoS mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J Bacteriol. 181(13):3890-7.

Sundin, G.W., Kidambi, S.P., Ullrich, M., Bender, C.L. 1996. Resistance to ultraviolet light *in Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. Gene. 177(1-2):77-81.

Tegova, R., Tover, A., Tarassova, K., Tark, M., Kivisaar, M. 2004. Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida.* J. Bacteriol. 186 (9): 2735-2744

Teich, A., Meyer, S., Lin, H.Y., Andersson, L., Enfors, S., Neubauer, P. 1999. Growth rate related concentration changes of the starvation response regulators sigmaS and ppGpp in glucose-limited fed-batch and continuous cultures of *Escherichia coli.* Biotechnol Prog. 15(1):123-9.

Tsui, H.C., Feng, G., Winkler, M.E. 1997. Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of Escherichia coli K-12. J Bacteriol. 179(23):7476-87.

van den Broek, D., Chin-A-Woeng, T.F., Bloemberg, G.V., Lugtenberg, B.J. 2005. Role of RpoS and MutS in phase variation of *Pseudomonas* sp. PCL1171. Microbiology. 151(Pt 5):1403-8.

Vijayakumar, S.R.V., Kirchhof, M.G., Patten, C.L., Schellhorn, H.E. 2004. RpoS-regulated genes of *Escherichia coli* identified by random *lacZ* fusion mutagenesis. J. Bacteriol. 186: 8499-8507

Visick, J.E., Clarke, S. 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpoS* mutations in common laboratory strains. J Bacteriol. 179(13):4158-63.

von Ossowski, I., Mulvey, M.R., Leco, P.A. 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. J Bacteriol. 173:514-20

Walker, C.G. 1996. The SOS of *Escherichia coli. Escherichia coli* and *Salmonella.* Cell Mol. Biol., Washington, DC, 1400-1412

Wick, L.M., Egli, T. 2004. Molecular components of physiological stress responses in *Escherichia coli.* Adv Biochem Eng Biotechnol. 89:1-45.

Winkler, M.E., Rosenberg, S.M. 1997. Mismatch repair protein MutL becomes limiting during stationary-phase mutation. Genes Dev. 11(18):2426-37.

Wolf, S.G., Frenkiel, D., Arad, T., Finkel, S.E., Kolter, R., Minsky, A. 1999. DNA protection by stress-induced biocrystallization. Nature 400: 83-85

Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., Cashel, M. 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J Biol Chem. 266(9):5980-90.

Yeiser, B., Pepper, E.D., Goodman, M.F., Finkel, S.E. 2002. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proc Natl Acad Sci USA. 99:8737-8741.

Zambrano, M.M., Siegele, D.A., Almiron, M., Tormo, A., Kolter, R. 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science. 259(5102):1757-60.

Zgurskaya, H.I., Keyhan, M., Matin, A. 1997. The sigma S level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. Mol Microbiol. 24(3):643-51.

Zhao, G-, Ceci, P., Ilari, A., Giangiacomo, L., Laue, T.M., Chiancone, E., Chasteen, N.D. 2002. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. J Biol Chem. 277(31):27689-96.

Zinser, E.R., Kolter, R. 1999. Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. J Bacteriol. 181(18):5800-7.

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Involvement of Error-Prone DNA Polymerase IV in Stationary-Phase Mutagenesis in *Pseudomonas putida*

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In this work we studied involvement of DNA polymerase IV (Pol IV) (encoded by the *dinB* gene) in stationary-phase mutagenesis in *Pseudomonas putida*. For this purpose we constructed a novel set of assay systems that allowed detection of different types of mutations (e.g., 1-bp deletions and different base substitutions) separately. A significant effect of Pol IV became apparent when the frequency of accumulation of 1-bp deletion mutations was compared in the *P. putida* wild-type strain and its Pol IV-defective *dinB* knockout derivative. Pol IV-dependent mutagenesis caused a remarkable increase (approximately 10-fold) in the frequency of accumulation of 1-bp deletion mutations on selective plates in wild-type *P. putida* populations starved for more than 1 week. No effect of Pol IV on the frequency of accumulation of base substitution mutations in starving *P. putida* cells was observed. The occurrence of 1-bp deletions in *P. putida* cells did not require a functional RecA protein. RecA independence of Pol IV-associated mutagenesis was also supported by data showing that transcription from the promoter of the *P. putida dinB* gene was not significantly influenced by the DNA damage-inducing agent mitomycin C. Therefore, we hypothesize that mechanisms different from the classical RecA-dependent SOS response could elevate Pol IV-dependent mutagenesis in starving *P. putida* cells.

During the past several years our understanding of mutation mechanisms has been expanded by the discovery of a new superfamily of DNA polymerases, called the Y family (46). The Y-family polymerases have been identified in prokaryotes, archaea, and eukaryotes. Members of this superfamily are devoid of 3′→5′ proofreading exonuclease activity and replicate undamaged DNA with low fidelity and low processivity; many of these enzymes can bypass DNA lesions that block chain elongation by replicative DNA polymerases (21–23). According to the concept of specialized polymerases some of these polymerases are able to copy cognate lesions with high genetic fidelity (22). On the other hand, the specialized DNA polymerases are involved in mutation processes when copying noncognate DNA lesions or normal DNA.

In a growth-restricting environment (e.g., during starvation), mutants arise that are able to take over bacterial populations by a process known as stationary-phase mutation (15). One widely discussed idea is that genetic adaptation of microbial populations under environmental stress might be accelerated by stress-induced activation of error-prone DNA polymerases (see, for example, references 16, 50, and 63). In *Escherichia coli*, two error-prone DNA polymerases, Pol V (UmuD′C) and Pol IV (DinB), and one high-fidelity DNA polymerase, Pol II, are upregulated during the SOS response (23). SOS induction has also been shown to occur spontaneously in static bacterial populations (62). It has been recently demonstrated that error-prone DNA polymerases Pol IV and Pol V are involved in stationary-phase mutagenesis in *E. coli* (4, 7, 42). The involvement of SOS-induced polymerases (Pol II, Pol IV, and Pol V) in stationary-phase mutagenesis has also been shown in the appearance of GASP (growth advantage in stationary phase) mutants of *E. coli* (70). *E. coli* mutants lacking one or more of these polymerases suffered considerable fitness reduction when competing with a wild-type strain under starvation conditions (70).

Although DinB-like polymerases have been identified in essentially all prokaryotic and eukaryotic organisms studied (46), with only a few exceptions (e.g., see reference 61), involvement of Pol IV homologues in stationary-phase mutagenesis in bacteria other than *E. coli* has not been investigated. Knowledge about mechanisms of Pol IV-dependent stationary-phase mutagenesis obtained using *E. coli*-based test systems includes studies of the 1-bp deletions occurring in an F′ plasmid (see, for example, references 38 and 42) and in the bacterial chromosome (7). In these cases, another copy of the *dinB* gene was present in the F′ plasmid and Pol IV-dependent stationary-phase mutations required the RecA protein. The genus *Pseudomonas* represents one of the most diverse and ecologically widely distributed groups of bacteria (58). Here, we studied the role of Pol IV in mutagenesis in *Pseudomonas putida*. We show that Pol IV is involved in generation of 1-bp deletions in starving cells. The occurrence of other types of stationary-phase mutations (e.g., base substitutions) does not require the presence of Pol IV. Pol IV-dependent mutagenesis in *P. putida* appears to be a RecA-independent process. Moreover, our results demonstrate that involvement of Pol IV in stationary-phase mutagenesis becomes essential only in long-term-starved populations of *P. putida*, which indicates that Pol IV-dependent mutagenesis can be induced by a so-far uncharacterized mechanism in cells that have suffered long-term starvation stress.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
Strains		
E. coli		
TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	10
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relAI* Δ(*ara-leu*) *araD* Δ*lacX74*	Invitrogen
CC118 λpir	*galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, λpir phage lysogen	30 30
P. putida		
PaW85	Wild type	3
PaW85 *dinB*::*tet*	*dinB*::*tet*	This work
PaW85 *recA*::*tet*	*recA*::*tet*	This work
PKS54	*rpoS*::*km*	47
Plasmids		
pBluescript KS(+)	Cloning vector (Ap^r)	Stratagene
pBR322	Cloning vector (Ap^r Tet^r)	6
pKT240	Medium-copy broad-host-range cloning vector (Ap^r Km^r)	2
pPR9TT	Single-copy broad-host-range vector (Cm^r Amp^r)	52
pPU1930	pUC19 carrying the *pheA* gene transcribed from constitutive promoter	45
pKSpheA56+A	pBluescript KS(+) containing PCR-amplified *pheA* sequence with A-nucleotide insertion cloned into the EcoRV-cleaved vector	This work
pPUpheA56+A	Segment of mutant *pheA* gene cloned as XbaI-BclI fragment from pKSpheA56 + A into pPU1930 to replace the original *pheA* sequence	This work
pKTpheA56+A	Mutant *pheA* gene transcribed from constitutive promoter cloned as SacI-PvuII fragment from pPUpheA56+A to pKT240	This work
pKTpheA22TGA	pKT240 carrying the *pheA* gene with TGA codon instead of CTG for Leu-22	This work
pKTpheA22TAA	Same as pKTpheA22TGA but contains TAA stop codon	This work
pKTpheA22TAG	Same as pKTpheA22TGA but contains TAG stop codon	This work
pKSdinB	pBluescript KS(+) containing PCR-amplified *P. putida dinB* gene cloned into EcoRV-cleaved vector	This work
pKSdinB::tet	Tet^r gene from pBR322 inserted into Van91-cleaved *dinB* gene in pKSdinB	This work
pGP704dinB::tet	*dinB*::*tet* sequence-containing Acc65-XbaI fragment from pKSdinB::tet in pGP704 L	This work
pKSrecA	pBluescript KS(+) containing PCR-amplified *P. putida recA* gene cloned into EcoRV-cleaved vector	This work
pKSrecA::tet	Tet^r gene from pBR322 inserted into BclI-cleaved *recA* gene in pKSrecA	This work
pGP704recA::tet	*recA*::*tet* sequence-containing XbaI-Ecl136II fragment from pKSrecA::tet in pGP704 L	This work
pGP704 L	Delivery plasmid for homologous recombination	48
pRK2013	Helper plasmid for conjugal transfer of pGP704 L	13
pKSATGdinB	pBluescript KS(+) containing PCR-amplified *P. putida dinB* sequence starting from ATG initiator codon cloned into EcoRV-cleaved vector	This work
pBRlacItac	Expression vector containing P_{tac} promoter and *lacI*^{q} repressor in pBR322	47
pBRlacItacdinB	*dinB* from pKSATGdinB as PstI-Acc65I fragment in pBRlacItac	This work
p9TTlacItacdinB	*dinB* expression cassette from pBRlacItacdinB cloned as NheI-Acc65I fragment into XbaI-Acc65I-cleaved pPR9TT	This work
pREP4	LacI repressor-expressing plasmid	QIAGEN
pKTlacI	pKT240 expressing LacI repressor cloned from pREP4 into Ecl136II site	This work
pKSPdinB	pBluescript KS(+) containing PCR-amplified *P. putida dinB* promoter P_{dinB} cloned into BamHI- and XhoI-cleaved vector	This work
pKSluxAB	pBluescript KS(+) containing *luxAB* reporter genes cloned from pGP704 L as BamHI fragment into BamHI-cleaved vector	This work
pKTluxAB	*luxAB* reporter-carrying SacI-XhoI fragment from pKSluxAB in pKT240	This work
pKTPdinBluxAB	P_{dinB}-carrying SmaI-Acc65I fragment from pKSPdinB in pKTluxAB	This work
p9TTPdinBluxAB	P_{dinB}-*luxAB* from pKTPdinBluxAB in NotI site of pPR9TT	This work
pKTlacZ	pKT240-derived promoter-probe vector (Ap^r)	31
pKTPdinBlacZ	P_{dinB}-carrying BamHI-XhoI fragment from pKSPdinB in pKTlacZ	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Complete medium was Luria-Bertani (LB) medium (43), and minimal medium was M9 (1). Phenol minimal plates with 1.5% Difco agar contained 2.5 mM phenol as a sole carbon and energy source. Antibiotics were added at the following final concentrations: for *E. coli*, ampicillin at 100 μg/ml and tetracycline at 10 μg/ml; for *P. putida*, carbenicillin at 1,000 to 3,000 μg/ml, chloramphenicol at 1,500 to 3,000 μg/ml, and tetracycline at 80 μg/ml; for both organisms, kanamycin at 50 μg/ml and rifampin at 100 μg/ml. *E. coli* was incubated at 37°C, and *P. putida* was incubated at 30°C. *E. coli* was transformed with plasmid DNA as described by Hanahan (24). *P. putida* was electrotransformed as described by Sharma and Schimke (54). *E. coli* strain TG1 or DH5α was used for the DNA cloning procedures.

Construction of test systems to study stationary-phase mutations in *P. putida*. An assay system to test 1-bp deletions (Fig. 1) was constructed by altering the phenol monooxygenase gene (*pheA*) coding sequence. Insertion of a single A nucleotide 5′ to the ACC codon for Thr-56 was performed by PCR amplification of the segment of the *pheA* gene from plasmid pPU1930 (45) with primer pheAup (5′-AAGGCGCTCCCGTAAGACA-3′), complementary to the sequence at nucleotides −40 to −22 relative to the coding sequence of the *pheA* gene, and the mutant primer pheA56+A (5′-GGTGATCATAATGTTGCTAA TGCCCTGGGTTCGACAGGAACATTGCTGCG-3′), complementary to the

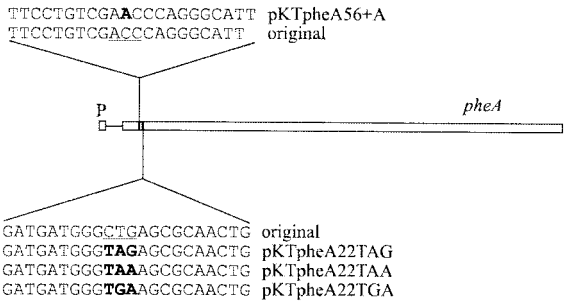

FIG. 1. Assay systems allowing measurement of 1-bp deletions (plasmid pKTpheA56+A) or different base substitutions (plasmids pKTpheA22TAG, pKTpheA22TAA, and pKTpheA22TGA) in *P. putida* stationary-phase cells. Segments of the original sequences and their mutant variants are shown. The test systems were constructed by altering the phenol monooxygenase gene (*pheA*) coding sequence. The +1 frameshift was constructed in the phenol monooxygenase gene *pheA* by inserting the A nucleotide (marked in bold) into the ACC codon for Thr-56 (underlined in the original sequence). Assay systems for the isolation of base substitutions were constructed by replacement of the CTG codon for Leu-22 (underlined in the original sequence) with a TGA, TAA, or TAG stop codon (indicated in boldface). Transcription from the *pheA* gene is initiated from the constitutively expressed promoter (45), indicated by P.

pheA coding sequence at nucleotides 147 to 195. The amplified DNA fragment was subcloned into the pBluescript KS(+) EcoRV site to obtain pKSpheA56+A. In addition to the generation of frameshift in the *pheA* coding sequence, this nucleotide insertion eliminated the SalI restriction site. The mutation was verified by DNA sequencing. The mutated DNA segment was thereafter inserted as the XbaI- and BclI-generated fragment from pKSpheA56+A (the XbaI site was provided by the pBluescript KS multicloning site) into pPU1930 by replacing the original *pheA* sequence located between the XbaI (the XbaI site is present upstream of the coding sequence of the *pheA* gene in pPU1930) and BclI sites to generate pPUpheA56+A. A pPU1930 derivative carrying the mutated sequence was first identified by the absence of the SalI restriction site in the *pheA* sequence. Finally, we cloned the SacI- and PvuII-generated fragment from pPUpheA56+A containing the constitutively expressed promoter and the mutated *pheA* gene into the broad-host-range plasmid pKT240 (2) to obtain pKTpheA56+A.

