Optimierung von *Pseudomonas putida* für die Produktion niedermolekularer Verbindungen

Von der Fakultät für Energie-, Verfahrens- und Biotechnik (Fakultät 4) der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Es wurden von mir nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Übernommenes Gedankengut wurde von mir als solches kenntlich gemacht.

Nadja Graf

Inhaltsverzeichnis

Abkürzungsverzeichnis

Abkürzung	Bedeutung
3,4-trans-CHD	3,4-trans Cyclohexadiendiol
5-FC	5-Fluorcytosin
5-FU	5-Fluoruracil
ACAR	Aromatische Carboxylat-Reduktase
Am (*bla*)	Ampicillin (Resistenzgen)
Ap (*aac(3)IV*)	Apramycin (Resistenzgen)
BAC	*Bacterial Artificial Chromosome*
BMBF	Bundesministerium für Bildung und Forschung
bp / bps	Basenpaare / *basepairs*
CCR	*Carbon Catabolite Repression*
cfu	*colony forming units*
CoA	Coenzym A
Cyo	Cytochrom-*o*-Ubiquinol-Oxidase
Δ	Deletion
DAHP	3-Desoxy-D-arabinoheptulosonat-7-phosphat
DMSO	Dimethylsulfoxid
DNA	*Deoxyribonucleic Acid*
dNTP(s)	desoxy-Nukleotidtriphosphat(e)
dTMP	desoxy-Thymidinmonophosphat
dUMP	desoxy-Uracilmonophosphat
dUTP	desoxy-Uraciltriphosphat
Ech	Enoyl-CoA-Hydratase/Aldolase
$FADH_2$	Flavin-Adenin-Dinukleotid (reduziert)
Fcs	Feruloyl-CoA-Synthetase
IPTG	Isopropyl-β-D-thiogalactosid
kb	Kilobasen
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
Km (*neo*)	Kanamycin (Resistenzgen)
LB	*Lysogeny Broth*
M.U.	*Miller Units*
MEK	Methylethylketon
MHK	Minimale Hemmkonzentration
mRNA	*messenger-RNA*
NADH	Nicotinamidadenindinukleotid (reduziert)
nt	Nukleotide
Ntr	*Nitrogen regulatory*
PCR	*Polymerase Chain Reaction*

Abkürzung	Bedeutung
PEP	Phosphoenolpyruvat
pH	negativer dekadischer Logarithmus der Protonen-Konzentration
PP_i	Pyrophosphat
PPTase	Phosphopantetheinyl-Transferase
PQQ	Pyrrolochinolinchinon
PRPP	Phosphoribosylpyrophosphat
PTS	Phosphotransferasesystem
RNA	*Ribonucleic Acid*
rRNA	ribosomale *RNA*
scFv	*single chain fragment variables*
sRNA	*small RNA*
SynOMT	Catechol-*O*-Methyltransferase aus *Synechocystis* sp. PCC 6803
tRNA	*transfer-RNA*
TS	Thymidylat-Synthase
UDP	Uracildiphosphat
UMP	Uracilmonophosphat
UPRTase	Uracilphosphoribosyl-Transferase

Zusammenfassung

Das Gram-negative Bakterium *Pseudomonas putida* ist aufgrund seines ausgeprägten Stoffwechsels für aliphatische und aromatische Kohlenwasserstoffe in den letzten Jahren zu einem der wichtigsten Wirtsorganismen in der Biotechnologie geworden. Für die gezielte Manipulation seines Genoms stehen gentechnische Methoden zur Verfügung, die jedoch ineffektiv und nur schwer zu handhaben sind. Basierend auf der Toxizität des Antimetaboliten 5-Fluoruracil und der Wirkung der Uracil-phosphoribosyl-Transferase wurde ein neues, effizientes Gegenselektionssystem für die Einführung genetischer Modifikationen etabliert. Damit konnte der Metabolismus von *P. putida* hinsichtlich der Produktion industrierelevanter Substanzen, wie Vanillin und Glyoxylsäure, erfolgreich beeinflusst werden.

Unter Verwendung dieses Systems wurde der vollständig sequenzierte *P. putida* Stamm KT2440 für die Biotransformation von Ferulasäure zu Vanillin genetisch so modifiziert, dass mit ruhenden Zellen eine hohe Ausbeute und Produktivität erzielt werden konnte. Da die Deletion des Gens *vdh* für die Vanillin-Dehydrogenase nicht ausreichend war, um den Vanillin-Abbau zu verhindern, wurde mit dieser Mutante eine Transposonmutagenese durchgeführt. Hierbei führte die Inaktivierung des Gens *modA*, welches für einen Molybdat-Transporter codiert, zu einem Stamm, der nicht mehr auf Vanillin als alleinige Kohlenstoffquelle wachsen kann. Durch zusätzliche Integration des starken *tac* Promotors konnte die Expression der chromosomalen Gene für die Feruloyl-CoA-Synthetase (*fcs*) und die Enoyl-CoA-Hydratase/Aldolase (*ech*) und damit die Umsatzrate von Ferulasäure zu Vanillin deutlich gesteigert werden. Weitere Optimierungen bezüglich geringerer Nebenproduktbildung führten zu einer molaren Ausbeute von bis zu 86% in nur 3 h. Dies stellt die höchste Produktivität dar, die bisher mit *Pseudomonas* Systemen beschrieben wurde.

Des Weiteren wurden im Rahmen dieser Arbeit für eine andere Arbeitsgruppe weitere Stämme konstruiert, mit denen Vanillin *de novo* aus Glucose bzw. Protocatechuat produziert werden soll.

Um *P. putida* als Plattform für die Produktion von Glyoxylsäure aus Ethylenglycol zu etablieren, wurde der Metabolismus zweier *P. putida* Stämme, KT2440 und JM37,

untersucht. Hierfür wurden in KT2440 verschiedene Gene deletiert und die erhaltenen Mutanten an andere Arbeitsgruppen übergeben, die auf dieser Basis weitere Analysen durchführen konnten. Hierbei konnten neben den Schlüsselenzymen auch Unterschiede zwischen den untersuchten Stämmen festgestellt werden. Während in JM37 die Expression der Gene für die Tartronatsemialdehydsynthase (*gcl*), Malatsynthase (*glcB*) und Isocitratlyase (*aceA*) durch Glyoxylsäure bzw. Ethylenglycol induziert wird, zeigt sich in *P. putida* KT2440 nur eine Induktion von *aceA*. Beide Stämme verwenden jedoch die periplasmatischen, Pyrrolochinolinchinon-abhängigen Dehydrogenasen PedE und PedH für den ersten Oxidationsschritt von Ethylenglycol. Mithilfe der erhaltenen Deletionsstämme konnte so der Ethylenglycol-Stoffwechselweg in *P. putida* aufgezeigt werden.

Pseudomonas Stämme dienen neben *Escherichia coli* und *Bacillus subtilis* als Wirtsorganismen für die Produktion heterologer Proteine. Allerdings sind die bisher vorhandenen Expressionssysteme nicht so stark und gut reguliert wie in *E. coli*. Daher wurde ein neues, Methylethylketon-induzierbares Expressionssystem auf Basis der Promotorregion P_{mekA} des *mekAB* Operons aus *P. veronii* MEK700 für *P. putida* und *E. coli* etabliert und charakterisiert. Der Transkriptionsstart von P_{mekA} wurde bestimmt und dabei eine potentielle Stamm-Schleife-Struktur am 5'-Ende der mRNA entdeckt. Im Gegensatz zu etablierten Systemen zeigt das neue System Vorteile hinsichtlich einer sehr niedrigen Basalexpression und einer hohen Induktionsrate. P_{mekA} wird positiv durch MekR, einem Mitglied der AraC/XylS-Familie, reguliert. Die Expression unterliegt außerdem einer Katabolitrepression durch Glucose, wobei eine explizite Wirkung der globalen Regulatoren Crc und PtsN nicht beobachtet werden konnte. Da MekR nur in unlöslicher und damit inaktiver Form gewonnen werden konnte, wurde seine Bindestelle durch Sequenzanalysen näher charakterisiert. Durch den Vergleich mit bekannten Bindestellen anderer Mitglieder der AraC/XylS-Familie konnte der mutmaßliche Operator von MekR als eine Wiederholung der Sequenz $CACCN_5CTTCAA$ mit einer 6 bp langen Zwischenregion definiert werden. Modifikationen dieser Region bestätigten ihre Bedeutung für die Initiation der Transkription. Aufgrund der Lage des mutmaßlichen Operators, der mit der -35-Region überlappt, wird für P_{mekA} eine Aktivierung der Klasse II vermutet.

Summary

The Gram-negative bacterium *Pseudomonas putida* emerged as a 'working horse' for the biotechnological industry due to its distinctive metabolism for aliphatic and aromatic compounds and several other advantages. Genetic methods for the directed manipulation of its genome are available. However, many of them are inefficient and often not easy to handle. Therefore, a negative counterselection system for *P. putida* based on the uracil phosphoribosyltransferase and the sensitivity against the antimetabolite 5-fluorouracil was developed, which allowed efficient and successive genetic modifications of the *P. putida* genome. Having this genetic tool at hand, the metabolism of *P. putida* could be efficiently affected concerning the production of industry relevant metabolites such as vanillin and glyoxylic acid.

Using this counterselection system, *P. putida* strain KT2440 was genetically optimized to convert ferulic acid to vanillin using resting cells. Since the deletion of the vanillin dehydrogenase gene (*vdh*) was not sufficient to prevent vanillin degradation, a transposon mutagenesis was conducted with this strain. Inactivation of the *modA* gene coding for a molybdate transporter led to a strain incapable to grow on vanillin as sole carbon source. Biotransformation of ferulic acid to vanillin was further optimized by enhanced chromosomal expression of the structural genes for feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*). This was achieved by integration of the strong *tac* promoter system upstream of these genes. Further genetic engineering to reduce unwanted by-product formation led to high initial conversion rates and molar vanillin yields of up to 86% within just 3 h representing the highest specific productivity and molar vanillin yield gained with a *Pseudomonas* strain so far.

Additionally, several strains were constructed in the context of this work for approaches of a *de novo* synthesis of vanillin out of glucose and protocatechuic acid conducted by another working group.