Assay systems allowing detection of base substitutions (Fig. 1) were constructed by using a two-step PCR amplification strategy. Mutant oligonucleotides contained specific base substitutions that replaced the CTG codon for Leu-22 in the *pheA* gene with a TGA, TAA, or TAG stop codon. In the first step, the PCR with oligonucleotides pheAup and pheA22TGA (5′-CGCTTCACCCATCATC AAAAATGACGCTA-3′), pheA22TAA (5′GCGCTTTACCCATCATCAAAA ATGACGCTA-3′), or pheA22TAG (5′-GCGCTCTACCCATCATCAAAAAT GACGCTA-3′), complementary to the positions 42 to 71 relative to the coding sequence of the *pheA* gene, was carried out. ExoI treatment followed, and the PCR products were purified and used in a second PCR with the oligonucleotide pheAts (5′-GTTCATGGGGGACTGCTTC-3′), complementary to *pheA* nucleotides 295 to 313. The amplified DNA fragments were cloned into the EcoRV site of pBluescript KS (+), and the mutations were verified by DNA sequencing. The next steps in the cloning strategy were identical to those used for the construction of the frameshift assay plasmid pKTpheA56+A. Finally, plasmids pKTpheA22TGA, pKTpheA22TAA, and pKTpheA22TAG were obtained.

Construction of *P. putida dinB and recA* knockout mutants. The *dinB* and *recA* gene sequences of *P. putida* KT2440 were obtained from The Institute for Genomic Research website (http://www.tigr.org). The *dinB* gene was amplified by PCR from genomic DNA of *P. putida* PaW85, which is isogenic to *P. putida* strain KT2440. Two primers, dinBFw (5′-GGCCTTTTCTTGAATCTGGTTGC G-3′), complementary to the sequence −518 to −496 upstream of the ATG initiator codon, and dinBRev (5′-GCGGATCCAGGCGTGCATTATTAG-3′), complementary to the sequence 24 to 47 nucleotides downstream of the TGA stop codon of the *dinB* gene, were used for PCR amplification. The amplified DNA fragment containing the *dinB* gene was subcloned into EcoRV-cleaved pBluescript KS (+), to obtain pKSdinB. The EcoRI- and Van9I-generated DNA fragment containing the Tet^r gene from pBR322 was inserted into the Van91-cleaved *dinB* gene. The resulting *dinB*::*tet* sequence from pKSdinB::tet was inserted into plasmid pGP704 L (48) by using Acc65I and XbaI sites. Plasmid pGP704dinB::tet was selected in *E. coli* strain CC118 λpir (30). The interrupted *dinB* gene was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGP704dinB::tet, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using a helper plasmid, pRK2013 (13). The PaW85 *dinB*::*tet* knockout strain was verified by PCR analysis.

For the construction of a *recA* knockout mutant of *P. putida*, the genomic DNA of PaW85 was amplified with oligonucleotides RecAFw (5′-AACAAGA AGCGCGCCTTGGTC-3′) and RecARev (5′-ATCAGCTTCAGCAGCAGCA GCTT-3′), complementary to the sequences −66 to −45 upstream of the ATG initiator codon and 37 to 60 nucleotides 5′ to the stop codon of the *recA* gene, respectively. The amplified DNA fragment was subcloned into EcoRV-cleaved pBluescript KS (+) to obtain pKSrecA. The EcoRI- and Van9I-cleaved DNA fragment containing the Tet^r gene from pBR322 was blunt ended and inserted into the BclI-cleaved *recA* gene (the BclI ends were blunt ended before the ligation as well), resulting in pKSrecA::tet. Finally, the DNA fragment containing the *recA*::*tet* sequence was inserted with XbaI and Ecl136II ends into pGP704 L to obtain pGP704recA::tet. The wild-type *recA* sequence of *P. putida* was replaced with the interrupted *recA*::*tet* sequence by homologous recombination using a procedure similar to that described above. The knockout mutant was verified by PCR analysis. Additionally, a UV sensitivity test (43) was performed to verify the RecA-defective strain PaW85 *recA*::*tet*.

DNA sequence analysis. In Phe$^+$ mutants, an approximately 350-bp DNA region covering the area of the *pheA* gene containing potential reversion mutations was analyzed by DNA sequencing. The DNA segment containing this region was amplified by PCR using the primers pheAup and pheAts. The nucleotide sequences were determined using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Inc). The oligonucleotides used in sequencing of the mutant DNA region were the same as those used in PCRs. The DNA sequencing reactions were analyzed with an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Overexpression of the *P. putida dinB* gene. The *dinB* gene lacking any regulatory sequences upstream of its ATG initiator codon was obtained by PCR amplification of *P. putida* PaW85 chromosomal DNA with primers dinBNde (5′-CATATGTCCTTGCGCAAGATCATCCA-3′), complementary to the nucleotides −3 to 23 relative to the *dinB* ATG initiator codon, and dinBRev. The amplified DNA fragment was cloned into the pBluescript KS (+) EcoRV site. We chose the construct carrying the *dinB* gene in the pBluescript vector in the opposite direction relative to the transcription direction from the *lac* promoter and named pKSATGdinB. By using the PstI and Acc65I restriction sites present in the pBluescript multicloning site, the *dinB* gene was further subcloned into the pBRlacItac plasmid (47) under the control of the P_{tac} promoter and *lacI*^{q} repressor to generate plasmid pBRlacItacdinB. In order to overexpress the *dinB* gene in *P. putida*, the *dinB* expression cassette was finally inserted as the NheI- and Acc65I-generated DNA fragment into the XbaI- and Acc65I-cleaved single-copy broad-host-range plasmid pPR9TT (52) to obtain p9TTlacItacdinB. To avoid a leaky transcription of the *dinB* gene from the P_{tac} promoter, which is deleterious to *P. putida* cells, plasmid pKTlacI carrying an additional copy of the *lacI* gene was constructed by inserting the DNA fragment containing the *lacI* gene from pREP4 (QIAGEN) with Eco47III- and SmaI-generated ends into the Ecl136II site of the pKT240 vector. The *dinB* overexpression studies with *E. coli* were performed with cells carrying plasmid pBRlacItacdinB; *P. putida* PaW85 carried two plasmids, p9TTlacItacdinB and pKTlacI. The expression level of *dinB* was artificially increased by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the growth medium of 1.5-ml separate cultures of exponentially growing bacteria. After 6 h of cultivation of bacteria, 0.1-ml samples were taken from the cultures and spread onto LB plates containing 100 μg of rifampin/ml. Colonies were counted on plates incubated for 24 h. The control experiments were performed using bacteria carrying the same vector plasmids lacking the *dinB* gene. The frequency of mutation to Rif^r per 10^9 cells was calculated for at least 35 independent cultures.

Studies of transcription from the *dinB promoter*. A DNA fragment containing a putative *dinB* promoter region was amplified by PCR with oligonucleotides dinBBamHI (5′-CCTTGGATCCAAGCTTTTTAACGGGCAAAGAAA-3′) and dinBXho (5′-ACGCTGCGATCGAGATGCGCTCGAGAAG-3′), complementary to the nucleotides at positions −328 to −350 and 40 to 58, respectively, relative to the ATG initiator codon of the *dinB* gene. The amplified DNA fragment was cut with BamHI and XhoI and inserted into BamHI- and XhoI-cleaved pBluescript KS (+) to obtain pKSPdinB. To construct the *luxAB* reporter plasmid replicating in *P. putida*, the promoterless *luxAB* genes were subcloned within the BamHI-cleaved DNA fragment from pGP704 L into pBlue-

script KS (+) to obtain pKSluxAB. Then, by using the SacI and XhoI restriction sites provided by the pBluescript vector multicloning sites, the *luxAB* genes were inserted into SacI- and XhoI-cleaved pKT240. The resulting plasmid, named pKTluxAB, was used as a multicopy promoter-probe vector for study of the transcription from the *dinB* promoter. The putative promoter region of the *dinB* gene was cloned from plasmid pKSPdinB into pKTluxAB, using restrictases SmaI and Acc65I. The resulting plasmid was named pKTPdinBluxAB. In order to study the regulation of transcription from a single copy of the *dinB* promoter, the P_{dinB}-*luxAB* expression cassette was subcloned from pKTPdinBluxAB into the NotI site of pPR9TT to obtain p9TTPdinBluxAB.

P. putida cells carrying pKTPdinBluxAB were grown exponentially in LB medium. In order to study whether DNA damage could induce transcription from the *dinB* promoter, mitomycin C (at a final concentration of 2 μg/ml) was added to cultures. Samples for luciferase assay were taken from *P. putida* exponential cultures grown in the presence or absence of mitomycin C. In all cases at least five independent measurements were made. The luciferase assay was performed as follows: 990 μl of phosphate buffer (100 mM Na_2HPO_4/KH_2PO_4 [pH 7]) and 10 μl of decanal (5 mM decanal in ethanol) were mixed in a test tube; 10 μl of bacterial culture was then added, and light emission was measured after 5 min of incubation with a luminometer (TD-20/20; Turner Designs).

To study the effect of the growth phase of bacteria on transcription from the *dinB* promoter, the promoter was subcloned from pKSPdinB into the promoter-probe vector pKTlacZ, using restrictases BamHI and XhoI, to obtain plasmid pKTPdinBlacZ. β-Galactosidase (β-Gal) activities in *P. putida* cells carrying pKTPdinBlacZ were measured by a modification of the standard protocol of Miller (43) as specified previously (64). Bacteria were grown in LB medium.

To map the transcription initiation site of the *P. putida dinB* gene, a reverse transcriptase reaction was carried out to identify the 5′ end of the *dinB*-specific mRNA. Total RNA was isolated from exponentially growing cells of *P. putida* using the QIAGEN RNeasy total RNA kit. Ten micrograms of purified RNA was used as a template in primer extension reactions with 10 pmol of [γ-^{32}P]ATP-labeled primer dinBXho. DNA sequencing reactions were performed using a Sequenase version 2.0 kit (U.S. Biochemicals) and the same primer, and the reaction mixtures were loaded onto a sequencing gel as size markers. A dried gel was exposed to a PhosphorImager screen (Molecular Dynamics).

Isolation of Phe$^+$ mutants. *P. putida* cells carrying different assay systems for the detection of Phe$^+$ revertants were grown overnight in LB medium. Cells sampled from the culture were harvested by centrifugation and washed in M9 solution. Approximately 5×10^8 to 1×10^9 cells were spread onto phenol minimal plates. A few Phe$^+$ colonies appearing on phenol minimal plates on day 2 contained mutations that occurred before the plating in a growing culture, whereas the colonies that emerged on selective plates on day 3 and later contained mutations that occurred after the cells were plated. To control whether the late-arising mutants could form a colony on phenol selective plates with a speed similar to that of those that emerged earlier, we performed reconstruction experiments. Plating of such mutants (approximately 100 cells) in the presence of 1×10^9 nonmutant cells onto phenol minimal plates demonstrated that all revertants tested were able to form visible Phe$^+$ colonies on day 2 after plating of the mixed cultures. No more Phe$^+$ colonies accumulated on these plates during the next days of incubation. This confirmed that the late revertants are truly stationary-phase mutants and that they are formed in *P. putida* populations after prolonged starvation. The pKT240-based plasmids carrying the test systems for detection of Phe$^+$ mutations in *P. putida* are derivatives of the broad-host-range plasmid RSF1010 (2). We have previously shown (33) that the copy number of RSF1010-based plasmids is not affected by the growth phase of the bacteria.

Measurement of viability of Pol IV-defective and RecA-defective *P. putida* on phenol minimal plates. The viability of bacteria was determined on the same plates that were used for the isolation of Phe$^+$ mutants. Using sterile 1-ml pipette tips, small plugs were cut out from the phenol-containing minimal plates, avoiding Phe$^+$ colonies. The plugs were suspended in M9 solution by shaking for 10 min. Approximately 10^5-fold dilutions were plated onto LB plates, and the number of CFU was determined for at least five independent starving cultures.

RESULTS

***P. putida* contains a DNA polymerase Pol IV homologue.** Analysis of complete genome sequences of *P. putida* KT2440 revealed that the putative Pol IV homologue in this strain has 50% identity with the *E. coli* Pol IV sequence. According to the literature, Pol IV overproduction in *E. coli* growing cells causes hypermutation, including −1 frameshifts and some base substitutions (35, 67). To study whether overexpression of the *P. putida dinB* gene would also elevate the frequency of occurrence of spontaneous mutations in growing cells, we amplified the *P. putida dinB* (*dinP*) gene, encoding Pol IV, by PCR from chromosomal DNA of *P. putida* PaW85 (this strain is identical to KT2440) and cloned the gene into plasmid pBRlacItac (47) under the control of the P_{tac} promoter. We found that overexpression of the *P. putida dinB* gene in growing cells of *E. coli* carrying pBRlacItacdinB led to an approximately 24-fold increase in the frequency of appearance of Rif-resistant mutants (Table 2), which indicates that *P. putida* Pol IV is functional in *E. coli*.

TABLE 2. Effect of *P. putida dinB* overexpression on frequency of mutations in growing cells[a]

Strain	*dinB* overexpression	Control[b]	*P*[c]
E. coli TG1	20.5	0.85	<0.0001
P. putida PaW85	1.70	0.30	<0.001

[a] The frequency of mutation of Rif^r per 10^9 cells was calculated using the Lea-Coulson method of the median (39, 49).
[b] Bacteria carried the same plasmid except lacking the *dinB* gene.
[c] The Mann-Whitney test (57) for the effect of *dinB* overexpression.

To study the effects of overexpression of Pol IV on the frequency of mutations in *P. putida*, the *dinB* gene placed under the control of the P_{tac} promoter and the *lacI*^{q} repressor gene was inserted into a broad-host-range single-copy plasmid, pPR9TT (52). In contrast to *E. coli*, *P. putida* cells did not tolerate this construct, possibly due to a leakiness of the P_{tac} promoter. The colonies of p9TTlacItacdinB-carrying transformants that appeared on selective plates remained tiny, and cells picked up from these colonies were not further culturable. This indicated that artificial overexpression of the *dinB* gene in *P. putida* cells strongly inhibits growth of the bacteria and is probably lethal to the cells. To overcome the deleterious effect of leakiness of transcription of the *dinB* gene to *P. putida* cells, additional copies of the *lacI*^{q} repressor gene, present in the pKT240-derivative pKTlacI, were introduced into *P. putida* carrying the *dinB* expression construct p9TTlacItacdinB. In this case, by manipulating the IPTG concentrations, we found that addition of IPTG at a 1 mM concentration (but not below or above that concentration) resulted in an increase in the frequency of appearance of Rif-resistant mutants in *P. putida* cells (Table 2).

Construction of novel test systems for the study of mechanisms of stationary-phase mutations in *P. putida*. *P. putida* PaW85 cells carrying the phenol monooxygenase gene, *pheA*, are able to grow on minimal medium containing phenol as the only carbon source (45). Selection of mutants able to grow on phenol minimal plates due to the activation of a silent *pheA* gene has been used by us to study stationary-phase mutagenesis in *P. putida* (33, 53). The assay system employed in our previous studies allowed isolation of different types of mutations, base substitutions, deletions, and insertions, which all led to the same outcome—generation of a functional promoter. In the current study, in order to study the effects of different genetic backgrounds on the frequency of different types of mutations separately, we constructed a novel set of assay sys-

tems. Details of the construction of test systems are presented in Materials and Methods. One of these systems measures reversion of +1 frameshift within the phenol monooxygenase gene, *pheA* (Fig. 1). Three other test systems, designed for the detection of base substitutions, contain different stop codons, TAG, TAA, or TGA, introduced into the same position (Leu-22) of the *pheA* coding sequence (Fig. 1).

To control whether the test systems constructed really detect the expected mutations, some of the Phe^+ revertants accumulating on phenol-containing selective plates were subjected to DNA sequence analysis. Sequence analysis of Phe^+ mutants emerging in *P. putida* carrying the assay system which should measure a reversion of +1 frameshift in plasmid pKTpheA56+A revealed that out of 15 mutants studied, 90% of frameshifts occurred at the neighboring site, in the CCC repeat flanking the inserted A nucleotide. Only 10% of the mutants contained an A nucleotide deletion. The −1 deletion at the CCC repeat restored the *pheA* reading frame by replacing the ACC codon (Thr) with the AAC codon (Asn).

Phe^+ mutants isolated using the assay systems carrying different stop codons instead of the codon for Leu-22 in the *pheA* sequence were true revertants. No suppressors were isolated among approximately 100 mutants analyzed per test system. The absence of suppressor mutations was confirmed by the finding that all mutants analyzed contained base substitutions in the *pheA* gene which eliminated the stop codon by replacing it with codons for different amino acids (Table 3). Also, in the case of every revertant studied, the Phe^+ phenotype was conjugatively transferred to the *P. putida* Phe^- recipient strain and the transconjugants carrying different mutant plasmids grew equally well on phenol minimal plates. Similar growth characteristics of different Phe^+ transconjugants on phenol minimal plates indicated that the amino acid Leu-22 in the PheA protein is replaceable with many other amino acids without changing the wild-type phenotype of this enzyme. Analysis of the Phe^+ revertants which appeared due to base substitutions revealed different spectra of mutations, depending on the sequence of the stop codon used in the particular assay system (Table 2). The spectrum of changes that occurred in the case of the assay system carrying the TAA stop codon (plasmid pKTpheA22TAA) was most homogeneous: among 43 mutants analyzed, 88% contained a T-to-C transition and the rest (12%) had a T-to-G transversion. The T-to-C transition was also dominant in the case of mutants isolated using the assay systems carrying either the TGA or TAG codon (plasmids pKTpheA22TGA and pKTpheA22TAG, respectively), although several other DNA changes were identified as well (Table 3).

Pol IV contributes to −1 deletions in starving cells of *P. putida*. In order to study possible involvement of the error-prone DNA polymerase Pol IV in occurrence of stationary-phase mutations in *P. putida*, we constructed a *dinB* knockout mutant of *P. putida* PaW85 (see Materials and Methods). Based on analysis of the sequence of the *P. putida* genome, the *dinB* gene does not belong to an operon of other genes. Therefore, possible negative effects of disruption of the *dinB* gene on transcription of other genes are excluded. Cells of wild-type *P. putida* PaW85 and its Pol IV-defective derivative PaW85 *dinB*::*tet* carrying different test systems (plasmid pKTpheA56+A, pKTpheA22TAA, pKTpheA22TGA, or pKTpheA22TAG) were plated onto phenol minimal plates containing phenol as the only carbon source. Results presented in Fig. 2A clearly demonstrate that the occurrence of 1-bp deletion mutations depends on the presence of the functional *dinB* gene in *P. putida*. Starting from day 9, the frequency of accumulation of Phe^+ mutants on selective plates increased remarkably, and on day 11 and later the accumulation frequency of the Phe^+ revertants in the *P. putida* wild-type strain was approximately 10 times higher than in the Pol IV-defective mutant. The lower frequency of accumulation of 1-bp deletion mutants in PaW85 *dinB*::*tet* could not be ascribed to a lower viability of *dinB*-defective cells under starvation conditions: during the 13 days studied, the Pol IV-defective strain survived as well as the wild-type strain (Fig. 2B). Thus, Pol IV-dependent mutagenesis could be elevated under conditions of long-term starvation of *P. putida* cells.

TABLE 3. Reversion of different nonsense mutations (TGA, TAA, and TAG) in independent Phe^+ mutants accumulating in starving cell populations of *P. putida* PaW85

Target[a]	DNA change	Occurrence(s) (%)	Reversion change	
			Codon	Amino acid
TGA	T → C	13 (54)	CGA	Arg
	T → G	5 (21)	GGA	Gly
	G → T	4 (17)	TTA	Leu
	A → C	2 (8)	TGC	Cys
TAA	T → C	38 (88)	CAA	Gln
	T → G	5 (12)	GAA	Glu
TAG	T → C	44 (61)	CAG	Gln
	A → G	11 (15)	TGG	Trp
	G → T	8 (11)	TAT	Tyr
	T → G	6 (8.5)	GAG	Glu
	G → C	1 (1.5)	TAC	Tyr
	A → T	1 (1.5)	TTG	Leu
	T → A	1 (1.5)	AAG	Glu

[a] In all cases the same codon, CTG for Leu 22, was altered in the wild-type *pheA* sequence.

No effect of the *dinB* knockout became apparent if we measured the frequency of appearance of Phe^+ mutants in starving cell populations of *P. putida* carrying test systems which allowed detection of base substitutions (data not shown). This indicated that Pol IV contributes only to a particular type of stationary-phase mutations in *P. putida*.

RecA is not required for Pol IV-dependent mutagenesis in *P. putida*. In *E. coli*, *dinB* is one of the genes induced in response to DNA damage (SOS response). After RecA-mediated cleavage of the LexA repressor, SOS regulon genes including *dinB* become upregulated (11, 38). DNA polymerase Pol IV-dependent stationary-phase mutations in the well-studied *E. coli* FC40 Lac^+ reversion system require a functional RecA protein (7, 42). In order to study whether the occurrence of *dinB*-dependent stationary-phase mutations are affected by RecA functionality in *P. putida*, we constructed a RecA-defective mutant of *P. putida* strain PaW85 (see Materials and Methods). Our experiments showed that the rate of accumulation of 1-bp deletion mutants was similar in the wild-type *P. putida* and its RecA-deficient derivative PaW85 *recA*::*tet* (Fig. 2A). This demonstrated that the occurrence of Pol IV-dependent sta-

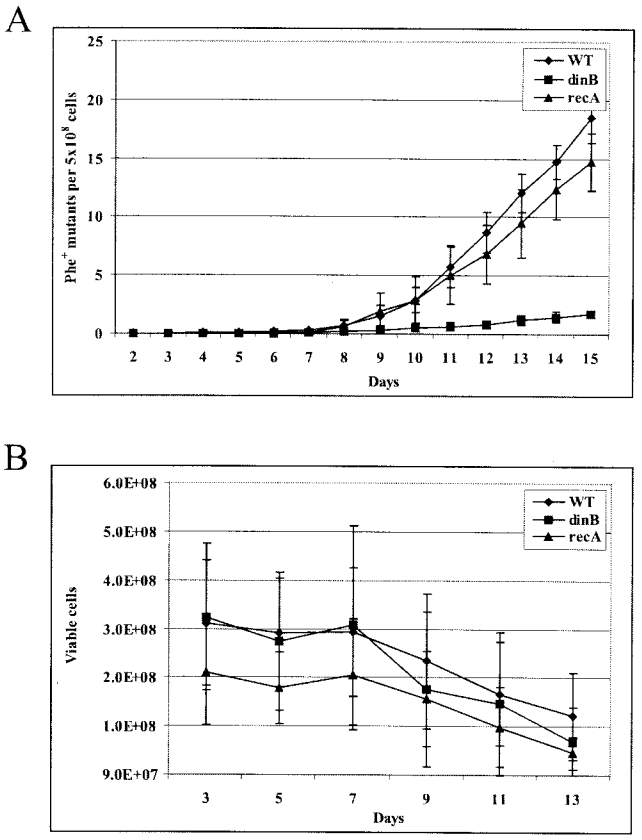

FIG. 2. (A) Accumulation of Phe^+ mutants on phenol minimal plates of *P. putida* wild-type strain PaW85 (WT) and in its *dinB*-defective (dinB) and *recA*-defective (recA) derivatives carrying plasmid pKTpheA56+A. About 5×10^8 *P. putida* cells were plated from overnight LB-grown cultures onto phenol minimal plates. Data for at least five parallel experiments are presented. Means ± standard deviations (error bars) for 10 plates calculated per 5×10^8 cells are shown. (B) Viability of *P. putida* wild-type strain PaW85 (WT) and in its *dinB*-defective (dinB) and *recA*-defective (recA) derivatives on phenol minimal plates. Means ± standard deviations for at least five cultures are shown. 1.0E + 08, for example, indicates 10^8 viable cells.

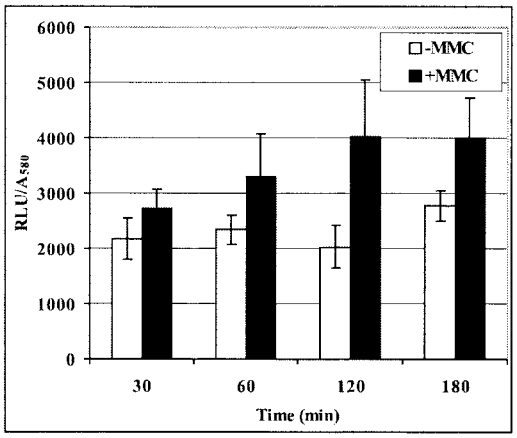

FIG. 3. Study of effects of DNA damage on transcription from the *dinB* promoter in *P. putida* PaW85. The promoter was cloned upstream of the reporter genes *luxAB*, encoding luciferase, and the expression of the transcriptional fusion was measured on pKT240-derived broad-host-range plasmid pKTPdinBluxAB. Transcription from the *dinB* promoter was assayed by measuring the luciferase activity (relative luciferase units/optical density unit at 580 nm) in cells grown in LB medium in the presence or absence of DNA-damaging agent mitomycin C (2 μg/ml). We determined that addition of higher concentrations of mitomycin C (up to 20 μg/ml) to the growth medium of the bacteria did not cause greater effects on transcription from the *dinB* promoter.

tionary-phase mutations observed in this study does not require the RecA protein.

Transcriptional analysis of the *P. putida dinB* promoter. In order to study transcription from the *dinB* promoter, the putative promoter region of the *dinB* gene was cloned upstream of the *luxAB* reporter into plasmid pKTluxAB. The resulting plasmid, pKTPdinBluxAB, expressed a high constitutive level of luciferase activity (Fig. 3). Mapping of the transcription initiation site by primer extension analysis localized the 5′end of the *dinB*-specific mRNA at the G nucleotide which is six nucleotides downstream from the putative promoter sequence, resembling the σ^{70}-type promoter consensus $TTGACAN_{16\text{-}18}$ TATAAT (Fig. 4). The sequence GTTTCA, resembling the −35 hexamer, and TACTAT, similar to the −10 hexamer, of this promoter were separated by a 17-nucleotide spacer. The *P. putida dinB* promoter region contains a sequence exhibiting similarity to the LexA binding consensus $CTG\text{-}N_{10}\text{-}CAG$ (69) (Fig. 4B). This sequence overlaps the −10 hexameric sequence of the *dinB* promoter by two nucleotides. Therefore, one may expect repression of this promoter by a LexA protein (the *P. putida* genome contains two different *lexA* gene homologues; see, e.g., http://www.tigr.org). However, despite the presence of putative LexA binding sites in the *dinB* promoter region, addition of the DNA damage-inducing agent mitomycin C to the growth medium of bacteria had only a minor increasing effect (up to twofold) on the level of transcription from the *dinB* promoter (Fig. 3). The similar up-to-twofold positive effect of addition of mitomycin C became evident when the level of transcription from the *dinB* promoter was monitored in a single-copy plasmid, p9TTPdinBluxAB (data not shown). The latter fact excludes the possibility that the cellular amount of LexA repressor might be too low to saturate all its binding sites when the *dinB* promoter is expressed in a medium-copy-number plasmid, pKTPdinBluxAB (approximately 10 copies per cell). Thus, transcription from the *dinB* promoter is poorly stimulated after DNA damage in *P. putida* cells.

Recently, Layton and Foster (38) demonstrated that in *E. coli*, the level of Pol IV is controlled by the stationary-phase sigma factor RpoS. RpoS is shown to also be a transcription regulator in *Pseudomonas*; however, it is less important in stress survival and has more specific roles related to virulence and colonization (reviewed in reference 66). In order to study whether the level of the transcription from the *dinB* promoter could be elevated in *P. putida* stationary-phase cells and to investigate possible effects of RpoS on transcription, we decided to compare expression of the reporter gene under the control of the *dinB* promoter in the wild type and RpoS-defective bacteria sampled from different time points of the batch culture. Because the *luxAB* reporter system is sensitive only in exponentially growing cells, we used another reporter, the *lacZ* gene, and monitored the level of β-Gal activity in *P. putida* cells carrying a *dinB* promoter-*lacZ* transcriptional fusion in the plasmid pKTPdinBlacZ during 100 h of cultivation

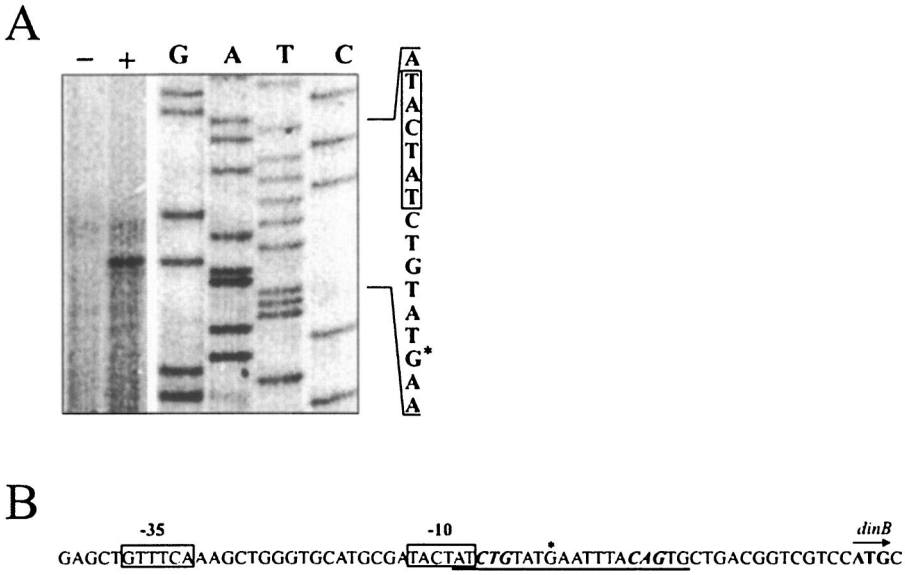

FIG. 4. (A) Mapping of the transcription initiation site for the *P. putida dinB* promoter by reverse transcriptase. Lanes G, A, T, and C show DNA-sequencing reactions of the *dinB* promoter region. Lanes marked by − and + represent primer extension reactions carried out with total RNA isolated from a *P. putida* PaW85 plasmid-free strain and from the same strain carrying extra copies of the *dinB* promoter region in medium-copy-number plasmid pKTPdinBluxAB, respectively. The sequence of the *dinB* promoter region, including the −10 sequence of the promoter (boxed) and the transcription start point (indicated by an asterisk), are shown on the right. (B) Nucleotide sequence of the *P. putida dinB* promoter region. The putative −35 and −10 hexamers of the promoter are boxed, and the 5′ end of the *dinB* mRNA is indicated by an asterisk. The potential LexA binding site is underlined (LexA binding consensus nucleotides [69] are in boldface). The translation start site of the *dinB* gene is indicated by an arrow.

of bacteria. Results shown in Fig. 5 demonstrate that transcription from the *dinB* promoter is not controlled by RpoS. In both strains studied (the wild type and the RpoS mutant), we observed a modest increase (approximately twofold) in the level of β-Gal expression if the activities from exponentially growing cells and stationary-phase cells sampled at h 12 were compared. During the prolonged incubation of bacteria in stationary phase the β-Gal activities increased but these changes were also small: an approximately threefold difference became apparent if transcription in exponentially growing and late-stationary-phase cells was compared. This experiment indicated that, although the transcription of the *dinB* gene in *P. putida* is affected by the growth conditions of the bacteria, RpoS does not play any role in *dinB* transcription.