In order to establish *P. putida* as a useful platform for the production of glyoxylic acid from ethylene glycol, the metabolism in the *P. putida* strains KT2440 and JM37 could be investigated after several genetic modifications with strain KT2440. These were

conducted in the context of this work using the developed negative counterselection method. The subsequent analyses of the strains, conducted by other working groups, revealed the key enzymes and differences between the two strains. In JM37 expression of the genes for tartronate semialdehyde synthase (*gcl*), malate synthase (*glcB*), and isocitrate lyase (*aceA*) is induced in the presence of ethylene glycol or glyoxylic acid, while in KT2440 only *aceA* expression is induced. Both strains use similar periplasmic dehydrogenases for the initial oxidation step of ethylene glycol, namely, the two redundant pyrroloquinoline quinone dependent enzymes PedE and PedH. From these results a new pathway for the metabolism of ethylene glycol in *P. putida* could be identified.

For the production of heterologous proteins, *P. putida* is regarded as an alternative host to *Escherichia coli* and *Bacillus subtilis*. However, expression systems are not as strong and well-regulated as in *E. coli*. Therefore, a new, methyl ethyl ketone (MEK)-inducible system based on the promoter region P_{mekA} of the MEK degradation operon of *P. veronii* MEK700 was established and characterized in *E. coli* and *P. putida*. The transcriptional start site of P_{mekA} was identified by primer extension, thereby revealing a potential stem-loop structure at the 5'-end of the mRNA. The system turned out advantageous over other established expression systems due to its extremely tight regulation accompanied by a three magnitudes fold increase of gene expression after treatment with MEK. It is positively regulated by MekR, a member of the AraC/XylS family of regulators, and is subject to carbon catabolite repression by glucose, which, however, could not be attributed to the single action of the global regulators Crc and PtsN. Since MekR was highly insoluble, its putative binding site was identified through sequence analysis. Taking known binding patterns of other members of the AraC/XylS family into consideration, the operator seems to be composed of a 15-bps tandem repeat ($CACCN_5CTTCAA$) separated by a 6-bps spacer region. Mutational modifications of this region confirmed its importance for transcriptional activation. As the -35 promoter element seems to be overlapped by the putative operator, a class II activation mechanism is assumed.

1 Einleitung

Das Gram-negative Bakterium *Pseudomonas putida* wurde in den letzten Jahren mehr und mehr zum „Arbeitstier" für Umweltforschung und Biotechnologie. Sein facettenreicher Metabolismus ermöglicht es ihm, zahlreiche aliphatische und aromatische Kohlenwasserstoffe abzubauen, und damit organische Schadstoffe und Lösungsmittel zu tolerieren. Diese Fähigkeiten machen es zu einem vielseitig anwendbaren Mikroorganismus.

1.1 Systematische Eingliederung

Die Gattung *Pseudomonas* ist systematisch in die Klasse der γ-Proteobakterien einzuordnen, zu der auch die Familie der Pseudomonadaceae gehört. Diese Gattung wurde erstmalig vom deutschen Botaniker Walter Migula im Jahre 1894 vorgeschlagen und verzeichnete in den darauf folgenden Jahrzehnten aufgrund einer unzureichenden Definition immer mehr Mitglieder (Migula, 1894; Palleroni, 2010). Erst mit der Einführung einer neuen Methodik, dem Sequenzvergleich der hochkonservierten 16S rRNA Moleküle (Woese und Fox, 1977), schrumpfte die Gattung auf nur noch ein Zehntel seiner ursprünglichen Größe. Gleichzeitig erfolgte eine Unterteilung in fünf rRNA Homologiegruppen (Palleroni *et al.*, 1973).

Die bekanntesten Vertreter, die alle der Homologiegruppe I angehören, haben sowohl ökologische als auch medizinische Bedeutung erlangt. Dazu gehören u.a. die human- bzw. pflanzenpathogenen Pseudomonaden *P. aeruginosa* und *P. syringae,* die durch ihre Virulenzfaktoren humane Zellmembranen bzw. pflanzliche Zellwände zerstören können. Des Weiteren gehören zu der Homologiegruppe I auch die mit Pflanzen in Symbiose lebenden Arten *P. fluorescens* und *P. putida*. Mit immer genauer werdenden Sequenzierungsmethoden wurden immer wieder neue 16S rRNA Analysen durchgeführt und *P. putida* schließlich als Namensgeber der „*P. putida* Gruppe" zugeordnet, zu der auch *P. mosselii*, *P. fulva*, *P. monteilii*, *P. oryzihabitans* und *P. plecoglossicida* gehören (Anzai *et al.*, 2000).

1.2 Allgemeine Merkmale von *P. putida*

Das ca. 0,5-1,0 µm mal 1,5-4,0 µm große, Gram-negative Bakterium *P. putida* weist eine Stäbchenform mit polarer Begeißelung auf (Abb. 1-1).

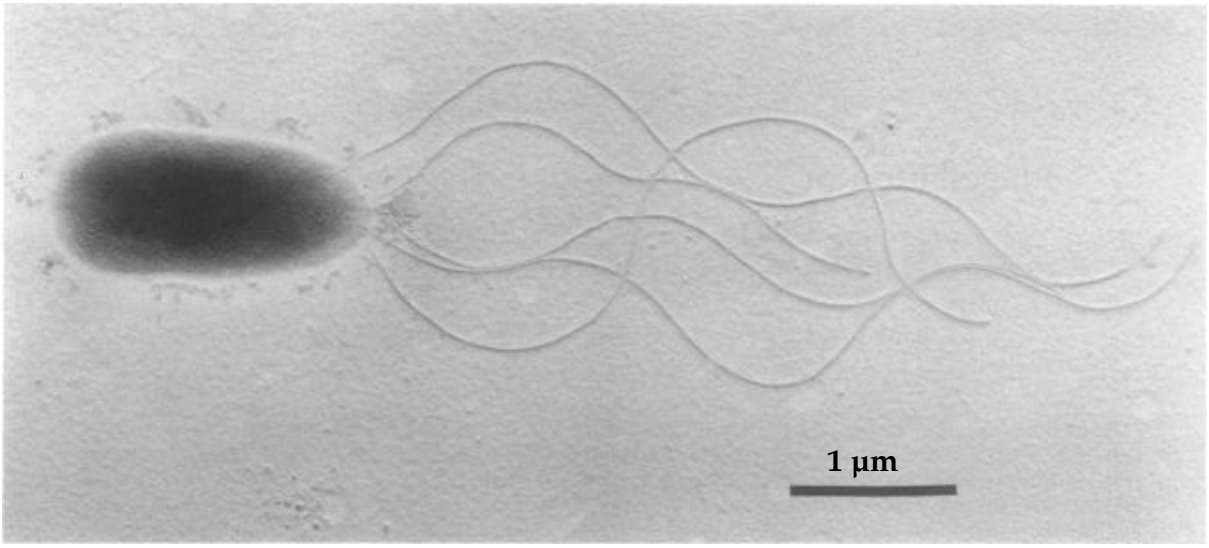


Abbildung 1-1: Elektronenmikroskopische Aufnahme von *P. putida* PRS2000 (verändert nach Harwood *et al*. 1989 - mit freundlicher Genehmigung durch die *American Society for Microbiology*, Copyright © 1989).

Durch seine Flagellen ist es in der Lage, sich chemotaktisch fortzubewegen (Harwood *et al.*, 1989). Es wächst am besten bei neutralem pH-Wert und bei Temperaturen im mesophilen Bereich [25-30 °C]. Für das Wachstum wird Sauerstoff benötigt, welcher als Elektronenakzeptor fungiert und damit die intrazellulären Energie- und Redoxbilanzen aufrechterhält. *P. putida* gehört außerdem zur taxonomisch uneinheitlichen Gruppe der Nonfermenter, d.h. Fermentationen finden in diesem Organismus nicht statt. Jedoch akzeptieren manche Stämme neben Sauerstoff auch Nitrat als alternativen Elektronenakzeptor (Carter *et al.*, 1995).

Bisher wurden insgesamt neun *P. putida* Stämme vollständig und weitere neun partiell sequenziert. Das zirkuläre Genom von *P. putida* KT2440, einem plasmidfreien Derivat von *P. putida* mt-2 (Nakazawa, 2002) wurde im Jahr 2002 von einem US-amerikanischen und deutschen Konsortium vollständig entschlüsselt (Nelson *et al.*,

2002). Es weist eine Größe von etwa 6,18 Megabasen mit einem GC-Gehalt von 61,5% auf (GenBank Accession Number: AE015451). Außerdem wurden 5.516 Gene identifiziert, davon codieren 5.350 für Proteine, 22 für rRNA sowie 74 für tRNA.

1.3 Stoffwechsel und ökologische Bedeutung

P. putida ist ubiquitär anzutreffen und kann leicht aus Boden- und Wasserproben isoliert werden (Timmis, 2002). Es lebt saprotroph, d.h. von abgestorbenem, organischem Material, und ist Teil der pflanzlichen Rhizosphäre. Dort fördert es z.B. als Symbiont synergistisch mit arbuskulären Mykorrhizapilzen das Wachstum von Klee und ernährt sich selbst von den Exsudaten der Pflanzenwurzel (Meyer und Linderman, 1986). Durch die Besiedlung der Rhizosphäre und Bildung von Siderophoren (eisenbindende Oligopeptide) können andere Pflanzenpathogene verdrängt (Liu *et al.*, 1995) und *P. putida* damit als potentielles Biopestizid eingesetzt werden (Espinosa-Urgel *et al.*, 2000).

Eines der charakteristischsten Merkmale von *P. putida* ist sein mannigfaltiger chemoorganotropher Stoffwechsel, und damit seine Fähigkeit ein weites Spektrum an organischen Verbindungen als Kohlenstoff- und Energiequelle nutzen zu können. Zucker werden durch *P. putida* über den Entner-Doudoroff-Weg abgebaut. Durch seine saprophytische Lebensweise spielt *P. putida* eine wichtige ökologische Rolle beim Abbau von löslichen Verbindungen, die bei der Zersetzung von pflanzlichem und tierischem Material entstehen. Ebenso konnte gezeigt werden, dass *P. putida* auch in der Lage ist, umweltschädliche Substanzen wie Nitrobenzen und Naphtalin abzubauen (Gomes *et al.*, 2005; Wang *et al.*, 2009).