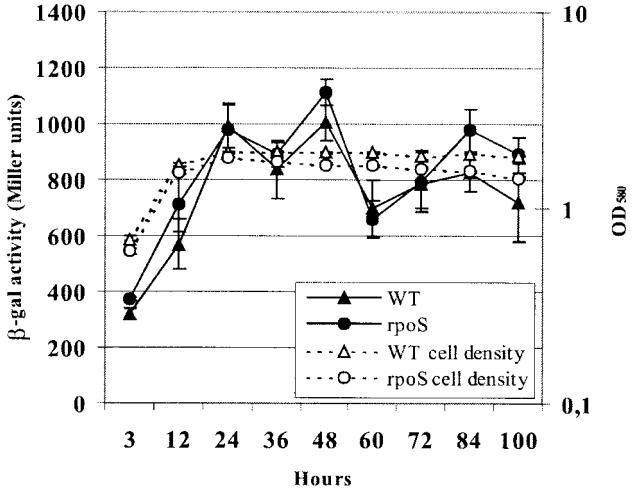

FIG. 5. Effect of growth phase of bacteria on transcription from the *dinB* promoter. β-Gal activity was measured in *P. putida* wild-type strain PaW85 (WT) and its RpoS-defective derivative PKS54 (rpoS) carrying the *dinB* promoter-*lacZ* fusion in plasmid pKTPdinBlacZ. Bacteria were grown in LB medium. Growth curves of bacteria are indicated by dashed lines. Results of four independent experiments are presented. The standard deviations are shown. OD_{580}, optical density at 580 nm.

DISCUSSION

Stationary-phase mutagenesis mechanisms have mostly been studied during short-term experiments, usually lasting less than 1 week. However, a large majority of microorganisms in their natural environments face far longer periods of starvation. Results of our recently published study (53) suggested that mutation processes in cells that have been starving for a short period are not entirely compatible with those of a prolonged starvation. The data presented by us herein demonstrate an increase in the frequency of accumulation of Phe^+ mutants in populations of *P. putida* during prolonged starvation. The test system used for the isolation of these mutants is based on reversion of +1 frameshift in the *pheA* gene. We found that the error-prone DNA polymerase Pol IV was specifically required for most 1-bp deletions detected by measuring the frequency of appearance of Phe^+ revertants in long-term-starved populations of *P. putida* (Fig. 2A). Meanwhile, this effect of Pol IV became clearly apparent only in cell populations that had already been starved for a carbon source for more than 7 days.

This implies that Pol IV-dependent mutagenesis could be induced under conditions of long-term environmental stress.

The idea that some mechanisms have probably evolved to control the mutation rate in a cell is a subject of intense scientific debate (see, for example, references 36, 50, 51, 55, and 63). Evidence supporting the hypothesis that stress-induced mutagenesis is a genetically programmed strategy has been found in different organisms, which indicates that regulation of stress-induced mutagenesis is general to all microorganisms. However, different control mechanisms may exist. For example, mutagenesis in resting organisms in structured environments (ROSE mutagenesis), which occurs in *E. coli* aging colonies, involves control by the SOS system and the catabolite repression system (62). Studies by Bjedov et al. (5) revealed that the frequency of mutations increased remarkably between 1-day- and 7-day-old colonies of most natural isolates of *E. coli*, from diverse habitats worldwide, and the mutagenesis in aging colonies, in one natural isolate tested, was genetically controlled by RpoS and carbon-sensing regulators. Sung and Yasbin (60) demonstrated that accumulation of prototrophic revertants among *Bacillus subtilis* cells required activity of genes that are involved in regulation of bacterial differentiation. In our previous study of *P. putida* (53), we demonstrated that the spectrum of stationary-phase mutations among early-arising mutants (picked up on days 3 and 4) differs from that of later-arising ones (picked up on days 6 and 7). The occurrence of mutations, the number of which started to increase later (e.g., 2- to 3-bp deletions), was dependent on the stationary-phase sigma factor RpoS (53). At the same time, the transposition of IS*1411*, which also increased with time of starvation and resulted in emergence of Phe^+ mutants, was under the negative control of RpoS (53). Most recently, Layton and Foster (38) provided evidence that the cellular amount of Pol IV in *E. coli* FC40 is controlled by RpoS, and Pol IV is required for stationary-phase mutation in that system (7, 42). Results presented here demonstrate that the frequency of accumulation of Pol IV-dependent mutants increases in *P. putida* populations during prolonged starvation. Whether RpoS is involved in Pol IV-dependent stationary-phase mutagenesis in the +1 frameshift reversion examined by us in the current study is difficult to determine because *P. putida* cells lacking functional RpoS die in cultures that have been starved more than 1 week (32).

Mechanisms of Pol IV-dependent stationary-phase mutations studied by us in *P. putida* differ in some aspects from those of the well-studied model organism *E. coli*. RecA is both a signal sensor/transducer molecule for the SOS response and a recombination protein (20). In the case of Pol IV-dependent mutagenesis in *E. coli* strain FC40, which has become a paradigm of stationary-phase mutation, the Lac^+ mutations that arise in starving-cell populations on lactose selective plates require RecA function and a RecBCD double-stranded-break repair system (8, 19, 27, 28). The recombination-dependent stationary-phase mutations are proposed to result from erroneous DNA replication at sites of double-stranded-break repair via homologous recombination (for a review of this model, see, for example, references 15, 17, and 50). The results reported by McKenzie et al. (41) indicate that the SOS activation function of RecA is also required for Lac^+ reversion in starving cells. DNA polymerase Pol IV, which is required specifically for 1-bp deletions in the Lac^+ reversion system in stationary-phase cells (but not in growing cells), is up-regulated during the SOS response (7, 42). An alternative amplification-mutagenesis model for RecA function in Pol IV-dependent Lac^+ reversion phenomena has also been proposed (29, 56). In the current study we showed that Pol IV-dependent mutagenesis in *P. putida* is RecA independent. Also, in contrast to *E. coli*, transcription from the *dinB* promoter in *P. putida* cells occurs at a high basal level both in the presence and absence of the DNA damage-inducing agent mitomycin C: we observed only a slight, maximum twofold increase after induction of DNA damage (Fig. 3). In comparison with *E. coli*, the SOS response in *P. putida* has been only poorly studied. However, the existence of a similar DNA damage-inducible response has been found by analyzing DNA damage-mediated induction of the *P. putida lexA* gene (9).

E. coli strains which have been used to study the role of Pol IV in stationary-phase mutations carry two copies of *dinB*, one in the chromosome and the other in the F′ plasmid (42). The higher expression of *dinB* in *E. coli* starving cells, resulting in stationary-phase mutagenesis, is hypothesized to be connected with sporadic amplification of the F′ plasmid DNA region carrying the *dinB* gene (37, 55). However, the increase in *dinB* expression in stationary phase shown by Layton and Foster (38) was observed in the absence of selection for *dinB* amplification. Differently from *E. coli*, the occurrence of stationary-phase mutations studied by us in *P. putida* depends on the presence of a single chromosomal copy of the *dinB* gene. If compared to exponentially growing cells, the level of transcription from the *dinB* promoter in *P. putida* was increased threefold in late-stationary-phase cells (Fig. 5). However, unlike expression of *dinB* in *E. coli*, the transcription of *dinB* in *P. putida* was not dependent on RpoS. This and other differences discussed above indicate that mechanisms distinct from those proposed to control expression of *dinB* in *E. coli* may up-regulate Pol IV-dependent mutagenesis under conditions of long-term environmental stress in *P. putida* starving cells.

There are at least three nonexclusive explanations of how the frequency of accumulation of Pol IV-dependent mutants can increase in long-term-starved cell populations: (i) some posttranscriptional/posttranslational mechanisms may control the activity of Pol IV in stressed *P. putida* cells; (ii) levels of Pol IV protein might increase in *P. putida* stationary-phase cells; and (iii) DNA repair can be depressed during prolonged starvation. Studies of stationary-phase mutagenesis in *E. coli* FC40 have demonstrated that defects in methyl-directed mismatch repair (MMR) resulted in a great increase in the number of Lac^+ mutants that arose with time after lactose selection (18). The spectra of Lac^+ reversion mutations observed in growing cells of MMR-deficient strains (1-bp deletions in small mononucleotide repeats) were indistinguishable from the spectrum of Lac^+ reversion mutations characterized in stationary-phase cells of the wild-type strain (40). MMR has been shown to be down-regulated in stationary phase (12, 65). Moreover, results by Harris et al. (26) imply that the MMR protein MutL becomes limiting during stationary-phase mutation. The role of down-regulation of MMR in accumulation of Lac^+ mutants in the FC40 system has stimulated an active dispute (14, 25). The idea that down-regulation of MMR might be involved in stationary-phase mutagenesis is supported by the finding that an

MMR-defective mutant did not show any significant elevation of mutagenesis in aging colonies (5). The 1-bp deletions measured in starving cells of FC40 which resulted in Lac^+ reversion were mostly produced by Pol IV (7, 42). Wagner and Nohmi (67) have also shown that many Pol IV-induced errors are corrected by MMR. Moreover, that report (67) demonstrates saturation of the MMR system as a result of accumulation of errors made by overproduced Pol IV. Hence, drawing parallels with data obtained in *E. coli*, we hypothesize that the increase in the frequency of Pol IV-dependent mutations observed by us might be caused by the malfunctioning of MMR in *P. putida* starving cells. Moreover, one may speculate that MMR might be disabled due to a higher activity of Pol IV in long-term-starved *P. putida*. Further and more straightforward investigations are needed to test these hypotheses.

Rifampin mutation assay scores only base substitutions, but the most frequently observed mutations associated with Pol IV activity in *E. coli* are frameshifts (35). Artificial overproduction of Pol IV in *P. putida* growing cells resulted in an elevated frequency of occurrence of Rif-resistant mutants, which indicates that Pol IV can contribute to base substitutions in this organism as well. However, the occurrence of base substitutions in starving *P. putida* did not require the activity of Pol IV. Depending upon the DNA lesion and its sequence context, different DNA polymerases are involved in generation of mutations in *E. coli* (22, 44, 68). Thus, if only a specific type of mutation in a specific sequence context is detectable in a given assay system, some assay systems may be less relevant for the detection of Pol IV-generated mutations than others. Base substitutions in the *rpoB* gene measured by us (Rif-resistant mutants in growing cell populations) and in the test systems detecting Phe^+ reversion mutations also occurred in different sequence contexts. Therefore, the failure to detect Pol IV-dependent base substitutions in starving cell populations in this study can be explained by the use of different assay systems for growing and stationary-phase cells. At the same time, results presented by Wagner and Nohmi (67) suggested a bias for Pol IV-generated mutations occurring in sequences with a guanine base at the 5′ position of the mutated base. Our test systems designed for the measurement of base substitutions in the *pheA* gene detected mostly T-to-C transitions either in the sequence 5′-GGGTAA-3′ or 5′-GGGTAG-3′. According to Wagner and Nohmi (67) both these sequences should be favored for base substitutions generated by Pol IV. Therefore, an alternative explanation for the phenomenon of why we did not observe any effect of Pol IV on generation of base substitutions in starving cells would be that the role of Pol IV in mutagenesis may vary between growing and stationary-phase cells.

It was recently shown that stationary-phase *E. coli* cells lacking one or more SOS-induced DNA polymerases (Pol II, Pol IV, and Pol V) are less fit when grown in the presence of wild-type cells under conditions in which the ability to generate GASP mutations (growth advantage in stationary phase) is under selection (70). Base pair substitution mutations occurring in stationary-phase cells of *E. coli* have been shown to be dependent on Pol V (4). Among gram-negative bacteria, *Pseudomonas* species examined so far, like many other nonenteric bacteria, lack chromosomally encoded Pol V. Although genes encoding Pol V homologues are frequently found in plasmids (34, 46, 59), bacteria lacking these plasmids accumulate stationary-phase mutations as well. Results of our current study demonstrate that only a particular type of stationary-phase mutations in *P. putida* requires Pol IV activity. Hence, the question arises whether these bacteria would express some other error-prone DNA polymerase activities involved in stationary-phase mutagenesis. Experiments to address this question are currently in progress.

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REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages, p. 445–447. Interscience Publishers Inc., New York, N.Y.
2. **Bagdasarian, M. M., E. Ammann, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida.* Construction of broad-host-range, controlled-expression vectors. Gene **26:**273–282.
3. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154:**203–204.
4. **Bhamre, S., B. B. Gadea, C. A. Koyama, S. J. White, and R. G. Fowler.** 2001. An aerobic *recA*, umuC-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli.* Mutat. Res. **473:**229–247.
5. **Bjedov, I., O. Tenaillon, B. Gérard, V. Souza, E. Denamur, M. Radman, F. Taddei, and I. Matic.** 2003. Stress-induced mutagenesis in bacteria. Science **300:**1404–1409.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
7. **Bull, H. J., M.-J. Lombardo, and S. M. Rosenberg.** 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proc. Natl. Acad. Sci. USA **98:**8334–8341.
8. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli.* Genetics **128:**695–701.
9. **Calero, S., X. Garriga, and J. Barbé.** 1993. Analysis of the DNA damage-mediated induction of *Pseudomonas putida* and *Pseudomonas aeruginosa lexA* genes. FEMS Microbiol. Lett. **110:**65–70.
10. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13:**4431–4443.
11. **Courcelle, J., A. Khodursky, B. Peter, P. O. Brown, and P. C. Hanawalt.** 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli.* Genetics **158:**41–64.
12. **Feng, G., H.-C. T. Tsui, and M. E. Winkler.** 1996. Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells. J. Bacteriol. **178:**2388–2396.
13. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
14. **Foster, P. L.** 1999. Are adaptive mutations due to decline in mismatch repair? The evidence is lacking. Mutat. Res. **436:**179–184.
15. **Foster, P. L.** 1999. Mechanisms of stationary phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. **33:**57–88.
16. **Foster, P. L.** 2000. Adaptive mutation in *Escherichia coli.* Cold Spring Harbor Symp. Quant. Biol. **65:**21–29.
17. **Foster, P. L.** 2001. Adaptive mutation: implications for evolution. BioEssays. **22:**1067–1074.
18. **Foster, P. L., and J. Cairns.** 1992. Mechanisms of directed mutation. Genetics **131:**783–789.
19. **Foster, P. L., J. M. Trimarchi, and R. A. Maurer.** 1996. Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli.* Genetics **142:**25–37.
20. **Friedberg, E. C., G. C. Walker, and W. Siede.** 1995. DNA repair and mutagenesis. ASM Press, Washington, D.C.
21. **Friedberg, E. C., P. L. Fischhaber, and C. Kisker.** 2001. Error-prone DNA polymerases: novel structures and the benefits of infidelity. Cell **107:**9–12.
22. **Friedberg, E. C., R. Wagner, and M. Radman.** 2002. Specialized DNA polymerases, cellular survival, and the genesis of mutations. Science **296:**1627–1630.
23. **Goodman, M. F.** 2002. Error-prone repair DNA polymerases in prokaryotes and eukaryotes. Annu. Rev. Biochem. **71:**17–50.