P. putida reagiert auf extreme Umweltbedingungen in seinen natürlichen Habitaten nicht mit Sporenbildung, sondern hat verschiedene Möglichkeiten entwickelt diese zu tolerieren (Dos Santos *et al.*, 2004). Dazu gehört zum einen die Ausbildung von Biofilmen (Brettar *et al.*, 1994), und zum anderen die Anpassung seiner Membranzusammensetzung (Ramos *et al.*, 1997), wodurch sogar organische Lösungsmittel wie Styrol, Xylol und Toluol toleriert werden können (Cruden *et al.*, 1992; Ramos *et al.*, 1995; Weber *et al.*, 1993). Stämme wie *P. putida* mt-2 oder *P. putida* GP01 tragen

Plasmide, mit denen sie in der Lage sind, Toluol bzw. Alkane als Kohlenstoffquelle zu verwerten und damit auf natürliche Weise zur Sanierung von mit Schadstoffen belasteten Böden beizutragen (van Beilen *et al.*, 1994; Williams und Murray, 1974).

1.4 Pathobiologische Bedeutung

P. putida zeigt hohe Sequenzhomologien (ca. 85%) zum humanpathogenen Keim *P. aeruginosa*, der z.B. bei Patienten mit Cystischer Fibrose (Mukoviszidose) als opportunistischer Erreger in Erscheinung tritt und Pneumonien auslösen kann. Virulenzfaktoren wie Exotoxine und Typ III Sekretionssysteme, die typisch für *P. aeruginosa* sind, wurden in *P. putida* nicht gefunden. Daher wurde *P. putida* im Jahre 1982 von den Instituten der US-amerikanischen Gesundheitsbehörde zunächst als sicher eingestuft. Im Laufe der Zeit wurden jedoch Fälle bei immunsupprimierten Menschen (Krebskranke und Frühgeborene) bekannt, bei denen *P. putida* als Erreger identifiziert wurde. In diesen Fällen erfolgte die Ansteckung über die Verwendung von kontaminierten, invasiven medizinischen Geräten (Perz *et al.*, 2005; Romney *et al.*, 2000; Yoshino *et al.*, 2011). Darüber hinaus konnte nachgewiesen werden, dass *P. putida* bei Fischen Weichteilinfektionen hervorrufen kann (Altinok *et al.*, 2006; Smolowitz *et al.*, 1998; Zhang *et al.*, 2012). Anhand der Erkenntnisse wachsender Sequenzdaten erfolgte daher 2012 in Deutschland eine neue Risikobewertung von *P. putida* als Spender- oder Empfängerorganismus für gentechnische Arbeiten gemäß § 5 Absatz 1 der Gentechnik-Sicherheitsverordnung. Mit Ausnahme von *P. putida* KT2440, der keinerlei Virulenzfaktoren aufweist und weiterhin in Labors der Sicherheitsstufe 1 für gentechnische Arbeiten verwendet werden darf, wurden alle anderen *P. putida* Stämme der Risikogruppe 2 zugeordnet.

1.5 Anwendung von *P. putida* in der Biotechnologie

Seine metabolische Vielseitigkeit und die Fähigkeit, auf Böden und in der Rhizosphäre zu wachsen, machen *P. putida* zu einem idealen Kandidaten für die Anwendung in der grünen und weißen Biotechnologie.

1.5.1 Allgemeine Anwendungsgebiete

In der grünen Biotechnologie sind *P. putida* Stämme als Biopestizide und Förderer von Pflanzenwachstum (Espinosa-Urgel *et al.*, 2000) sowie für die biologische Bodensanierung (Glick, 2003; Holden und Firestone, 1997; Khan *et al.*, 2009; McDermott *et al.*, 1989; Sašek *et al.*, 2003) in den Fokus der Forschung gerückt.

An der Schnittstelle von grüner zu weißer Biotechnologie finden *P. putida* Zellen Anwendung in der Herstellung des biologisch abbaubaren Biopolymers Poly-3-hydroxyalkanoat aus der aromatischen Kohlenwasserstoffverbindung Styrol (Ward *et al.*, 2005).

In der weißen Biotechnologie kommt *P. putida* als Ganzzellkatalysator für die Biotransformation von Ausgangsstoffen zur Herstellung verschiedenster aromatischer Verbindungen für die Lebensmittel- und Kosmetikindustrie zur Anwendung, wie z.B. für die Herstellung von natürlichem Vanillin aus Eugenol bzw. Ferulasäure (Muheim und Lerch, 1999; Overhage *et al.*, 1999b).

1.5.2 Produktion von natürlichem Vanillin

Vanillin [4-Hydroxy-3-methoxybenzaldehyd] ist die organoleptische Komponente des Vanille-Aromas und der in Nahrungsmitteln und Kosmetikprodukten weltweit am häufigsten eingesetzte Aromastoff. Aufgrund des steigenden Konsumbewusstseins in der Bevölkerung ist die Nachfrage nach „naturidentischem" Vanillin, welches bisher durch die chemische Umsetzung von Guajacol oder Lignin synthetisiert wurde, in den letzten Jahren gesunken (Ramachandra und Ravishankar, 2000). Die Nachfrage nach natürlichem Vanillin ist dafür stetig gewachsen und kann mittlerweile, obwohl Bemühungen unternommen wurden *in vitro* kultivierte *Vanilla*

planifolia Zellen zu verwenden (Davidonis und Knorr, 1991), bei Weitem nicht mehr durch die botanische Quelle gedeckt werden. Aus diesem Grund rückte die biotechnologische Produktion von Vanillin, das als „natürlich" deklariert werden darf, immer mehr in den Fokus (Krings und Berger, 1998; Priefert *et al.*, 2001). Die Biotransformation mit isolierten Enzymen oder ganzen, ruhenden Zellen gilt dabei als der vielversprechendste Ansatz (Berger, 2009; Havkin-Frenkel und Belanger, 2008).

Als Ausgangsstoffe eignen sich besonders Lignin, phenolische Stilbene wie Eugenol [2-Methoxy-4-allylphenol], und Ferulasäure [4-Hydroxy-3-methoxyzimt-säure] (Priefert *et al.*, 2001; Rosazza *et al.*, 1995). Ferulasäure ist als Teil vieler Pflanzenzellwände reichlich in der Natur vorhanden und eignet sich damit besonders für die Biotransformation, da im Gegensatz zu Lignin und Eugenol deutlich weniger Schritte notwendig sind, um Vanillin zu synthetisieren (Escott-Watson und Marais, 1992; Ishii, 1997; Ishikawa *et al.*, 1963; Oosterveld *et al.*, 2000).

Für die Biotransformation wurden bereits viele verschiedene Mikroorganismen herangezogen, dabei auch Stämme von *P. fluorescens* und *P. putida*, die sich aufgrund des ausgeprägten Aromatenstoffwechsels und ihrer Lösungsmitteltoleranzen besonders eignen (Clarke, 1982; Di Gioia *et al.*, 2010; Muheim und Lerch, 1999; Okeke und Venturi, 1999). Der Abbau von Ferulasäure wurde in den Stämmen *Pseudomonas* sp. HR199, *P. fluorescens* BF13 und *P. putida* KT2440 beschrieben (Calisti *et al.*, 2008; Gasson *et al.*, 1998; Narbad und Gasson, 1998; Overhage *et al.*, 1999b; Plaggenborg *et al.*, 2003) und verläuft über einen Coenzym A [CoA]-abhängigen Stoffwechselweg (Abb. 1-2).

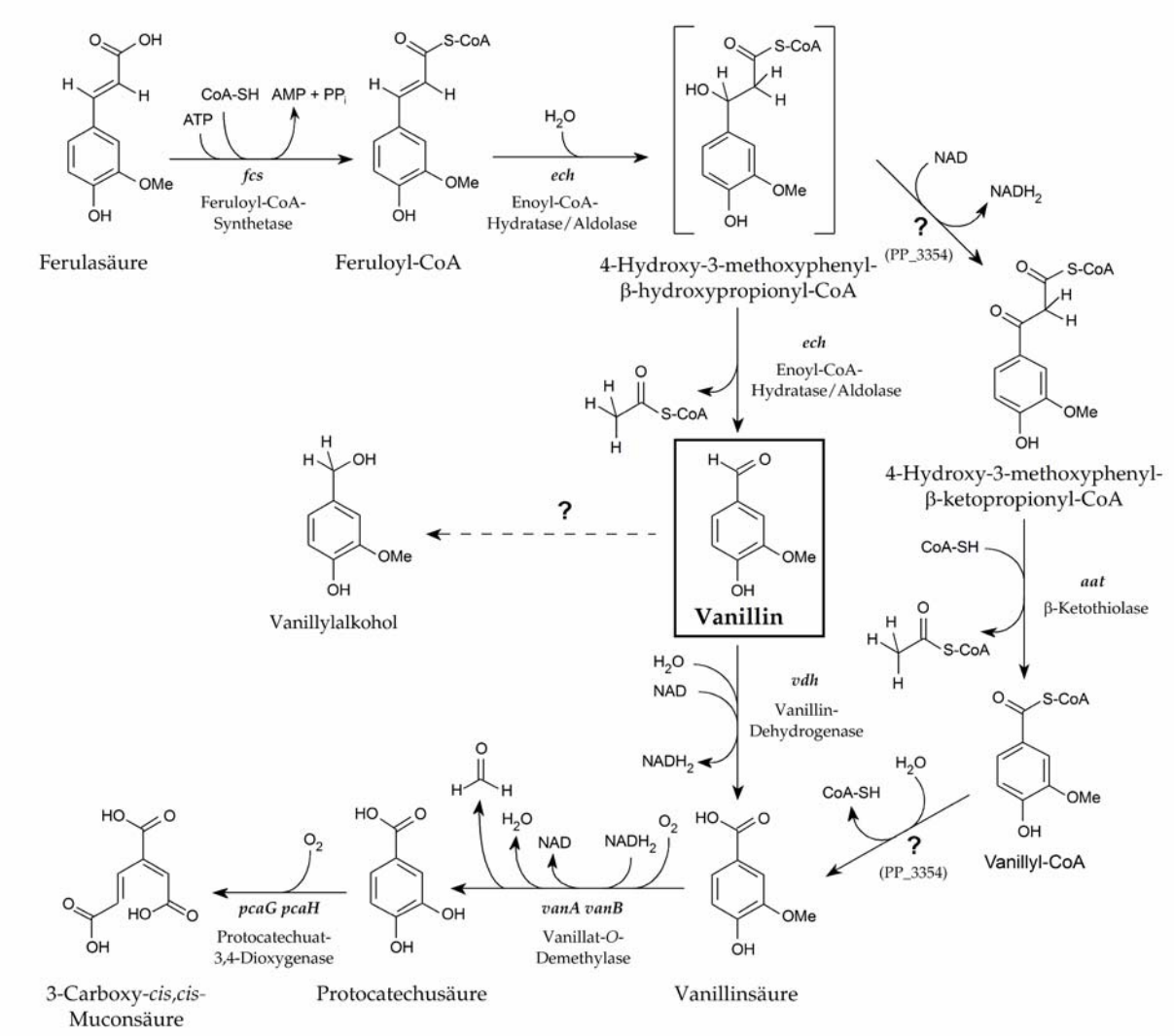


Abbildung 1-2: Vorgeschlagener Stoffwechselweg inkl. alternativer Route (rechts) von Ferulasäure über Vanillin in *Pseudomonas* sp. (verändert nach Overhage *et al.*, 1999b). Die Reduktion von Vanillin zu Vanillylalkohol ist durch einen gestrichelten Pfeil gekennzeichnet. Fragezeichen wurden verwendet, wenn es sich um bisher nicht nachgewiesene Enzymfunktionen handelt.

Die Ferulasäure wird zunächst durch die Feruloyl-CoA-Synthetase [EC 6.2.1.34; codiert durch *fcs*] zu Feruloyl-CoA aktiviert. Der Thioester wird anschließend hydrolytisch durch die Enoyl-CoA Hydratase/Aldolase [EC 4.2.1.101; codiert durch *ech*] zu Vanillin und Acetyl-CoA gespalten. Vanillin wird wiederum durch die Vanillin-Dehydrogenase [EC 1.2.1.67; codiert durch *vdh*] zu Vanillinsäure [4-Hydroxy-3-methoxybenzoesäure] oxidiert, welche durch die Vanillat-*O*-Demethylase [EC 1.14.13.82; codiert durch *vanAB*] weiter zu Protocatechusäure [3,4-Dihydroxybenzoesäure] abgebaut wird. Vanillin scheint außerdem durch die Wirkung einer bisher unbekannten Dehydrogenase zu Vanillylalkohol [4-Hydroxy-3-methoxy-

benzylalkohol] umgesetzt zu werden. Overhage *et al.* (1999b) schlugen außerdem einen zweiten Stoffwechselweg über 4-Hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA und Vanillyl-CoA vor, der durch die von den Genen PP_3354 und PP_3355 (*aat*) codierten Enzyme katalysiert werden könnte.

Die molaren Ausbeuten, Umsetzungsraten und damit die Produktivitäten bei der Biotransformation von Ferulasäure zu Vanillin mit *P. putida* Zellen sind jedoch nach wie vor gering. Neben ungenügender heterologer Genexpression und Plasmidinstabilität ist dies v.a. auf die zytotoxische Natur von Vanillin zurückzuführen (Krings und Berger, 1998). Ein weiteres Problem ist der Abbau des produzierten Vanillins zu Vanillylalkohol und Vanillinsäure (Bonnin *et al.*, 1999; Civolani *et al.*, 2000; Oddou *et al.*, 1999; Stentelaire *et al.*, 1997). Di Gioia *et al*. entwickelten 2010 eine plasmidbasierte Biotransformationsmethode mit rekombinanten *P. fluorescens* Zellen, mit der es ihnen gelungen ist, eine molare Ausbeute an Vanillin aus Ferulasäure von 84,1% zu erhalten. Dies ist bisher der höchste Wert, der mit *Pseudomonas* erhalten wurde. Da die Umsetzung von 10 mM Ferulasäure in diesem System allerdings bis zu 24 h in Anspruch nimmt, ist auch hier die Produktivität äußerst gering.

1.5.3 *P. putida* als möglicher Produzent von Glyoxylsäure

Die Glyoxylsäure ist ein wichtiger Baustein für die Synthese von vielen verschiedenen, industriell hergestellten Produkten. Durch Erhitzen der Glyoxylsäure mit Harnstoff wird beispielsweise das in der Kosmetikindustrie häufig eingesetzte Allantoin dargestellt. Auch beim ersten Schritt der technischen Synthese von „naturidentischem“ Vanillin spielt Glyoxylsäure bei der Substitutionsreaktion von Guajacol eine wichtige Rolle. Glyoxylsäure wird außerdem für die Synthese des Breitbandantibiotikums Amoxicillin benötigt und findet Anwendung im Herstellungsprozess des Breitbandherbizids Glyphosat, welches aufgrund seiner Ähnlichkeit zu Phosphoenolpyruvat das zur Synthese der aromatischen Aminosäuren benötigte Enzym 5-Enolpyruvyl-shikimat-3-phosphat-Synthase blockiert.

Glyoxylsäure wird auf verschiedenen Wegen bisher nur chemisch hergestellt (Mattioda und Christidis, 2000). Die Ausbeuten dieser Prozesse sind jedoch eher

gering, so dass die Biokatalyse einen geeigneten Ansatz darstellt, diese Schwierigkeiten zu umgehen. So konnte beispielsweise Glyoxylsäure mithilfe der Glycolsäure-Oxidase aus Spinat [EC 1.1.3.15] in einem Ganzzellsystem mit einer Ausbeute von bis zu 98% hergestellt werden (Gavagan *et al.*, 1995). Problematisch ist hierbei jedoch die mögliche Bildung von Peroxiden durch die Oxidase, die aufgrund dessen mit der Zeit denaturiert werden könnte.

Eine vielversprechende Route, Glyoxylsäure biotechnologisch herzustellen, wäre eine Biotransformation von Ethylenglycol über die Zwischenprodukte Glycolaldehyd und Glycolsäure (Abb. 1-3).

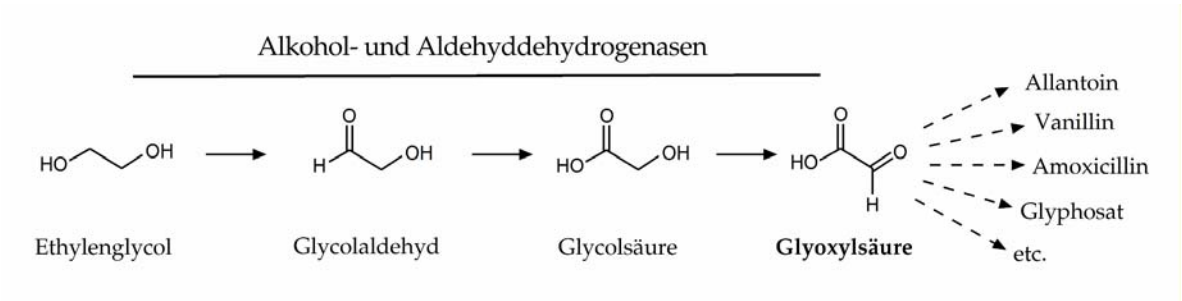


Abbildung 1-3: Mögliche biotechnologische Route zur Produktion von Glyoxylsäure ausgehend von Ethylenglycol über Glycolaldehyd und Glycolsäure, katalysiert von Alkohol- und Aldehyd-Dehydrogenasen von *P. putida*.

Die Pyrrolochinolinchinon [PQQ]-abhängige Alkoholdehydrogenase QEDH aus *P. aeruginosa* kann ein breites Spektrum an Substraten erkennen und umsetzen, darunter auch sekundäre Alkohole und Diole wie Ethylenglycol (Chattopadhyay *et al.*, 2010). Für diesen Prozess wäre jedoch eine Cofaktor-Regenerierung erforderlich und damit ein Ganzzellsystem zu bevorzugen. Da sich aber *P. aeruginosa* aufgrund seiner Humanpathogenität dazu nicht eignet, müssten Alternativen gefunden werden. *P. putida* KT2440 wäre z.B. eine solche Alternative, da mit PP_2674 und PP_2679 zwei homologe Gene zu *qedH* identifiziert werden konnten (Nelson *et al.*, 2002), deren Produkte zu 84 bzw. 52% mit der Aminosäuresequenz von QEDH übereinstimmen. *P. putida* KT2440 besitzt außerdem 37 Gene für Aldehyd-Dehydrogenasen (Nelson *et al.*, 2002), die im Ethylenglycol-Stoffwechsel eine Rolle spielen könnten.

1.6 Genetische Manipulation von *P. putida*

Die vollständige Sequenzierung mikrobieller Genome lieferte in den letzten Jahren zahlreiche neue Informationen und Möglichkeiten, Mikroorganismen für die Herstellung gewünschter Produkte genetisch zu optimieren. Seit der Sequenzierung von *P. putida* KT2440 (Nelson *et al.*, 2002) rückte auch die Erforschung einzelner Genfunktionen dieses Stamms immer mehr in den Fokus, so dass infolgedessen bereits viele Methoden für die Deletion chromosomaler Gene sowie für die chromosomale Integration von Genen entwickelt wurden.

1.6.1 Klassische Methoden für die chromosomale Gendeletion

In der Regel erfolgt die Inaktivierung chromosomaler Gene durch Resistenzgene, welche nach erfolgter homologer Rekombination die ursprüngliche Gensequenz unterbrechen (Schweizer und Hoang, 1995). Um mehrere Gene in ein und demselben Stamm sukzessive zu inaktivieren, ist diese Methode aufgrund der geringen Zahl an geeigneten Markern allerdings sehr eingeschränkt. Aus diesem Grunde wurden Deletionsmethoden entwickelt, bei denen die Resistenzkassetten z.B. mit Erkennungssequenzen für ortsspezifische Rekombinasen, wie Flp/FRT und Cre/*loxP*, flankiert werden, und nach der chromosomalen Integration wieder präzise aus dem Genom entfernt werden können (Hoang *et al.*, 1998; Marx und Lidstrom, 2002). Allerdings hinterlassen diese Methoden einzelne Erkennungssequenzen im Genom, und diese können, falls die Methode häufiger angewendet wird, zu chromosomalen Deletionen und Inversionen führen. Ein erster Ansatz, dies zu verhindern, ist die Entwicklung und Verwendung von chimären Erkennungssequenzen, wie z.B. *mroxP*, einer hybriden Struktur aus *loxP* und *mrpS* Erkennungssequenzen (Warth und Altenbuchner, 2013). Probleme dieser Methoden sind allerdings die Kurierung derjenigen Plasmide, mit denen die ortsspezifischen Rekombinasen exprimiert werden, und der damit verbundene Zeitaufwand.