24. **Hanahan, D.** 1983. Studies on the transformation of *E. coli* with plasmids. J. Mol. Biol. **166:**577–580.
25. **Harris, R. S., G. Feng, K. J. Ross, R. Sidhu, C. Thulin, S. Longerich, S. K. Szigety, P. J. Hastings, M. E. Winkler, and S. M. Rosenberg.** 1999. Mismatch repair is diminished during stationary-phase mutation. Mutat. Res. **437:**51–60.
26. **Harris, R. S., G. Feng, K. J. Ross, R. Sidhu, C. Thulin, S. Longerich, S. K. Szigety, M. E. Winkler, and S. M. Rosenberg.** 1997. Mismatch repair protein MutL becomes limiting during stationary-phase mutation. Genes Dev. **11:** 2426–2437.
27. **Harris, R. S., S. Longerich, and S. M. Rosenberg.** 1994. Recombination in adaptive mutation. Science **264:**258–260.
28. **Harris, R. S., K. J. Ross, and S. M. Rosenberg.** 1996. Opposing roles of the Holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. Genetics **142:**681–691.
29. **Hendrickson, H., E. S. Slechta, U. Bergthorsson, D. I. Andersson, and J. R. Roth.** 2002. Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. Proc. Natl. Acad. Sci. USA **99:**2164–2169.
30. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
31. **Hőrak, R., and M. Kivisaar.** 1998. Expression of the transposase gene *tnpA* of Tn*4652* is positively affected by integration host factor. J. Bacteriol. **180:** 2822–2829.
32. **Ilves, H., R. Hőrak, and M. Kivisaar.** 2001. Involvement of σ^S in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652.* J. Bacteriol. **183:**5445–5448.
33. **Kasak, L., R. Hőrak, and M. Kivisaar.** 1997. Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria. Proc. Natl. Acad. Sci. USA **94:**3134–3139.
34. **Kim, J. J., and G. W. Sundin.** 2000. Regulation of the *rulAB* mutagenic DNA repair operon of *Pseudomonas syringae* by UV-B (290 to 320 nanometers) radiation and analysis of *rulAB*-mediated mutability in vitro in planta. J. Bacteriol. **182:**6137–6144.
35. **Kim, S.-R., G. Maenhaut-Michel, M. Yamada, Y. Yamamoto, K. Matsui, T. Sofuni, T. Nohmi, and H. Ohmori.** 1997. Multiple pathways for SOS-induced mutagenesis in *Eschericha coli*: an overexpression of *dinB*/*dinP* results in strongly enhancing mutagenesis in the absence of any exogeneous treatment to damage DNA. Proc. Natl. Acad. Sci. USA **94:**13792–13797.
36. **Kivisaar, M.** 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environ. Microbiol. **5:**814–827.
37. **Kofoid, E., U. Bergthorsson, E. S. Slechta, and J. R. Roth.** 2003. Formation of an F′ plasmid by recombination between imperfectly repeated chromosomal Rep sequences: a closer look at an old friend (F'_{128} *pro lac*). J. Bacteriol. **185:**660–663.
38. **Layton, J. C., and P. L. Foster.** 2003. Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli.* Mol. Microbiol. **50:**549–561.
39. **Lea, D. E., and C. A. Coulson.** 1949. The distribution of the numbers of mutants in bacterial populations. J. Genet. **49:**264–265.
40. **Longerich, S., A. M. Galloway, R. S. Harris, C. Wong, and S. M. Rosenberg.** 1995. Adaptive mutation sequences reproduced by mismatch repair deficiency. Proc. Natl. Acad. Sci. USA **92:**12017–12020.
41. **McKenzie, G. J., R. S. Harris, P. L. Lee, and S. M. Rosenberg.** 2000. The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. USA **97:**6646–6651.
42. **McKenzie, G. J., P. L. Lee, M.-J. Lombardo, P. J. Hastings, and S. M. Rosenberg.** 2001. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell **7:**571–579.
43. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Napolitano, R., R. Janel-Bintz, J. Wagner, and R. P. P. Fuchs.** 2000. All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis. EMBO J. **19:**6259–6265.
45. **Nurk, A., L. Kasak, and M. Kivisaar.** 1991. Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: expression in *Escherichia coli* and *Pseudomonas putida.* Gene **102:**13–18.
46. **Ohmori, H., E. C. Friedberg, R. P. P. Fuchs, M. F. Goodman, F. Hanaoka, D. Hinkle, T. A. Kunkel, C. W. Lawrence, Z. Livneh, T. Nohmi, L. Prakash, S. Prakash, T. Todo, G. C. Walker, Z. Wang, and R. Woodgate.** 2001. The Y-family of DNA polymerases. Mol. Cell **8:**7–8.
47. **Ojangu, E.-L., A. Tover, R. Teras, and M. Kivisaar.** 2000. Effects of combination of different −10 hexamers and downstream sequences on stationary-phase-specific sigma factor σ^S-dependent transcription in *Pseudomonas putida.* J. Bacteriol. **182:**6707–6713.
48. **Pavel, H., M. Forsman, and V. Shingler.** 1994. An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on para-substituted methylphenols. J. Bacteriol. **176:**7550–7557.
49. **Rosche, W. A., and P. L. Foster.** 2000. Determining mutation rates in bacterial populations. Methods **20:**4–17.
50. **Rosenberg, S. M.** 2001. Evolving responsively: adaptive mutation. Nat. Rev. Genet. **2:**504–515.
51. **Roth, J. R., E. Kofoid, F. P. Roth, O. G. Berg, J. Seger, and D. I. Andersson.** 2003. Regulating general mutation rates: examination of the hypermutable state model for cairnsian adaptive mutation. Genetics **163:**1483–1496.
52. **Santos, P. M., I. Di Bartolo, J. M. Blatny, E. Zennaro, and S. Valla.** 2001. New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. FEMS Microbiol. Lett. **195:**91–96.
53. **Saumaa, S., A. Tover, L. Kasak, and M. Kivisaar.** 2002. Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and stationary-phase sigma factor RpoS. J. Bacteriol. **184:**6957–6965.
54. **Sharma, R. C., and R. T. Schimke.** 1996. Preparation of electro-competent *E. coli* using salt-free growth medium. BioTechniques **20:**42–44.
55. **Slechta, E. S., K. L. Bunny, E. Kugelberg, E. Kofoid, D. I. Andersson, and J. R. Roth.** 2003. Adaptive mutation: general mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac.* Proc. Natl. Acad. Sci. USA **100:**12847–12852.
56. **Slechta, E. S., J. Liu, D. I. Andersson, and J. R. Roth.** 2002. Evidence that selected amplification of a bacterial *lac* frameshift allele stimulates Lac^+ reversion (adaptive mutation) with or without general hypermutability. Genetics **161:**945–956.
57. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry. W. H. Freeman, San Francisco, Calif.
58. **Spiers, A. J., A. Buckling, and P. B. Rainey.** 2000. The causes of *Pseudomonas* diversity. Microbiology **146:**2345–2350.
59. **Sundin, G. W., S. P. Kidambi, M. Ullrich, and C. L. Bender.** 1996. Resistance to ultraviolet light in *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rulAB* genes. Gene **177:**77–81.
60. **Sung, H.-M., and R. E. Yasbin.** 2002. Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in *Bacillus subtilis.* J. Bacteriol. **184:**5641–5653.
61. **Sung, H.-M., G. Yeamans, C. A. Ross, and R. E. Yasbin.** 2003. Roles of YgjH and YgjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis.* J. Bacteriol. **185:**2153–2160.
62. **Taddei, F., I. Matic, and M. Radman.** 1995. cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. Proc. Natl. Acad. Sci. USA **92:**11736–11740.
63. **Tenaillon, O., F. Taddei, M. Radman, and I. Matic.** 2001. Second-order selection in bacterial evolution: selection acting on mutation and recombination rates in the course of adaptation. Res. Microbiol. **152:**11–16.
64. **Tover, A., E.-L. Ojangu, and M. Kivisaar.** 2001. Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida.* Microbiology **147:**2149–2156.
65. **Tsui, H.-C. T., G. Feng, and M. E. Winkler.** 1997. Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. J. Bacteriol. **179:**7476–7487.
66. **Venturi, V.** 2003. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol. Microbiol. **49:**1–9.
67. **Wagner, J., and T. Nohmi.** 2000. *Escherichia coli* DNA polymerase IV mutator activity: genetic requirements and mutational specificity. J. Bacteriol. **182:**4587–4595.
68. **Wagner, J., H. Etienne, R. Janel-Bintz, and R. P. Fuchs.** 2002. Genetics of mutagenesis in *E. coli*: various combinations of translesion polymerases (Pol II, IV and V) deal with lesion/sequence context diversity. DNA Repair **1:**159–167.
69. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli.* Microbiol. Rev. **48:**60–93.
70. **Yeiser, B., E. D. Pepper, M. F. Goodman, and S. E. Finkel.** 2002. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proc. Natl. Acad. Sci. USA **99:**8737–8741.

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Brief report

Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida*

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ABSTRACT

One of the popular ideas is that decline in methyl-directed mismatch repair (MMR) in carbon-starved bacteria might facilitate occurrence of stationary-phase mutations. We compared the frequency of accumulation of stationary-phase mutations in carbon-starved *Pseudomonas putida* wild-type and MMR-defective strains and found that knockout of MMR system increased significantly emergence of base substitutions in starving *P. putida*. At the same time, the appearance of 1-bp deletion mutations was less affected by MMR in this bacterium. The spectrum of base substitution mutations which occurred in starving populations of *P. putida* wild-type strain was distinct from mutation spectrum identified in MMR-defective strains. The spectrum of base substitutions differed also in this case when mutants emerged in starved populations of MutS or MutL-defective strains were comparatively analyzed. Based on our results we suppose that other mechanisms than malfunctioning of MMR system in resting cells might be considered to explain the accumulation of stationary-phase mutations in *P. putida*. To further characterize populations of *P. putida* starved on selective plates, we stained bacteria with LIVE/DEAD kit in situ on agar plates. We found that although the overall number of colony forming units (CFU) did not decline in long-term-starved populations, these populations were very heterogeneous on the plates and contained many dead cells. Our results imply that slow growth of subpopulation of cells at the expenses of dead cells on selective plates might be important for the generation of stationary-phase mutations in *P. putida*. Additionally, the different survival patterns of *P. putida* on the same selective plates hint that competitive interactions taking place under conditions of prolonged starvation of microbial populations on semi-solid surfaces might be more complicated than previously assumed.

1. Introduction

Due to intense competition for nutrients, bacteria spend the majority of their lives under starvation conditions. Evolution occurs very rapidly in starved populations and takes advantage of different types of mutations. Apparently

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static microbial populations under the nonlethal selective pressure accumulate mutations called as adaptive mutations or stationary-phase mutations (reviewed in Refs. [1,2]). In addition to the appearance of mutants able to utilize novel growth substrates, starvation selects mutants with enhanced abilities to scavenge amino acids released from dead cells in bacterial populations (e.g. [3,4]). Cells with growth advantage in stationary-phase (GASP) phenotype either co-exist with the parental majority or displace the parent [3,5]. Foster [1] has pointed out that the rate of mutation under starvation conditions is too high to be accounted for by the amount of DNA synthesis if it is assumed that DNA synthesis in stationary-phase cells involves the whole genome and has an overall error rate similar to that of DNA replication in growing cells. However, some published reports [6,7] have implied that there was considerably more DNA synthesis under the starvation conditions than might have been assumed.

Starvation conditions encountered during stationary-phase incubation may permit a transient increase in the rate of mutation. Transient mutability induced by starvation has been shown in most natural isolates of *Escherichia coli* [8]. Also, results by Loewe et al. [9] suggest a correlation between deleterious mutation rate and the time bacteria spend in the stationary-phase. It is known, for example, that DNA damage, such as the formation of the 7,8-dihydro-8-oxoguanine (GO) can give rise to stationary-phase mutations [6,7,10]. Additionally, error-prone DNA polymerases including the members of DinB/UmuDC superfamily are implicated in stationary-phase mutations [11–15]. It is also possible that methyl-directed mismatch repair (MMR) might be disabled transiently during stationary-phase mutagenesis. This idea, although it has met controversy [16,17], was initially supported by the finding that the mutation spectrum observed in Lac$^+$ revertants emerging in a starving *E. coli* population was reproduced by MMR deficiency in growing cells [18]. Subsequent studies [19,20] demonstrated that MutS and MutH decline to levels appropriate for decreased DNA synthesis in stationary-phase *E. coli*, whereas functional MutL is limiting for MMR specifically during stationary-phase [20]. Some other studies, originally proposed by Schaaper and Radman [21], suggest that MMR deficiency is caused by saturation of the MMR system with an excess of DNA replication errors.

The genus *Pseudomonas* represents one of the largest groups of bacteria including both pathogenic and non-pathogenic species. Bacteria from the genus *Pseudomonas* are known for their ability to colonize multiple habitats and to adapt rapidly to new environments. The results of our recently published studies of stationary-phase mutagenesis in *Pseudomonas putida* suggest that mutation processes in cells that have been starved for a short period are not entirely compatible with those from a prolonged starvation [15,22]. In the current study we have investigated whether the mechanisms of occurrence of stationary-phase mutations in populations of long-term-starved *P. putida* could be explained by malfunctioning of MMR in this bacterium under starvation conditions. Our results indicated that MMR is not entirely disabled in starving *P. putida*. Specifically, we found that the frequency of accumulation of base substitution mutations was higher in MMR-deficient *P. putida* compared to the wild-type during the 2-week starvation period studied. Moreover, the spectrum of base substitutions which occurred in starved *P. putida* wild-type strain was distinct from that identified in MMR-defective strains. Characterization of populations of *P. putida* starved on selective plates by staining bacteria with LIVE/DEAD kit in situ on agar plates revealed that the ratio of living to dead cells varied regionally on the same plate whereas the proportion of dead cells increased with time of starvation. Hence, we suppose that the death of a subpopulation of cells under carbon starvation conditions allows slow growth of the other cells and this might be necessary for DNA replication and generation of mutations in long-term-starved populations of *P. putida*. Involvement of different stress-induced DNA polymerases in stationary-phase mutagenesis in *P. putida* will be also discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are described in Table 1. Complete medium was Luria–Bertani (LB) medium [23]. M9 minimal medium [24] was supplemented with solution of trace salts [25] at final concentration 2.5 ml/l. The content of this solution was following: 10.75 g MgO, 2 g $CaCO_3$, 4.5 g $FeSO_4{\cdot}7H_2O$, 1.44 g $ZnSO_4{\cdot}7H_2O$, 1.12 g $MnSO_4{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.28 g $CoSO_4{\cdot}7H_2O$, and 0.06 g H_3BO_4 dissolved in 1 l water, supplemented by 51.3 ml concentrated HCl. Solid medium contained 1.5% Difco agar. Casamino acids (CAA) and glucose were added to the minimal medium at final concentrations 0.4% and 0.2%, respectively. Phenol minimal plates contained 2.5 mM phenol as a sole carbon and energy source. Antibiotics were added at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml; tetracycline at 80 μg/ml; carbenicillin at 1000–3000 μg/ml and rifampin at 100 μg/ml. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. *E. coli* was transformed with plasmid DNA as described by Hanahan [26]. *P. putida* was electrotransformed as described by Sharma and Schimke [27]. *E. coli* strain TG1 [28] or DH5α (Invitrogen) was used for the DNA cloning procedures.