1.6.2 Gegenselektionssysteme

Um den Zeitaufwand für die Selektion gezielter chromosomaler Veränderungen zu reduzieren, wurden Gegenselektionssysteme entwickelt. Hierbei erfolgt die Selektion auf die Integration eines Vektors mit Deletionskassette anhand eines Resistenzmarkers. Die nachfolgende Gegenselektion zur Exzision des Vektors erfolgt mithilfe eines weiteren plasmidcodierten Markers, der zur Wachstumsinhibierung des Stamms führt. Damit kann die Exzision auf einfache Weise selektioniert werden, wobei in ca. 50% der kurierten Zellen gleichzeitig auch der gewünschte Genabschnitt verloren geht. Die leistungsfähigsten Gegenselektionssysteme basieren auf Sensitivitäten gegen Fusarsäure (*tetAR*), Streptomycin (*rpsL*) und Saccharose (*sacB*) (Reyrat *et al.*, 1998).

Die durch *sacB* codierte Levansucrase aus *Bacillus subtilis* bildet aus Saccharose das Fructan Levan, dessen toxische Wirkung bisher noch nicht genau verstanden ist. Da die Levansucrase in *Escherichia coli* periplasmatisch aktiv ist, wurde ursprünglich angenommen, dass Levan das Periplasma verstopfen und damit die Nährstoffaufnahme erschweren bzw. verhindern könnte (Steinmetz *et al.*, 1983). Aus diesem Grund wurde das *sacB*/Saccharose-Gegenselektionssystem zunächst nur bei Gram-negativen Bakterien angewendet (Gay *et al.*, 1985). Es konnte jedoch gezeigt werden, dass *sacB* auch bei einigen Gram-positiven Bakterien, wie beispielsweise *Corynebacterium glutamicum, Mycobacterium smegmatis* und *Mycobacterium bovis,* zu einer Saccharose-Sensitivität führt (Jäger *et al.*, 1992; Pelicic *et al.*, 1996). Stämme mit SacB-Aktivität können in Gegenwart von Saccharose nicht wachsen, wohingegen Stämme, welche die Resistenzkassette und damit auch den Gegenselektionsmarker *sacB* verloren haben, wieder in dessen Gegenwart wachsen können. In Kombination mit dem λ-Red-Rekombinationssystem konnte so eine effiziente Methode für die sequentielle Gendeletion in *P. aeruginosa* entwickelt werden (Liang und Liu, 2010).

Das Toxin CcdB aus *E. coli* ist ein weiterer Gegenselektionsmarker, der mit der DNA-Gyrase [GyrA] einen kovalenten Komplex bildet und damit deren Enzymfunktion, die Entwindung der DNA bei der Replikation, inhibiert (Bernard *et al.*, 1994). In *P. aeruginosa* wurde für die Einführung chromosomaler Modifikationen dieses *ccdB*-

System mit dem *sacB*-System gekoppelt (Choi und Schweizer, 2005). Solche Kombinationen haben den Vorteil, das Auftreten von falsch-positiven Klonen zu reduzieren.

Die Endonuklease I-*Sce*I der Hefe *Saccharomyces cerevisiae* stellt ein weiteres Werkzeug für die Einführung markerfreier, chromosomaler Modifikationen dar. Sie erkennt eine 18 bp lange Sequenz, die damit - statistisch gesehen - in den meisten bakteriellen Genomen nicht vorkommt (Monteilhet *et al.*, 1990). I-*Sce*I führt zu DNA-Doppelstrangbrüchen, die letal für die Zellen sind. Im ersten Schritt erfolgt durch homologe Rekombination die Insertion der I-*Sce*I-Erkennungssequenz zusammen mit einem positiven Selektionsmarker. Nach Selektion auf diesen Marker soll im zweiten Schritt über eine erneute homologe Rekombination die gewünschte Mutation eingeführt und dabei die I-*Sce*I-Erkennungssequenz zusammen mit dem positiven Selektionsmarker wieder entfernt werden. Diese Rekombination wird durch transiente Expression der Endonuklease I-*Sce*I quasi erzwungen, da diejenigen Klone, die die I-*Sce*I-Erkennungssequenz wieder verloren haben, direkt als überlebende Zellen selektioniert werden können. In Kombination mit dem λ-Red-Rekombinationssystem wurde so für den speziellen *E. coli* Stamm GS1783, der in seinem Genom das λ-Red-System unter Kontrolle eines temperatursensitiven Repressors sowie I-*Sce*I unter Kontrolle eines Arabinose-induzierbaren Promotors trägt, ein effizientes System für die Manipulation von BACs [engl. *Bacterial Artificial Chromosomes*] etabliert (Tischer *et al.*, 2010). Das λ-Red-Rekombinationssystem konnte zwar in *P. aeruginosa* (Lesic und Rahme, 2008; Liang und Liu, 2010), bisher jedoch nicht in *P. putida* erfolgreich angewendet werden. Um *P. putida* KT2440 mithilfe des I-*Sce*I-Gegenselektionssystems genetisch zu modifizieren, wurde daher ein Integrationsvektor mit homologen Regionen und der I-*Sce*I-Erkennungssequenz verwendet (Martínez-García und de Lorenzo, 2011). Die streng regulierte Expression von I-*Sce*I erfolgte in diesem System unter Zuhilfenahme eines RK2-basierten, instabilen Expressionsvektors. Dessen Kurierung sowie die Basalexpression des Gens für die Endonuklease stellen allerdings die wesentlichen Probleme dieses Systems dar.

1.6.3 Das 5-FU/*upp*-Gegenselektionssystem

Auf der Suche nach neuen Gegenselektionsmarkern wurde für *B. subtilis* ein weiteres System entwickelt (Fabret *et al.*, 2002), das inzwischen auch schon für einige andere Mikroorganismen adaptiert werden konnte (Goh *et al.*, 2009; Keller *et al.*, 2009; Kristich *et al.*, 2005). Dieses System basiert auf der Wirkungsweise der Uracil-phosphoribosyl-Transferase [UPRTase; EC 2.4.2.9] und der Toxizität von 5-Fluor-uracil [5-FU]. Die UPRTase spielt bei der Wiederverwertung von Pyrimidinen eine Rolle und generiert UMP aus Uracil und Phosphoribosylpyrophosphat [PRPP] (Neuhard, 1983) (Abb. 1-4).

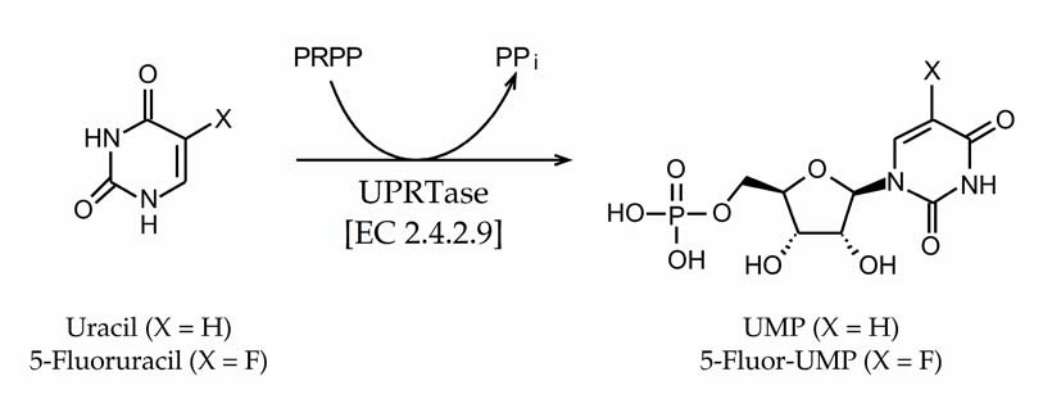


Abbildung 1-4: Katalysierte Reaktion der Uracilphosphoribosyl-Transferase [UPRTase]. PRPP = Phosphoribosylpyrophosphat, PP_i = Pyrophosphat.

5-FU findet als Warzen-Therapeutikum (Verrumal®, Almirall Hermal GmbH) vor allem aber als Zytostatikum (Prodrug: Capecitabin, Xeloda®, Hoffmann-La Roche AG) bei der Chemotherapie von Krebspatienten medizinische Anwendung. Es wird in der Leber zu ca. 80% durch die Dihydropyrimidin-Dehydrogenase in 5,6-Dihydrofluoruracil umgewandelt und so unschädlich gemacht (Longley *et al.*, 2003). Die restlichen 20% werden von der UPRTase als Substrat erkannt und in 5-Fluor-UMP umgewandelt. Über die Umsetzungsraten von 5-FU durch die Dihydro-pyrimidin-Dehydrogenase bzw. UPRTase in Bakterien sind keine genauen Daten bekannt.

Die Zytotoxizität beruht auf der Umwandlung von 5-Fluor-UMP zu 5-Fluor-UTP und 5-Fluor-dUMP. Durch den Einbau von 5-Fluor-UTP in die RNA kommt es zu irreparablen Schäden, während 5-Fluor-dUMP die Thymidylat-Synthase [TS; EC 2.1.1.45] irreversibel hemmt (Selbstmordinhibierung). Dies erfolgt, indem 5-Fluor-dUMP mit dem Cofaktor N^5,N^{10}-Methylentetrahydrofolat und der TS einen stabilen, kovalenten Komplex bildet, der aufgrund der hohen Elektronegativität des Fluoratoms nicht durch Abspalten eines F^+-Ions aufgelöst werden kann (Abb. 1-5).