2.2. Construction of P. putida DNA mismatch repair-deficient strains

The part of the *mutS* gene of *P. putida* strain KT2440 lacking 796 nucleotides from 5′ end of the original 2571 nucleotide-long *mutS* (PP1626) was obtained from cosmid pMIR13415 [29]. The 5-kb BamHI-ClaI DNA fragment containing sequence of the *mutS* gene was cloned from cosmid pMIR13415 into the BamHI- and EcoRV-cleaved pBluescript SK (+) to obtain pSKrpoSmutS. The ClaI ends were blunt ended before the ligation. Then we deleted approximately 2.5-kb ClaI-EcoRI DNA fragment containing DNA sequences locating downstream of the *mutS* gene in pSKrpoSmutS to obtain pSKmutS. To interrupt the *mutS* sequence with antibiotic resistance gene, the Eco47III-cleaved DNA fragment containing the Km^r gene from pUTmini-Tn5 Km2 [30] was inserted into the SmaI-cleaved pSKmutS. The XbaI- and HincII-generated DNA fragment from pSKmutS::km was subsequently cloned into plasmid pGP704 L [31] by using XbaI and Ecl136II sites.

Table 1 – Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype or construction	Source or reference
Strains		
E. coli		
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	[28]
DH5α	*supE44* Δ*lacU169*(f80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1* λpir phage lysogen	[32]
P. putida		
PaW85	Wild type	[33]
PaWMutS	*mutS*::*km*	This study
PaWMutL	Δ*mutL*::*km*	This study
Plasmids		
pBluescript KS (+)	Cloning vector (Ap^r)	Stratagene
pBluescript SK (+)	Cloning vector (Ap^r)	Stratagene
pUTmini-Tn5 Km2	Delivery plasmid for mini-Tn5 Km2 (Ap^r Km^r)	[30]
pGP704 L	Delivery plasmid for homologous recombination (Ap^r)	[31]
pRK2013	Helper plasmid for conjugal transfer of pGP704 L (Km^r)	[34]
pMIR13415	Cosmid carrying partial sequence of the *mutS* gene from *P. putida*	[29]
pSKrpoSmutS	pBluescript SK (+) containing BamHI-ClaI fragment from pMIR13415 cloned into BamHI-EcoRV-cleaved vector	This study
pSKmutS	pSKrpoSmutS with deletion of 2.5-kb ClaI-EcoRI fragment	This study
pSKmutS::km	*mutS* in pSKmutS is interrupted with Km^r gene from pUTmini-Tn5 Km2	This study
pGP704mutS::km	pGP704 L with XbaI-HincII fragment of *mutS*::*km* from pSKmutS::km in XbaI-Ecl136II-cleaved vector	This study
pSKmutLlopp	pBluescript SK (+) containing PCR-amplified *mutL* DNA region encoding C-terminal part of MutL inserted as EcoRV-XhoI fragment	This study
pSKmutL	pSKmutLlopp containing PCR-amplified *mutL* DNA region encoding N-terminal part of MutL inserted as AviII-XhoI fragment in XhoI site	This study
pSKΔmutL::km	*mutL* in pSKmutL is interrupted with Km^r gene from pUTmini-Tn5 Km2 by replacing AatII-Bsp119I-generated fragment from *mutL* with Km^r gene	This study
pGP704ΔmutL::km	pGP704 L with XbaI-Bpu1102I fragment of Δ*mutL*::*km* from pSKΔmutL::km in EcoRV-cleaved vector	This study
pKTpheA56+A	Test system for detection of Phe$^+$ revertants occurring due to 1-bp deletions	[15]
pKTpheA22TGA	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTpheA22TAA	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTpheA22TAG	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTluxAB	pKT240-derivative carrying *luxAB* genes (Ap^r)	[15]

Plasmid pGP704mutS::km was selected in *E. coli* strain CC118 λpir [32]. The interrupted *mutS* gene was inserted into the chromosome of *P. putida* PaW85 [33] by homologous recombination. Plasmid pGP704mutS::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013 [34]. The PaW85 *mutS*::*km* knockout strain PaWMutS was verified by PCR analysis using primers PpmutSNdeI (5′-CATATGTCAGATCTTTCCGCACAC-3′), complementary to the sequences −3 to +21 relatively to the ATG initiator codon of the *mutS* gene, and KmOc (5′-TCGAGCAAGACGTTTCCC-3′), complementary to the sequences 34–16 nucleotides downstream of the ATG initiator codon of Km^r gene, respectively. Additionally, we could confirm that the spontaneous frequency of mutation to rifampin resistance was about 1000-fold higher in MutS-defective bacteria compared to the wild-type strain.

The *mutL* (PP4896) gene sequence of *P. putida* KT2440 was obtained from The Institute for Genomic Research website (http://www.tigr.org). The *mutL* gene was amplified by PCR from genomic DNA of *P. putida* PaW85, which is isogenic to *P. putida* strain KT2440. Four primers were used for the amplification of the *mutL* gene. Firstly, two primers, MutLloppRev (5′-GCCGGTGCGGGTGGGCAGCG-3′), complementary to the sequence +193 to +174 downstream of the TGA stop codon and MutLloppFW (5′-GGCACCTTGCACCGTGCCGTT-3′), complementary to the sequence +978 to +998 nucleotides downstream of the ATG initiator codon of the *mutL* gene, were used to amplify 1125-nucleotide DNA region of the 1896 nucleotide-long *mutL* gene. The PCR product was cleaved with EcoRV and XhoI enzymes generating 905-bp DNA fragment which was subsequently subcloned into EcoRV- and XhoI-cleaved pBluescript SK (+), to obtain pSKmutLlopp. The second half of the *mutL* gene was amplified using two primers, MutLalgusRev (5′-GTCGCCCTGGCTTTCGGGCAG-3′) complementary to the sequence +1313 to +1293 downstream of the ATG initiator codon and MutLalgusFW (5′-ACCGTACGCCCTGGCGAAACC-3′) complementary to the sequence −149 to −129 upstream of the ATG initiator codon of the *mutL* gene. The amplified 1500-bp DNA fragment containing the second half of the *mutL* gene was cleaved with AviII and XhoI, and the obtained 1212-bp DNA fragment was inserted into XhoI-cleaved pSKmutLlopp resulting in plasmid pSKmutL. The Km^r gene was amplified by PCR from plasmid pUTmini-Tn5 Km2 by using the primer KmSac [35]. The Ecl136II-cleaved DNA fragment containing the Km^r gene was used to replace the AatII- and Bsp119I-generated 880-bp fragment in the *mutL* gene in plasmid pSKmutL.

The resulting ΔmutL::km sequence from pSKΔmutL::km was inserted into EcoRV-cleaved plasmid pGP704 L by using XbaI and Bpu1102I sites. The XbaI and Bpu1102I ends were blunt ended before the ligation. Plasmid pGP704ΔmutL::km was selected in *E. coli* strain CC118 λpir. The interrupted *mutL* gene containing the internal deletion was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGP704ΔmutL::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013. The PaW85 ΔmutL::km knockout strain PaWMutL was verified by PCR analysis using primers KmOc and MutLalgusFW and by measuring the spontaneous frequency of mutation to rifampin resistance. Similarly to the PaWMutS, the frequency of mutation of Rif^r was increased about 1000-fold in PaWMutL compared to the wild-type strain.

2.3. Isolation and analysis of Phe^+ revertants

Test systems used for detection of different point mutations were the same as previously described [15]. When phenol monooxygenase *pheA*-expressing plasmid is introduced into phenol-nondegrading *P. putida* strain PaW85 lacking this gene in a chromosome, bacteria gain the ability to utilize phenol as a sole carbon source. Sequence of the reporter gene *pheA* encoding phenol monooxygenase was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation (plasmid pKTpheA56+A) or by introducing different translational stop codons into the same position (Leu-22) of the *pheA* sequence (plasmids pKTpheA22TAA, pKTpheA22TGA, or pKTpheA22TAG). Incubation of *P. putida* strain carrying any of these tester plasmids with mutated *pheA* sequence on minimal selective plates containing phenol as an only carbon source enables to isolate phenol-growing (Phe^+) revertants. Independent cultures of *P. putida* strains carrying different tester plasmids for the detection of Phe^+ revertants [15] were generated by growing cells to late logarithmic growth phase in M9 medium containing glucose and CAA, diluting this culture by 10^5 into fresh glucose and CAA-containing M9 medium, dispensing 2-ml aliquots into test tubes and allowing cells to reach saturation by growing cells for 18–20 h. Cells sampled from the culture were harvested by centrifugation and washed in M9 solution. Approximately 5×10^8 cells of *P. putida* wild-type and 2×10^8 cells of MMR-defective strains with test system measuring frameshift mutations, or 2×10^7 cells of MMR-defective strains carrying test systems allowing detection of base substitution mutations were spread onto phenol minimal plates containing 1500 μg/ml carbenicillin. When fewer amounts of cells of the tester strains were plated onto selective plates, they were plated with approximately 5×10^8 scavenger cells. Scavenger cells (*P. putida* PaW85 carrying plasmid pKTluxAB [15]) were grown to saturation in M9 minimal medium containing glucose and CAA. In order to avoid inhibition of accumulation of mutants caused by the presence of earlier-emerged mutant colonies on selective plates [22], all counted colonies were removed, as soon as they appeared, by cutting them off on small agar plugs. We have previously shown [15,36] that Phe^+ colonies appearing on phenol minimal plates on day 2 contained mutations that occurred before the plating in a growing culture, whereas colonies that emerged on selective plates on day 3 and later contained mutations that occurred after the cells were plated. The latter were called as stationary-phase mutations. Based on our previous studies [15,36] we can confirm that the copy number of the tester plasmids is not affected by the growth phase of the bacteria. Here, we addressed the same question by comparing the number of the plasmid copies in MMR-defective and wild-type strains but no differences were found.

In Phe^+ mutants, an approximately 350-bp DNA region covering the area of the *pheA* gene containing potential reversion mutations was analyzed by DNA sequencing as described previously [15]. The DNA sequencing reactions were analyzed with an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

2.4. Measurement of number of colony forming units (CFU) in starving *P. putida* cultures incubated on phenol minimal plates

In order to control whether bacteria would die during starvation on the selective medium, we measured the number of colony forming units (CFU) for at least five independent starving cultures of wild-type or MMR-defective strains. The viability of bacteria was determined on the same plates that were used for the isolation of Phe^+ revertants. Using sterile 1-ml pipette tips, small plugs were cut out from the phenol-containing minimal plates avoiding Phe^+ colonies. Bacteria from the plugs were suspended in M9 solution by shaking. Thereafter, appropriate dilutions were made and plated onto LB plates to determine the number of CFU. The similar results were obtained despite CFU determinations were performed by plating bacteria onto LB or M9 minimal glucose medium in the presence or absence of carbenicillin. As the resistance to carbenicillin is provided by plasmids carrying the test systems for detection of point mutations, these results confirm that the tester plasmids are stably maintained in cells survived in *P. putida* starving populations.

2.5. Cell staining and microscopic examination of *P. putida* starved populations on phenol minimal plates

We cut approximately 1 cm^2 agar pieces containing starved *P. putida* cells from phenol-containing selective plates and placed these pieces onto a glass slide. Thereafter, we added 10 μl of diluted LIVE/DEAD *Bac*Light 7012 kit (Molecular Probes, Inc., Eugene, OR, USA) stain mixture of SYTO 9 and propidium iodide at final concentrations of 10 and 60 μM, respectively, onto the surface of the agar containing the cell lawn and covered gently with coverslip. The samples were incubated with stain mixture in the dark for 10 min at room temperature. Stained cell populations were observed under an Olympus BX 41 epifluorescent microscope equipped with a mercury lamp and with the appropriate filters. The samples were excited at 470 nm allowing simultaneous viewing red and green fluorescent cells. Green fluorescent cells were considered to be alive; other cells were considered to be dead [37]. In all cases, a 100× objective was used with immersion oil, giving a total magnification of 1000×. Images were captured with an Olympus U-TV0.5XC camera and were processed with DP Controller 1.2.1. 108 and DP Manager 1.2.1. 107. A minimum of 20 fields selected at random were examined on each sample.

3. Results and discussion

3.1. *Mismatch repair is involved in avoidance of stationary-phase mutations both in growing and starving populations of P. putida*

One of the widely disputed ideas is that MMR is active and essential for mutation avoidance in growing bacteria but its malfunctioning in starving cells might facilitate occurrence of stationary-phase mutations [16,17]. Here, we studied whether the occurrence of stationary-phase mutations in *P. putida* could be explained by malfunctioning of MMR during prolonged starvation of bacteria. For that purpose, we constructed *P. putida* MMR-defective strains PaWMutS and PaWMutL either defective in MutS or MutL and compared the frequency of occurrence of mutants in these strains with that in the wild-type strain PaW85. The test systems used for the selection of mutants measured appearance of phenol-degrading (Phe^+) revertants as previously described [15]. No differences were observed in viability between the wild-type strain PaW85 and MMR-deficient strains PaWMutS and PaWMutL even during prolonged incubation of bacteria under starvation conditions, and the number of CFU did not decline in starving populations (Fig. 1A).

The results presented in Fig. 1B show the accumulation of the Phe^+ revertants which occurred in starving populations of *P. putida* wild-type strain and MMR-defective strains due to base substitution mutations eliminating TAG stop codon. The similar accumulation curves appeared when we used two other assay systems detecting changes removing either TAA or TGA stop codon (data not shown). Base substitution mutants accumulated in *P. putida* wild-type strain at well-detectable level already at the beginning of starvation and the frequency of appearance of these revertants remained constant during the 2-week-starvation period studied. The similar accumulation dynamics of the base substitution mutants became evident also with MMR-defective strains PaWMutS and PaWMutL (Fig. 1B). At the same time, the frequency of accumulation of base substitution mutants was approximately 25 times elevated in starved populations of PaWMutS and PaWMutL strains compared to the wild-type strain throughout all starvation period studied (Fig. 1B). This indicated that the efficiency of MMR in repairing base substitutions did not decline with time of starvation of *P. putida*.