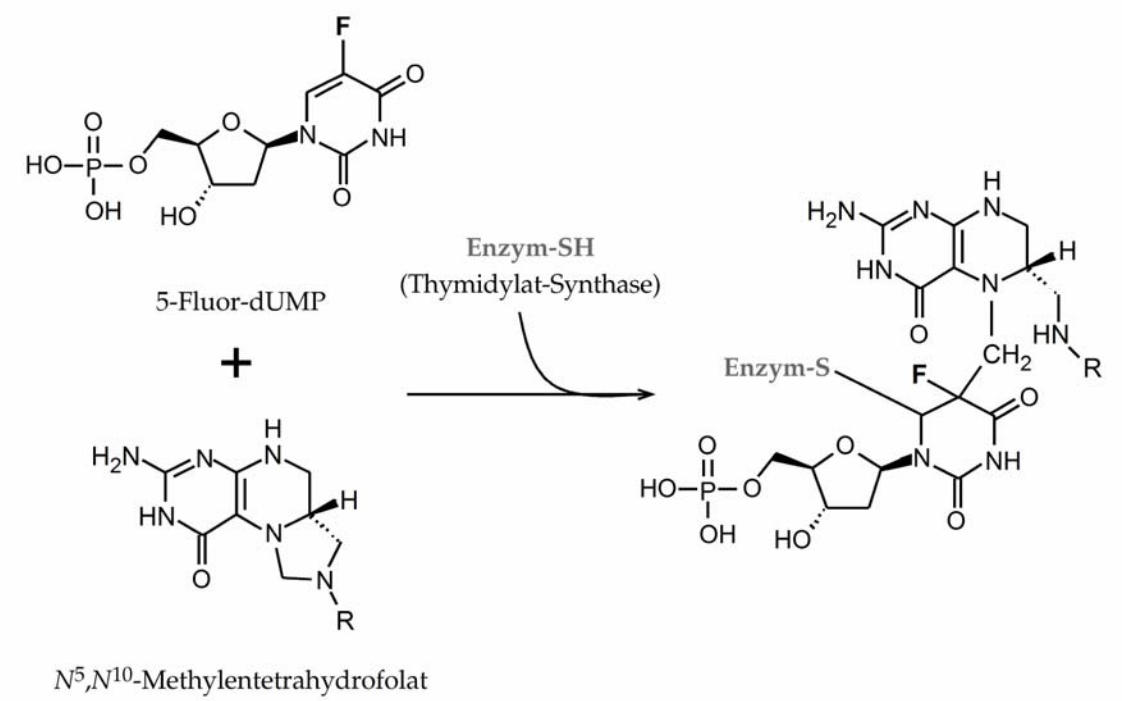
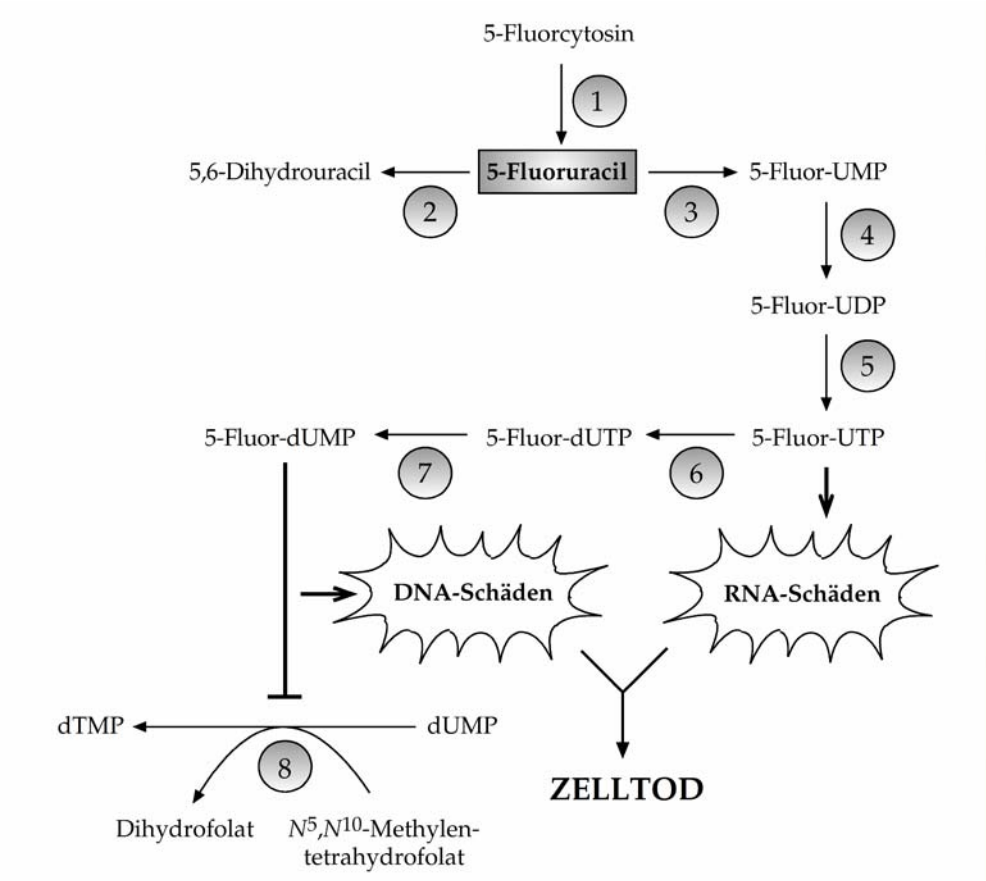


Abbildung 1-5: Selbstmordinhibierung der Thymidylat-Synthase durch kovalente Bindung von 5-Fluor-dUMP ans aktive Zentrum (Cystein-Sulfhydrylgruppe) und an den Cofaktor N^5,N^{10}-Methylentetrahydrofolat. R = p-Aminobenzoesäure- und Glutaminsäure-Rest.

Durch Inaktivierung der TS kommt es zu einem Ungleichgewicht des dNTP-Pools in der Zelle, welches von einer intrazellulären Erhöhung der dUTP-Konzentration begleitet wird. Beides zusammen führt zu irreparablen DNA-Schäden und zusammen mit den RNA-Schäden zum Zelltod (Longley *et al.*, 2003). Mikroorganismen mit UPRTase-Aktivität sind demnach sensitiv gegen 5-FU. Auch in *P. putida* KT2440 wurde die UPRTase, die durch das Gen *upp* (PP_0746) codiert wird, identifiziert (Nelson *et al.*, 2002). Dadurch kann der Einsatz des 5-FU/*upp*-Gegenselektionssystems auch für *Pseudomonas* in Betracht gezogen werden.

Der Stoffwechselweg des 5-FUs in *P. putida* KT2440 wurde anhand der Einträge in die KEGG Datenbank [Kyoto Encyclopedia of Genes and Genomes][1] in der Abbildung 1-6 schematisch dargestellt.



	Enzym	EC-Nummer	Gen
1	Cytosin-Deaminase	EC 3.5.4.1	PP_3189 (*codA*)
2	Dihydropyrimidin-Dehydrogenase	EC 1.3.1.2	PP_4038
3	Uracilphosphoribosyl-Transferase	EC 2.4.2.9	PP_0746 (*upp*)
4	Uridylat-Kinase	EC 2.7.4.22	PP_1593 (*pyrH*)
5	Nukleosiddiphosphat-Kinase	EC 2.7.4.6	PP_0849 (*ndk*)
6	Thioredoxin-Reduktase	EC 1.8.1.9	PP_0786 (*trxB*)
7	dUTP-Pyrophosphatase	EC 3.6.1.23	PP_5586 (*dut*)
8	Thymidylat-Synthase	EC 2.1.1.45	PP_5141 (*thyA*)

Abbildung 1-6: 5-Fluorcytosin / 5-Fluoruracil Metabolismus in *P. putida* KT2440. Der Einbau von 5-Fluor-UTP führt zu RNA-Schäden. 5-Fluor-dUMP inhibiert irreversibel die Thymidylat-Synthase (8). Das entstehende Ungleichgewicht im dNTP-Pool sowie steigende, intrazelluläre dUTP-Konzentrationen führen zu irreparablen DNA-Schäden und letztendlich zum Zelltod.

[1] http://www.genome.jp/kegg-bin/show_pathway?org_name=ppu&mapno=00240&mapscale=&show_description=hide

Das 5-FU/*upp*-Gegenselektionssystem verlangt jedoch eine Inaktivierung der natürlichen UPRTase-Aktivität und damit die chromosomale Deletion von *upp*. Durch die Verwendung von 5-Fluorcytosin [5-FC] kann in manchen Fällen eine genetische Anpassung verhindert werden, da die Gene für die Cytosin-Deaminase [CodA, EC 3.5.4.1], die Cytosin zu Uracil bzw. 5-FC zu 5-FU konvertiert (Abb. 1-6), in Prokaryoten nicht so häufig anzutreffen sind wie die Gene für die UPRTase. Auch höheren Eukaryoten, wie Pflanzen, fehlt die Cytosin-Deaminase-Aktivität. Durch die Verwendung von *codA* aus *E. coli* konnten so in *Streptomyces* und *Arabidopsis* effektive 5-FC/*codA*-Gegenselektionssysteme ohne vorherige, genetische Modifikation des Wirtsorganismus etabliert werden (Dubeau *et al.*, 2009; Perera *et al.*, 1993).

1.7 *P. putida* als Expressionsplattform

Obwohl sich *E. coli* K-12 Systeme für die Produktion heterologer Proteine etabliert haben und häufig auch am geeignetsten sind, sind die Produktausbeuten oftmals nicht ausreichend und alternative Wirte müssen gefunden werden. Pseudomonaden wie *P. putida* aber auch *P. fluorescens* (Retallack *et al.*, 2012) haben sich in den letzten Jahren als Wirte für die Produktion rekombinanter Proteine bewährt. So konnten beispielsweise mit *P. putida* KT2440 Zellen scFv [engl. *single chain fragment variables*]-Antikörperfragmente in hoher Ausbeute hergestellt werden (Dammeyer *et al.*, 2011). Für die Anwendung von Expressionsvektoren in alternativen Wirtsorganismen wie *P. putida* sind jedoch funktionierende Replikationsregionen [Replicons], Selektionsmarker und effiziente Promotorsysteme erforderlich.

1.7.1 Replicons

Aufgrund der einfacheren genetischen Manipulationen in *E. coli* sind die meisten Vektoren für *P. putida* KT2440 Shuttle-Vektoren, d.h. sie können in beiden Organismen replizieren. Der gleichzeitige Einsatz von zwei verschiedenen Replicons, je eines für *E. coli* und *P. putida*, hat den Nachteil, dass dadurch die Größe der Vektoren stark ansteigt, und damit wiederum die Handhabbarkeit bei Klonierungen erschwert wird. Daher kommen immer häufiger Vektoren zum Einsatz, die mit einem einzigen

Replicon für beide Spezies auskommen. Die bekanntesten und am häufigsten verwendeten Replicons sind die der Plasmide RSF1010 (Scholz *et al.*, 1989), RK2 (Pansegrau *et al.*, 1994), pRO1600 (Olsen *et al.*, 1982) und pBBR1 (Antoine und Locht, 1992), auf deren Grundlage Vektoren wie z.B. pBBR1MCS konstruiert wurden, um Werkzeuge für eine einfache und schnelle genetische Manipulation zur Hand zu haben (Kovach *et al.*, 1994).

1.7.2 Selektionsmarker

Als Selektionsmarker kommen in *Pseudomonas* v.a. Resistenzgene gegen Antibiotika oder Schwermetalle zum Einsatz. Allerdings verleihen hoch effiziente Efflux-Systeme sowie eine chromosomal codierte β-Lactamase (PP_2876) (Nelson *et al.*, 2002) *P. putida* eine natürliche Resistenz gegen die Breitbandantibiotika Chloramphenicol und Ampicillin (Fernández *et al.*, 2012; Fukumori *et al.*, 1998). Als Alternative wurden metabolische Marker wie z.B. die *lac*-Gene von *E. coli* erfolgreich eingesetzt, wodurch *P. putida* die normalerweise nicht nutzbare Lactose verwerten kann (Kok *et al.*, 1994).

1.7.3 Negativ regulierte Expressionssysteme

Viele verschiedene Promotorsysteme, die ursprünglich für *E. coli* entwickelt wurden, konnten auch in Pseudomonaden erfolgreich eingesetzt werden. Dazu gehören die negativ regulierten Systeme mit *lacUV5-tac*-Tandem-Promotor sowie Promotoren der Bakteriophagen λ und T7 (Morales *et al.*, 1991; Schweizer, 2001; Suh *et al.*, 2004). Diese haben jedoch den Nachteil einer unzureichenden Repression und damit eine hohe Basalexpression. Dadurch wird die Produktion von Proteinen erschwert, die schädlich für die Zelle sein können. So wird im Falle des für die Produktion von toxischen Proteinen häufig eingesetzten T7-Expressionssystems z.B. ein zusätzlicher Vektor mit dem Regulatorgen für das T7-Lysozym benötigt, um die durch die Basalexpression vorhandene T7-RNA-Polymerase abzufangen (Moffatt und Studier, 1987; Stano und Patel, 2004; Studier, 1991).

1.7.4 Positiv regulierte Expressionssysteme durch Transkriptionsaktivatoren der AraC/XylS-Familie

Aufgrund ihrer niedrigeren Basalexpression wird positiv regulierten Promotoren häufig der Vorzug gegenüber negativ regulierten Systemen gewährt. Zu den bekanntesten Beispielen gehören die Promotoren *rhaP*$_{BAD}$ und *araP*$_{BAD}$ aus *E. coli* und die Pm-, Pu- und Psal-Promotoren der *P. putida* Plasmide TOL und NAH, sowie der Promotor des Mannitol-Operons aus *P. fluorescens* DSM 50106, die bisher in *E. coli* als auch in *Pseudomonas* sp. angewendet wurden (Brünker *et al.*, 1998; Jeske und Altenbuchner, 2010; Qiu *et al.*, 2008; Schweizer, 2001). Die Regulation der genannten Beispiele erfolgt dabei durch die allosterische Bindung von Effektoren an Transkriptionsaktivatoren der AraC/XylS-Familie, die wiederum an die entsprechenden Operatorregionen binden.

Zur AraC/XylS-Familie zählen zurzeit ca. 2.000 Mitglieder, aber nur wenige davon fanden bisher biotechnologische Anwendung (Ibarra *et al.*, 2008; Schüller *et al.*, 2012; Tobes und Ramos, 2002). Sie haben typischerweise eine Länge von 250 bis 300 Aminosäuren und besitzen zwei Domänen. Neben einer nichtkonservierten N-terminalen Domäne, die vermutlich bei der allosterischen Bindung der Effektormoleküle und der Dimerisierung eine Rolle spielt, findet man eine hochkonservierte C-terminale Domäne mit einer typischen Abfolge von 100 Aminosäuren, die zwei Helix-Turn-Helix-Motive für die DNA-Bindung ausbilden (Gallegos *et al.*, 1997). Die typische Operatorstruktur dieser Regulatoren besteht aus zwei sich direkt wiederholenden, zweigeteilten DNA-Bindemotiven, einem sogenannten Tandem-Repeat, bei dem die beiden Motive vermutlich von jeweils einem Monomer gebunden werden (Domínguez-Cuevas *et al.*, 2008; Gallegos *et al.*, 1997). So sind für die Aktivierung des *meta*-Operonpromotors Pm des TOL Plasmids pWW0 aus *P. putida* durch XylS beispielsweise zwei 15-bp lange direkte Sequenzwiederholungen erforderlich, die durch 6 bp voneinander getrennt sind und mit der -35 Region überlappen (Kaldalu *et al.*, 1996).

Aufgrund der äußerst geringen Löslichkeit dieser Regulatoren (Egan und Schleif, 1994; Jair *et al.*, 1995; Michan *et al.*, 1995; Munson und Scott, 2000; Timmes *et al.*, 2004), konnten nur ein paar wenige aufgereinigt und anschließend deren dreidimensionale Struktur ermittelt werden (Kwon *et al.*, 2000; Rhee *et al.*, 1998).

Ein weiteres, vielversprechendes System für die Anwendung in *E. coli* und *P. putida* basiert auf dem Abbau von Methylethylketon [MEK] und wurde in *P. veronii* MEK700 entdeckt (Onaca *et al.*, 2007). Dieser Stamm wurde aus Abluftfiltern isoliert, die zur Aufreinigung von mit MEK versetzter Luft verwendet wurden. Die essentiellen Gene, die der Stamm für den MEK-Abbau benötigt, sind in einem Operon organisiert (GenBank Accession-Number DQ855566). Dieses besteht aus den Strukturgenen *mekA* und *mekB*, deren Produkte, eine Baeyer-Villiger-Monooxygenase und eine Homoserin-Acetyltransferase-ähnliche Esterase, bereits detailliert beschrieben wurden (Onaca *et al.*, 2007; Völker *et al.*, 2008). Das Gen für den Regulator MekR (GenBank accession number ABI15713), bei dem es sich ebenfalls um ein Mitglied der AraC/XylS-Familie handelt, wurde unmittelbar stromabwärts des *mekAB* Operons lokalisiert (Onaca *et al.*, 2007). Über das Expressionsverhalten, die genauen Wirkungsmechanismen von MekR und inwiefern das System global reguliert wird, lagen bisher jedoch noch keine Daten vor.

1.7.5 Katabolitrepression in *P. putida*

Pseudomonaden können eine Vielzahl an Substraten als Kohlenstoffquelle und zur Energiegewinnung verwerten. Dabei zeigt sich eine Rangfolge, mit der die einzelnen Substrate bevorzugt verwertet werden. Organische Säuren, wie Aminosäuren oder Succinat, stehen in der Hierarchie von *Pseudomonas* oben, gefolgt von Kohlenhydraten (z.B. Glucose) und diversen Kohlenwasserstoffen (Collier *et al.*, 1996; Rojo, 2010). Der regulatorische Prozess dahinter wird als Katabolitrepression [engl. *carbon catabolite repression*, CCR] bezeichnet. Obwohl die CCR in *Pseudomonas* nicht so gut untersucht und verstanden ist wie in *E. coli* oder *B. subtilis*, konnten einige der regulatorischen Schlüsselfaktoren identifiziert werden: Dies sind der globale

Regulator Crc, das Stickstoff-Phosphotransferasesystem PTS^{Ntr} und die Cytochrom-*o*-Ubiquinol-Oxidase (Rojo, 2010).

Am besten untersucht wurde die CCR am Beispiel des Pm-Promotors des TOL-Plasmids pWW0 aus *P. putida*, welcher durch den Transkriptionsaktivator XylS reguliert wird (Kessler *et al.*, 1994). Das XylS/Pm-Expressionssystem unterliegt einer Crc-abhängigen CCR, wenn in Vollmedium wachsenden Zellen Aminosäuren zur Verfügung gestellt werden (Moreno *et al.*, 2010). Der Regulator Crc ist ein RNA-Bindeprotein (Moreno *et al.*, 2007), welches trotz seiner strukturellen Ähnlichkeit zu Endo- und Exonukleasen keine Nuklease-Aktivität besitzt (Ruiz-Manzano *et al.*, 2005). Crc reguliert die Genexpression posttranskriptional, indem es bevorzugt an das 5'-Ende der mRNA mit der A-reichen Konsensussequenz AA(C/U)AA(C/U)AA nahe der Shine-Dalgarno-Sequenz bindet und damit die Translation inhibiert (Moreno *et al.*, 2007). Es wird davon ausgegangen, dass Crc die Ausbildung des ternären 30S-$tRNA^{Met}$-RNA-Komplexes verhindert, der für die Initiation der Translation benötigt wird (Moreno *et al.*, 2009). Crc inhibiert die Expression von über 134 Genen, von denen die meisten an der Aufnahme von Zuckern und Aminosäuren beteiligt sind (Moreno *et al.*, 2009). Crc inhibiert jedoch nur die Stoffwechselwege von einigen Aminosäuren, und gewährleistet damit eine Hierarchie für die Aufnahme der bevorzugten Aminosäuren (Rojo, 2010). Es sorgt ebenfalls dafür, dass weniger bevorzugte Kohlenstoffquellen wie Benzoat und Alkane erst verstoffwechselt werden, wenn keine Substrate mehr vorhanden sind, die in der Hierarchie weiter oben stehen (Morales *et al.*, 2004; Yuste und Rojo, 2001). In diesen Fällen inhibiert Crc nicht nur die Translation von Regulatoren, wie z. B. BenR und AlkS (Moreno *et al.*, 2007; Moreno und Rojo, 2008), sondern auch die Translation derjenigen Strukturgene, die für das erste Enzym im jeweiligen Stoffwechselweg codieren (Hernández-Arranz *et al.*, 2013). Die Stärke des Repressionseffekts von Crc variiert je nach verwendeter Kohlenstoffquelle. Bis heute ist aber immer noch nicht geklärt, welches die Signale sind, die Crc funktionell beeinflussen. Bekannt ist hingegen der Einfluss von kleinen RNA Molekülen (sRNA), die Crc binden, wenn es nicht benötigt wird (Rojo, 2010). In *P. aeruginosa* wurde mit CrcZ eine 407 nt lange sRNA identifiziert, die insgesamt fünf AANAANAA-Motive besitzt und Crc *in vitro* effektiv bindet