We have previously shown that the frequency of accumulation of 1-bp deletion mutants significantly increased in long-term-starved *P. putida* populations [15]. The results presented in Fig. 1C demonstrated that during the first 7 days of carbon starvation, the absence of MutL or MutS in *P. putida* increased the frequency of accumulation of 1-bp deletants approximately four and seven times, respectively, compared to the wild-type populations. Similarly to the wild-type strain, the frequency of appearance of the Phe^+ revertants increased significantly with time of starvation in MMR-defective strains as well. For example, during the shorter period of starvation (days 3–7) in average 0.44 deletion mutants appeared per day per 5×10^8 cells of MutS-defective strain, whereas on longer starvation period of PaWMutS (days 11–15) the frequency of accumulation of the revertants was elevated 10 times (in average 4.2 mutants per day per 5×10^8 cells). The remarkable increase in the rate of the accumulation of the revertants (about 15-fold) appeared also in this case when we used PaWMutL. At the same time, these results demonstrated that the lack of the MMR system has less influence upon rate of the occurrence of 1-bp deletion mutants compared to the emergence of base substitution mutants in starving *P. putida* populations (compare Fig. 1B and C). Moreover, if we compared

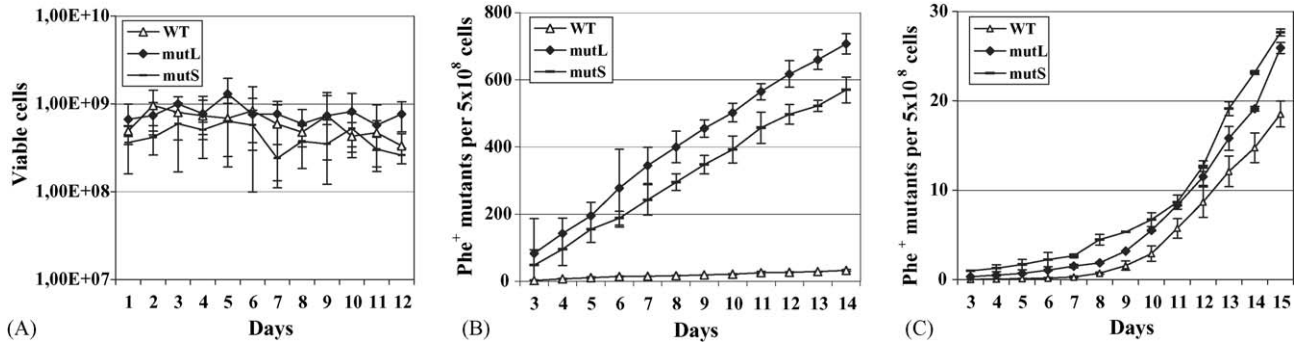

Fig. 1 – (A) Viability of *P. putida* wild-type strain PaW85 (WT), and its MMR-defective derivatives PaWMutS (mutS) and PaWMutL (mutL) on phenol minimal plates. Mean ± standard deviation (error bars) for at least five independent determinations are shown. 1.0E + 08, for example, indicates 10^8 viable cells. (B) Accumulation of Phe^+ revertants on phenol minimal plates in *P. putida* wild-type strain PaW85, and in its MMR-defective derivatives PaWMutS and PaWMutL carrying the tester plasmid pKTpheA22TAG which measures elimination of TAG stop codon by the base substitutions within the *pheA* gene. About 5×10^8 *P. putida* wild-type cells or 2×10^7 MMR-defective cells with 5×10^8 scavenger cells (PaW85 carrying pKTluxAB) were plated from overnight in liquid M9 medium-grown independent cultures onto phenol minimal plates. Such amounts of tester plasmid-carrying cells produced no more than two colonies per plate per day in average. (C) Accumulation of Phe^+ mutants on phenol minimal plates in *P. putida* wild-type strain PaW85, and in its MMR-defective derivatives carrying the tester plasmid pKTpheA56+A which measures reversion of +1 frameshift within the *pheA* gene. About 5×10^8 *P. putida* wild-type cells or 2×10^8 MMR-defective cells with 5×10^8 scavenger cells (PaW85 carrying pKTluxAB) were plated from overnight in liquid M9 medium-grown independent cultures onto phenol minimal plates. In all cases, mean ± standard deviations (error bars) for at least 10 plates calculated per 5×10^8 are shown.

the frequency of accumulation of 1-bp deletion mutations in the MMR-defective strains to that in the wild-type on day 10 and later, we did not detect remarkable differences at all. It is possible that different types of errors are corrected by MMR with different efficiency in starving *P. putida*. Also, our previous results demonstrated that *dinB* (PP1203)-encoded Pol IV homologue is required for most 1-bp deletions detected in long-term-starved populations of *P. putida* [15]. As the frequency of accumulation of the revertants increased with time of starvation also in MMR-defective strains, this data implied that the increased accumulation rate of the DinB-dependent mutations in the long-term-starved *P. putida* wild-type populations might be (at least partially) ascribed to increase in the activity and/or amount of DinB. At the same time, some studies, originally proposed by Schaaper and Radman [21], suggest that MMR deficiency is caused by saturation of the MMR system with an excess of DNA replication errors. Indeed, the results by Wagner and Nohmi [38] have demonstrated saturation of the MMR system as a result of accumulation of errors made by overproduction of Pol IV in *E. coli*. The spectrum of stationary-phase mutations of Lac$^+$ revertants in *E. coli* FC40 system which also measures frameshift mutations resembled mutations that appear in MMR-deficient cells [18], whereas Pol IV was required specifically for 1-bp deletions in stationary-phase cells but not in growing cells [12,13]. Hence, drawing parallels with these reports, and comparing the results presented herein we cannot exclude the possibility that the appearance of 1-bp deletion mutants in long-term-starved *P. putida* wild-type strain is in some extent facilitated by partial titration of MMR due to increase in DinB-caused replication errors in starving *P. putida*.

3.2. *Spectrum of base substitution mutations is affected by growth phase of P. putida and the presence of DNA repair functions in bacteria*

In order to study whether and how the spectrum of base substitution mutations could be affected by the activity of MMR enzymes MutS and MutL in *P. putida* and whether the growth phase of bacteria could influence the frequency of occurrence of different nucleotide changes, we analyzed the phenol monooxygenase gene *pheA* sequence of the Phe$^+$ revertants either collected from growing or stationary-phase populations of the wild-type and MMR-defective strains. The results of the DNA sequence analysis of the Phe$^+$ mutants which occurred due to elimination of TAG, TAA or TGA stop codons are summarized in Table 2. The T-to-C transition was the most prominent change in all cases studied. However, the spectra of mutations which eliminated either TGA or TAG stop codon in the *pheA* gene were more heterogeneous than the spectrum of substitutions which resulted in elimination of TAA codon. Although the usage of TAA codon did not allow detection of A-to-G transitions due to the generation of other stop codons TGA or TAG, we did not notice other changes (e.g., substitutions T-to-A; A-to-C; A-to-T) that potentially could also eliminate this stop codon.

The spectra of base substitutions identified among revertants isolated in growing cultures were distinct from those characterized for mutants accumulated in starving populations. These spectra were also dependent on the sequence of the stop codon used in the particular assay system. In the wild-type strain, the mutants isolated in growing cultures revealed the most heterogeneous spectrum of changes with the test system which measured the elimination of TGA stop codon. At the same time, if bacteria were starved, the TAG codon was eliminated with wider spectrum of mutations than the elimination of TGA or TAA codons.

The spectra of the base substitutions identified in starving populations of the wild-type strain were distinct from those characterized in MMR-defective strains. Presumably, if the MMR system is non-functional in starving bacteria, then the spectrum of mutations described in the starving wild-type strain should resemble spectrum observed in the MMR-defective strains. We can confirm that the mutation spectra characterized in the wild-type and MMR-defective strains differed from each other also in this case if mutants emerged in more than 1-week-starved populations were analyzed separately from the earlier arisen mutants (data not shown). Thus, the results of the analysis of spectra of base substitution mutations in different genetic backgrounds support the idea that the MMR system is functional at least during 2-week carbon starvation period in stationary-phase *P. putida*. Both types of base substitutions, transversions and transitions, were represented among the Phe$^+$ revertants which were isolated in the wild-type *P. putida* populations. However, the revertants which were collected in populations of MMR-defective strains contained mostly two types of transitions, T-to-C or A-to-G. Thus, similarly to *E. coli* MMR system [39], the MMR pathway in *P. putida* preferentially avoids transition mutations.

Surprisingly, spectrum of mutations which restored the functional *pheA* sequence in MMR-defective bacteria was also dependent on which component of MMR was inactivated. This difference appeared only in this case when we characterized stationary-phase mutations. The dissimilarity was the most significant between the spectra of mutations which eliminated the TAG stop codon in the *pheA* coding sequence (Table 2). In the MutS-defective strain the proportion of the A-to-G transitions increased from 18% in growing cells to 70% in starved populations, but the change was less significant when the revertants collected in the MutL defective strain were analyzed (14% A-to-G transitions in growing cells versus 33% in starved bacteria). One possible explanation of this phenomenon is that the absence of MutS or MutL in cells might influence differently DNA replication. Data from the literature indicate that different DNA polymerases may create different spectra of mutations within the same DNA sequence [40]. In *E. coli* it has been shown that the β processivity clamp of the replicative DNA polymerase Pol III communicates directly with multiple proteins to promote DNA replication and DNA repair [41]. Besides interacting with different DNA polymerases, *E. coli* β processivity clamp binds the MMR protein MutS and ligase [42]. The finding that the clamp loader and all the polymerases present in *E. coli* interact with the β clamp at the same location has suggested that clamp binding may be competitive and regulated [43]. A pentapeptide motif QL[SD]LF which is sufficient to enable interaction of these proteins with an archetypical *E. coli* β clamp is present in most sequenced members of eubacterial DNA polymerases and MutS proteins [44]. As *P. putida* MutS protein also contains this motif, it is very likely that the MutS protein interacts with β clamp in this organism. Thus, one may

Table 2 – Reversion of different nonsense mutations (TGA, TAA, TAG) in Phe+ mutants accumulating in *P. putida* wild-type strain and its MutS- and MutL-defective derivatives[a]

Target[b]	DNA change	Occurrences						Reversion	Amino acid change
		Wild type growing cells	Starving cells	*mutS* Growing cells	Starving cells	*mutL* Growing cells	Starving cells		
TGA	T → C	38 (69%)	13 (54%)	65(92%)	117 (76%)	25 (93%)	82 (81%)	CGA	Arg
	T → G	3 (5.5%)	5 (21%)	0	0	0	3 (3%)	GGA	Gly
	T → A	1 (2%)	0	0	0	0	1 (1%)	AGA	Arg
	G → C	4 (7%)	0	0	0	0	0	TCA	Ser
	G → T	3 (5.5%)	4 (17%)	0	0	0	0	TTA	Leu
	A → C	4 (7%)	2 (8%)	3 (4%)	0	0	0	TGC	Cys
	A → G	0	0	3 (4%)	37 (24%)	2 (7%)	15 (15%)	TGG	Trp
	A → T	2 (4%)	0	0	0	0	0	TGT	Cys
TAA	T → C	56 (100%)	38 (88%)	63 (100%)	144 (99%)	17 (100%)	114 (100%)	CAA	Gln
	T → G	0	5 (12%)	0	1 (1%)	0	0	GAA	Glu
TAG	T → C	48 (98%)	44 (61%)	60 (82%)	26 (28%)	18 (86%)	62 (67%)	CAG	Gln
	T → G	0	6 (8.5%)	0	0	0	0	GAG	Glu
	T → A	0	1 (1.5%)	0	0	0	0	AAG	Lys
	G → C	0	1 (1.5%)	0	0	0	0	TAC	Tyr
	G → T	0	8 (11%)	0	0	0	0	TAT	Tyr
	A → C	0	0	0	2 (2%)	0	0	TCG	Ser
	A → G	0	11 (15%)	13 (18%)	66 (70%)	3 (14%)	30 (33%)	TGG	Trp
	A → T	1 (2%)	1 (1.5%)	0	0	0	0	TTG	Leu

[a] The spectrum of base substitutions occurring in growing bacteria was derived from analysis of DNA sequence of Phe+ revertants appeared onto selective plates on day 2. Mutant colonies used for identification of stationary-phase mutations were picked up on days 3–12. Approximately 10–15 mutants were analyzed per each day. We did not notice remarkable changes in the spectrum of mutations in revertants either derived from earlier or later period of starvation (days 3–7 or days 8–12, respectively).

[b] In all cases the same codon, CTG for Leu 22, was altered in the wild-type *pheA* sequence.

hypothesize that the absence of MutS may somehow influence competitive interactions of different DNA polymerases with the β clamp.

In *E. coli*, the involvement of Pol V in stationary-phase mutagenesis has demonstrated in assaying reversion of base pair substitutions [11] but not in test systems that measure frameshift mutations [11-13]. Unlike *E. coli*, *Pseudomonas* species do not harbor chromosomally encoded Pol V. Instead, the genome of *P. putida* like the vast majority of recently sequenced bacterial genomes carries a second copy of the *dnaE* gene (PP3119) (http://www.tigr.org). The deduced amino acid sequence of this gene exhibits 30% identity to the deduced amino acid sequence of *dnaE* gene PP1606 encoding catalytic subunit of Pol III. Here we decided to name this *dnaE* homologue (PP3119) in *P. putida* as *dnaE2*. In the pathogen *Mycobacterium tuberculosis* DnaE1 is the replicative polymerase but DnaE2 mediates SOS mutagenesis and contributes to the emergence of drug resistance in vivo [45]. In *Caulobacter crescentus* an operon composed of two hypothetical genes and *dnaE2* is damage-inducible in a *recA*-dependent manner and is responsible for most DNA damage-induced mutations [46]. This widespread operon has been shown to be regulated by LexA also in *P. putida* [47]. Our preliminary results (not shown in this paper) have demonstrated that inactivation of *dnaE2* in MutS-defective strain resulted in reduction in the fraction of A-to-G transitions to the level similar to that observed in MutL-defective strain PaWMutL. Therefore, it is possible that DnaE2 might be involved in stationary-phase mutagenesis, this polymerase may create different spectrum of mutations than other DNA polymerase(s) and MutS may somehow suppress access of DnaE2 to the replication apparatus. Further studies are necessary to examine this intriguing possibility.

3.3. *Starving populations of P. putida are highly heterogeneous*

Our results presented above demonstrated that the frequency of accumulation of stationary-phase mutations was higher in MMR knockout strains PaWMutS and PaWMutL compared to the wild-type strain. According to the model that only a few cells in a stationary-phase population differentiate into a hypermutable state [48–51] one may argue that MMR becomes limiting only in a small subpopulation and therefore the overall frequency of mutations in starving wild-type population cannot be as high as in experiments performed with MMR-defective strains. However, the facts that the spectra of base substitution mutations characterized in starving populations of *P. putida* wild-type strain were distinct from those identified in the MMR-defective strains and that the frequency of frameshift mutations increased with time of starvation also in MMR-defective strains do not support this idea. Thus, the occurrence of stationary-phase mutations in *P. putida* cannot be explained simply by malfunctioning of MMR in long-term-starved bacteria.

To further characterize starving *P. putida* populations on phenol selective plates, we stained bacteria in situ on agar medium with LIVE/DEAD kit and visualized cells by epifluorescent microscopy using a 1000-fold magnification. The staining kit contains two nucleic acid stains and distinguishes live bacterial cells from dead by means of membrane integrity. The

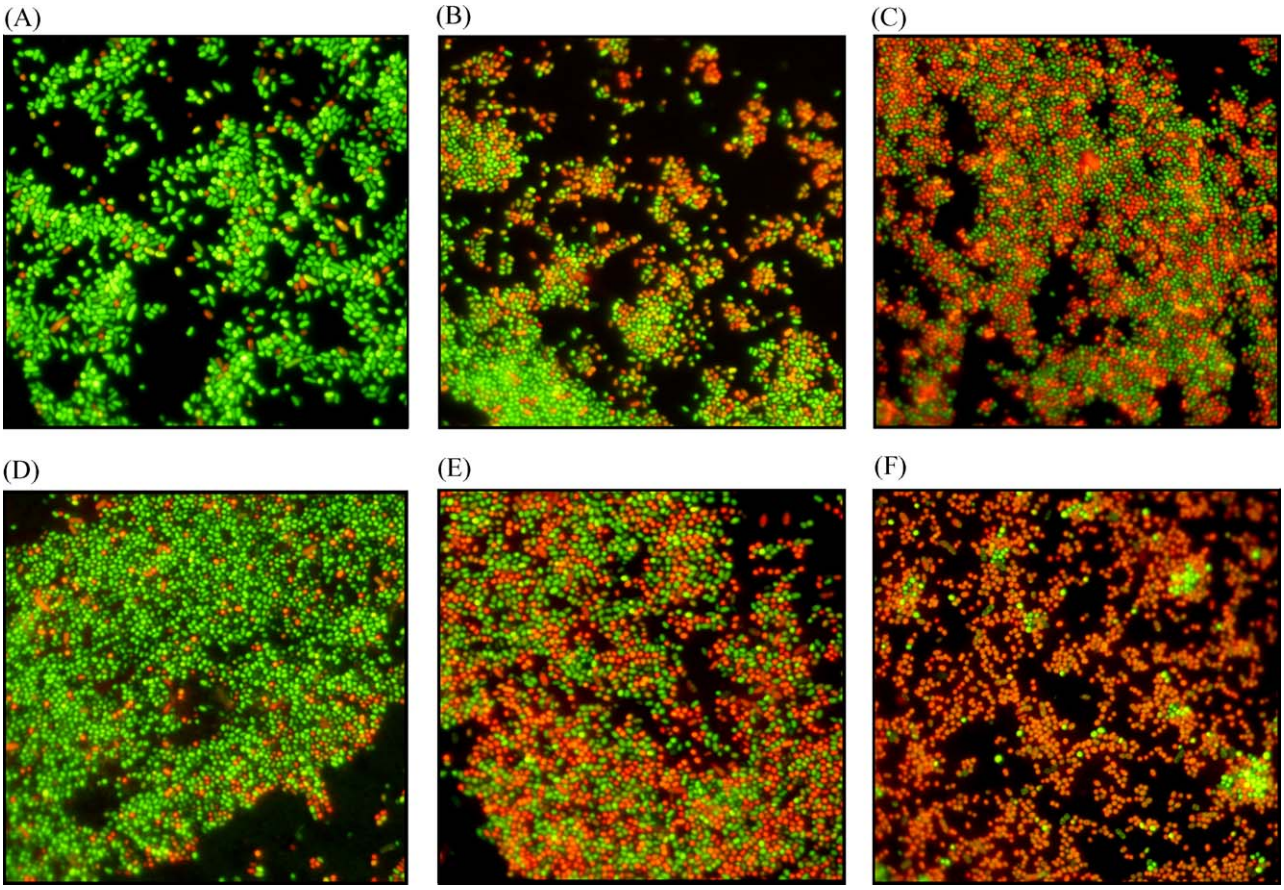

Fig. 2 – Characterization of *P. putida* populations on phenol minimal plates. Bacteria were stained with the LIVE/DEAD kit and visualized by epifluorescent microscopy using 1000-fold magnification. Samples are taken at different time points. (A) Cells immediately after plating; (B) and (C) 1-day-starved populations; (D), (E) and (F) 1-week-starved populations.

green fluorochrome (SYTO 9) is a small molecule that can penetrate intact plasma membranes while the larger red fluorochrome propidium iodide penetrates only compromised membranes. Bacterial suspensions incubated in the two stains simultaneously and then excited at 470 nm contain red and green fluorescent cells, depending on whether the bacteria are dead or live [37]. The most representative images of *P. putida* populations stained in situ on selective plates with LIVE/DEAD kit at different stages of starvation are shown in Fig. 2. Fig. 2A shows the image of *P. putida* wild-type cells that were stained directly after plating of 5×10^8 cells from stationary-phase liquid cultures onto phenol minimal plates. In this case, the majority of cells were green-colored. However, a small fraction of the cells was colored red which indicated that the plated bacterial populations contained some dead cells as well. During the next days of incubation of bacteria on selective plates the starving populations became significantly heterogeneous. The differences appeared already in this case when we examined 1-day-starved populations. The proportion of red-colored cells was increased in all studied fields. However, some fields contained more red-colored cells than the others (Fig. 2B and C) which indicated that cells incubated at the same selective plate may reside in different microenvironments. Visualization of *P. putida* populations starved for 1 week on phenol-containing minimal agar plates revealed the presence of many red-colored cells in populations. However, the ratio of green-to red-colored cells varied regionally on the same plate (Fig. 2D–F). Based on such variation, three different sub-classes were distinguished. Fig. 2D represents fields that mostly contained green-colored cells and red-colored cells were in minority. Fig. 2F depicts an opposite situation where majority of cells were red-colored. An intermediate survival pattern is visualized in Fig. 2E. Screening at random at least 20–30 fields on the same sample revealed that these three sub-classes were almost equally represented. The similar heterogeneity appeared by visualization of *P. putida* populations when we stained 2-week-starved cells or when we examined starved populations of MMR-defective strains (data not shown). At the same time, as already noted above (Fig. 1A), the number of CFU did not decline in *P. putida* populations with time of starvation. This discrepancy is difficult to explain. One may speculate that just after the plating bacteria can multiply a few generations on selective plates but this remains undetected because a fraction of cells which are colored green at the beginning of starvation are metabolically active but unable to form colony. Later, with time of starvation membranes of these cells become more compromised, these cells die and are colored red. Further experiments are needed to evaluate this intriguing possibility. However, the presence of many dead cells in long-term-starved population implies that a fraction of

cells in *P. putida* starving populations can grow at the expenses of nutrients released from the dead ones. Hence, we suppose that slow growth of fraction of cells might be necessary for DNA replication and generation of mutations in long-term-starved populations of *P. putida*.

Populations of stationary-phase cells have been shown to be highly dynamic: waves of fitter mutants constantly arise and take over previous populations [52]. The growth advantage in stationary-phase (GASP) phenotype has been observed in many different bacterial species [4,52]. So far, the appearance of GASP mutants has been studied in homogeneous liquid media. However, in natural habitats bacteria are living in a structured environment: they usually grow as biofilms, organised communities of cells embedded in an extracellular polysaccharide matrix and attached to a surface [53]. Our results of in situ staining of bacteria on selective plates (Fig. 2) provide insight into the processes of development of heterogeneity in starving microbial populations on semi-solid surface. We have noticed unequal distribution of living and dying cells in the different regions on the same selective plate. This implies that in the structured environment (e.g., on agar plates), the dying of bacteria may not occur randomly but is stimulated under certain conditions provided by a local environment. It is also possible that the emergence of individual GASP mutants on semi-solid surface may locally affect living conditions of the remaining cells, leading to different survival patterns at territory of the whole population. Thus, competitive interactions taking place in microbial populations might be more complicated than are currently appreciated.

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REFERENCES

[1] P.L. Foster, Adaptive mutation: the uses of adversity, Annu. Rev. Microbiol. 47 (1993) 467–504.
[2] P.L. Foster, Mechanisms of stationary phase mutation: a decade of adaptive mutation, Annu. Rev. Genet. 33 (1999) 57–88.
[3] M.M. Zambrano, D.A. Siegele, M. Almiron, A. Tormo, R. Kolter, Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures, Science 259 (1993) 1757–1760.
[4] E.R. Zinser, R. Kolter, *Escherichia coli* evolution during stationary phase, Res. Microbiol. 155 (2004) 328–336.
[5] B. Yeiser, E.D. Pepper, M.F. Goodman, S.E. Finkel, SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 8737–8741.
[6] B.A. Bridges, Elevated mutation rate in *mutT* bacteria during starvation: evidence for DNA turnover? J. Bacteriol. 178 (1996) 2709–2711.
[7] B.A. Bridges, S. Ereira, DNA synthesis and viability of a *mutT* derivative of *Escherichia coli* WP2 under conditions of amino acid starvation and relation to stationary-phase (adaptive) mutation, J. Bacteriol. 180 (1998) 2906–2910.
[8] I. Bjedov, O. Tenaillon, B. Gérard, V. Souza, E. Denamur, M. Radman, F. Taddei, I. Matic, Stress-induced mutagenesis in bacteria, Science 300 (2003) 1404–1409.
[9] L. Loewe, V. Textor, S. Scherer, High deleterious genomic mutation rate in stationary phase of *Escherichia coli*, Science 302 (2003) 1558–1560.
[10] B.A. Bridges, Spontaneous mutation in stationary-phase *Escherichia coli* WP2 carrying various DNA repair alleles, Mutat. Res. 302 (1993) 173–176.
[11] S. Bhamre, B.B. Gadea, C.A. Koyama, S.J. White, R.G. Fowler, An aerobic *recA*, *umuC*-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli*, Mutat. Res. 473 (2001) 229–247.
[12] H.J. Bull, M.-J. Lombardo, S.M. Rosenberg, Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8334–8341.
[13] G.J. McKenzie, P.L. Lee, M.-J. Lombardo, P.J. Hastings, S.M. Rosenberg, SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification, Mol. Cell 7 (2001) 571–579.
[14] H.-M. Sung, G. Yeamans, C.A. Ross, R.E. Yasbin, Roles of YgjH and YgjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*, J. Bacteriol. 185 (2003) 2153–2160.
[15] R. Tegova, A. Tover, K. Tarassova, M. Tark, M. Kivisaar, Involvement of error-prone DNA polymerase pol IV on stationary phase mutagenesis in *Pseudomonas putida*, J. Bacteriol. 186 (2004) 2735–2744.
[16] P.L. Foster, Are adaptive mutations due to decline in mismatch repair? The evidence is lacking, Mutat. Res. 436 (1999) 179–184.
[17] R.S. Harris, G. Feng, K.J. Ross, R. Sidhu, C. Thulin, S. Longerich, S.K. Szigety, P.J. Hastings, M.E. Winkler, S.M. Rosenberg, Mismatch repair is diminished during stationary-phase mutation, Mutat. Res. 437 (1999) 51–60.
[18] S. Longerich, A.M. Galloway, R.S. Harris, C. Wong, S.M. Rosenberg, Adaptive mutation sequences reproduced by mismatch repair deficiency, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 12017–12020.
[19] G. Feng, H.-C.T. Tsui, M.E. Winkler, Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells, J. Bacteriol. 178 (1996) 2388–2396.
[20] R.S. Harris, G. Feng, K.J. Ross, R. Sidhu, C. Thulin, S. Longerich, S.K. Szigety, M.E. Winkler, S.M. Rosenberg, Mismatch repair protein MutL becomes limiting during stationary-phase mutation, Genes Dev. 11 (1997) 2426–2437.
[21] R.M. Schaaper, M. Radman, The extreme mutator effect of *Escherichia coli mutD5* results from saturation of mismatch repair by excessive DNA replication errors, EMBO J. 8 (1989) 3511–3516.
[22] S. Saumaa, A. Tover, L. Kasak, M. Kivisaar, Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and stationary-phase sigma factor RpoS, J. Bacteriol. 184 (2002) 6957–6965.
[23] J.H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[24] M.H. Adams, Bacteriophages, Interscience Publishers Inc., New York, 1959, pp. 445–447.
[25] T. Bauchop, S.R. Elsden, The growth of micro-organisms in relation to their energy supply, J. Gen. Microbiol. 23 (1960) 457–469.

[26] D. Hanahan, Studies on the transformation of *E. coli* with plasmids, J. Mol. Biol. 166 (1983) 577–580.
[27] R.C. Sharma, R.T. Schimke, Preparation of electro-competent *E. coli* using salt-free growth medium, BioTechniques 20 (1996) 42–44.
[28] P. Carter, H. Bedouelle, G. Winter, Improved oligonucleotide site-directed mutagenesis using M13 vectors, Nucl. Acids Res. 13 (1985) 4431–4443.
[29] M.I. Ramos-González, S. Molin, Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440, J. Bacteriol. 180 (1998) 3421–3431.
[30] V. de Lorenzo, M. Herrero, U. Jakubzik, K.N. Timmis, Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria, J. Bacteriol. 172 (1990) 6568–6572.
[31] H. Pavel, M. Forsman, V. Shingler, An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on para-substituted methylphenols, J. Bacteriol. 176 (1994) 7550–7557.
[32] M. Herrero, V. de Lorenzo, K.N. Timmis, Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria, J. Bacteriol. 172 (1990) 6557–6567.
[33] S.A. Bayley, C.J. Duggleby, M.J. Worsey, P.A. Williams, K.G. Hardy, P. Broda, Two modes of loss of the TOL function from *Pseudomonas putida* mt-2, Mol. Gen. Genet. 154 (1977) 203–204.
[34] D.H. Figurski, D.R. Helinski, Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 1648–1652.
[35] R. Hõrak, H. Ilves, P. Pruunsild, M. Kuljus, M. Kivisaar, The ColR-ColS two-component signal transduction system is involved in regulation of Tn4652 transposition in *Pseudomonas putida* under starvation conditions, Mol. Microbiol. 54 (2004) 795–807.
[36] L. Kasak, R. Hõrak, M. Kivisaar, Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3134–3139.
[37] R.P. Haugland, Assays for cells viability, proliferation and function, in: M.T.Z. Spence (Ed.), Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc., Eugene, 1996, pp. 365–398.
[38] J. Wagner, T. Nohmi, *Escherichia coli* DNA polymerase IV mutator activity: genetic requirements and mutational specificity, J. Bacteriol. 182 (2000) 4587–4595.
[39] R.M. Schaaper, Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*, J. Biol. Chem. 268 (1993) 23762–23765.
[40] E.C. Friedberg, R. Wagner, M. Radman, Specialized DNA polymerases, cellular survival, and the genesis of mutations, Science 296 (2002) 1627–1630.
[41] M.D. Sutton, G.C. Walker, Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8342–8349.
[42] F.J. López de Saro, M. O'Donnell, Interaction of the β sliding clamp with MutS, ligase, and DNA polymerase I, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8376–8380.
[43] F.J. López de Saro, R.E. Georgescu, M.F. Goodman, M. O'Donnell, Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair, EMBO J. 22 (2003) 6408–6418.
[44] B. Dalrymple, K. Kongsuwan, G. Wijffels, N.E. Dixon, P.A. Jennings, A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 11627–11632.
[45] H.I.M. Boshoff, M.B. Reed, C.E. Barry III, V. Mizrahi, DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*, Cell 113 (2003) 183–193.
[46] R.S. Galhardo, R.P. Rocha, M.V. Marques, C.F.M. Menck, An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*, Nucl. Acids Res. 33 (2005) 2603–2614.
[47] M. Abella, I. Erill, M. Jara, G. Mazón, S. Campoy, J. Barbé, Widespread distribution of a *lexA*-regulated DNA damage-inducible multiple gene cassette in the Proteobacteria phylum, Mol. Microbiol. 54 (2004) 212–222.
[48] B.G. Hall, Spontaneous point mutations that occur more often when they are advantageous than when they are neutral, Genetics 126 (1990) 5–16.
[49] B.A. Bridges, Hypermutation under stress, Nature 387 (1997) 557–558.
[50] P.L. Foster, Nonadaptive mutations occur on the F'episome during adaptive mutation conditions in *Escherichia coli*, J. Bacteriol. 179 (1997) 1550–1554.
[51] J. Torkelson, R.S. Harris, M.-J. Lombardo, J. Nagendran, C. Thulin, S.M. Rosenberg, Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation, EMBO J. 16 (1997) 3303–3311.
[52] S.E. Finkel, E.R. Zinser, R. Kolter, Long-term survival and evolution in the stationary phase, in: G. Storz, R. Hengge-Aronis (Eds.), Bacterial Stress Responses, American Society for Microbiology, Washington, DC, 2000, pp. 231–238.
[53] J.W. Costerton, Z. Lewandowski, D.E. Caldwell, D.R. Korber, H.M. Lappin-Scott, Microbial biofilms, Annu. Rev. Microbiol. 49 (1995) 711–745.