(Sonnleitner *et al.*, 2009). Die *crcZ* Expression wird bei *P. aeruginosa* wiederum durch CbrB des Zwei-Komponenten-Systems CbrA/CbrB, welches die Nutzung von einigen Kohlenstoff- und Stickstoffquellen kontrolliert (Zhang und Rainey, 2008), sowie durch den alternativen Sigmafaktor σ^{54} (RpoN) reguliert (Sonnleitner *et al.*, 2009). Im Gegensatz zu *P. aeruginosa* besitzt *P. putida* zwei solcher sRNAs, CrcZ und CrcY (Moreno *et al.*, 2012). Crc scheint damit eine wesentliche Rolle bei der Koordination des Gesamtstoffwechsels zu spielen. Außerdem konnte gezeigt werden, dass die Inaktivierung von *crc* in *P. putida* KT2442 (Rifampicin-resistente Variante von KT2440) zu einem um ca. 8% langsameren Wachstum in Vollmedium führt (Moreno *et al.*, 2009), und in *P. aeruginosa* die Ausbildung von funktionellen Typ IV Pili und damit dessen Fortbewegung und Fähigkeit zur Ausbildung von Biofilmen verhindert (O'Toole *et al.*, 2000).

Wie viele andere Eubakterien, besitzt *P. putida* ebenfalls ein alternatives Phosphotransferase-System [PTS], welches aus den Komponenten PtsP (EI^{Ntr}), PtsO (NPr) und PtsN ($EIIA^{Ntr}$) besteht. Das PTS^{Ntr} System besitzt zwar alle Komponenten und Domänen für die Phosphorylgruppenübertragung, jedoch keine Permease-Komponente. Obwohl der direkte Nachweis der Phosphorylgruppenübertragung von PtsN auf ein bestimmtes Substrat noch nicht erbracht werden konnte, wird davon ausgegangen, dass PTS^{Ntr} eine Rolle bei der Signaltransduktion spielt. Die Gene *ptsO* und *ptsN* gehören dem *rpoN* Operon an. RpoN ist der alternative Sigmafaktor σ^{54}, der u.a. für die Expression einer Reihe von Genen für den Kohlenstoff- und Stickstoffmetabolismus benötigt wird (Cases *et al.*, 2003; Reitzer und Schneider, 2001). In *P. aeruginosa* wird PtsN als Co-Induktor für einige dieser σ^{54}-abhängigen Gene benötigt (Jin *et al.*, 1994). In *P. putida* verändert sich der Phosphorylierungszustand von PtsN in Abhängigkeit von der Stickstoffquelle und scheint damit das regulatorische Signal darzustellen (Pflüger und de Lorenzo, 2007). PtsN spielt bei der C/N Balance in der Zelle eine wesentliche Rolle, da dessen Inaktivierung in *P. putida* die Nutzung von Lysin, Arginin und Glycerin als Kohlenstoffquelle, sowie die Nutzung von Aminosäuren und Dipeptiden als Stickstoffquelle verhindert (Daniels *et al.*, 2010). Auch spielt PtsN bei der CCR durch Glucose eine Rolle (Rojo, 2010). Das XylR/Pu-Expressionssystem des TOL-Plasmids

aus *P. putida* unterliegt beispielsweise einer PTS^{Ntr}-abhängigen CCR, die durch Glucose und Succinat verursacht wird (del Castillo und Ramos, 2007; Duetz *et al.*, 1994; Duetz *et al.*, 1996; Holtel *et al.*, 1994). In *P. putida* ist PtsN zwar ein globaler Regulator, trotzdem scheint er nicht allein für die CCR durch Glucose verantwortlich zu sein (Cases *et al.*, 2001). Studien mit *E. coli* haben gezeigt, dass PTS^{Ntr} außerdem den Influx von K^+-Ionen über direkte Wechselwirkung mit den K^+-Transportern TrkA und KdpFABC kontrolliert (Lee *et al.*, 2007; Lüttmann *et al.*, 2009). Es konnte außerdem gezeigt werden, dass der intrazelluläre K^+-Level in *E. coli* und damit auch PtsN Einfluss auf die Bindung der Sigmafaktoren σ^{70} und σ^{38} an das Core-Enzym der RNA-Polymerase nehmen (Lee *et al.*, 2010) und somit die berichteten Stickstoff-abhängigen Phänotypen möglicherweise nur Artefakte sein könnten (Reaves und Rabinowitz, 2011). Ferner berichten neuere Studien über einen Einfluss von PTS^{Ntr} auf die Phosphat-Homöostase in *E. coli*, indem PtsN direkt mit der Histidin-Kinase PhoR des PhoR/PhoB-Zweikomponentensystems interagiert, und damit Einfluss auf die Expression der 30 Gene des *pho* Regulons nimmt (Lüttmann *et al.*, 2012). Inwiefern diese Erkenntnisse auch auf die Rolle von PtsN in *P. putida* zutreffen, ist allerdings bisher ungeklärt.

Durch Zufallsmutagenese wurden *P. putida* Stämme mit Mutationen im *cyoABCDE* Gencluster isoliert, das für die Cytochrom-*o*-Ubiquinol-Oxidase [Cyo] codiert, bei denen keine CCR durch Succinat oder Aminosäuren mehr auftritt (Dinamarca *et al.*, 2002; Petruschka *et al.*, 2001). Cyo ist Teil der Atmungskette und einer der terminalen Elektronenakzeptoren in *P. putida*. Außerdem reguliert es auf mRNA-Ebene die Expression von mehr als 100 Genen, von denen die meisten für Porine und Transportproteine codieren (Morales *et al.*, 2006). Es wird vermutet, dass Cyo den Energiestatus der Zelle registriert und Signale auf bisher unbekannte Weise weiterleitet. Der intrazelluläre Cyo Level ist abhängig von der Kohlenstoffquelle sowie vom Sauerstoffgehalt und korreliert mit der Stärke der CCR (Dinamarca *et al.*, 2003; Morales *et al.*, 2006). Die Inaktivierung von Cyo in *P. putida* verhindert die Nutzung von einigen Aminosäuren, Benzoat, Fructose und verschiedenen organischen Säuren als Kohlenstoffquelle, sowie die Nutzung von Aminosäuren und Harnstoff als Stickstoffquelle (Daniels *et al.*, 2010).

1.8 Zielsetzungen

Die vorliegende Arbeit wurde im Rahmen des BMBF-Forschungsprojekts „*Systembiologie in Pseudomonas für die industrielle Biokatalyse*“ durchgeführt und untergliedert sich in unterschiedliche Teilprojekte, von denen einige komplett eigenständig bearbeitet oder für die Zuarbeiten in Form von gentechnisch veränderten Stämmen geleistet wurden:

- Essentieller Bestandteil dieser Arbeit war die gezielte Manipulation des Genoms von *P. putida* KT2440, um neue Syntheseschritte oder Stoffwechselwege generieren zu können. Daher sollte eine neue, effiziente Methode entwickelt werden, um mehrere Gene bzw. größere chromosomale Abschnitte sukzessive zu deletieren bzw. um neue Gene / Gencluster chromosomal zu integrieren.
- Im Teilprojekt „Biotransformation von Ferulasäure zu Vanillin“ sollte *P. putida* KT2440 genetisch so modifiziert werden, dass mit ruhenden, plasmidfreien Zellen Vanillin mit möglichst hoher Ausbeute und Produktivität aus Ferulasäure produziert werden kann.
- Das Teilprojekt „*De novo* Biosynthese von Vanillin mit *P. putida*“ wurde von der Arbeitsgruppe um Prof. Dr. G. Sprenger am Institut für Mikrobiologie der Universität Stuttgart bearbeitet. Hierfür sollten modifizierte *P. putida* Stämme konstruiert werden.
- Für die heterologe Proteinexpression in *P. putida* KT2440 sollte ein neues Expressionssystem auf Basis des MEK-induzierbaren Promotors aus *P. veronii* MEK700 etabliert werden. Dabei stand neben der Regulation die Charakterisierung der Promotorregion im Vordergrund. Des Weiteren sollte dieses System auf einen möglichen Einsatz in *E. coli* getestet werden.
- Auch für das Teilprojekt „Wege zur Synthese von Glyoxylsäure aus Ethylenglycol“, welches hauptsächlich von den Arbeitsgruppen um Prof. Dr. B. Hauer am Institut für Technische Biochemie der Universität Stuttgart sowie um Prof. Dr. A. Huber am Institut für Physiologie der Universität Hohenheim bearbeitet wurde, sollten modifizierte *P. putida* Stämme konstruiert werden.

2 Ergebnisse und Diskussion

Die Hintergründe, Ziele und Ergebnisse der bearbeiteten Teilprojekte des BMBF-Forschungsprojekts „*Systembiologie in Pseudomonas für die industrielle Biokatalyse*“ werden in den nachfolgenden Abschnitten zusammengefasst dargestellt und diskutiert. Die jeweils zugehörigen Publikationen sind Kapitel 4 zu entnehmen.

2.1 Genetische Manipulation von *P. putida* KT2440 - Entwicklung eines Gegenselektionssystems für markerlose Deletionen und Integrationen

Die genetische Manipulation von *P. putida* KT2440 stand im zentralen Mittelpunkt des gesamten BMBF-Forschungsprojekts. Trotz der mittlerweile vielen genetischen Werkzeuge für Pseudomonaden (Schweizer und de Lorenzo, 2004; Suh *et al.*, 2004), stellt die Isolation der gewünschten Klone noch immer einen mühsamen Prozess dar. Die Entwicklung von Gegenselektionssystemen bringt zwar eine deutliche Vereinfachung mit sich, allerdings kann deren Anwendung auch mit Problemen verbunden sein. So ist beispielsweise bei der Arbeit mit dem Saccharose/*sacB*-System ein exaktes Timing notwendig, da gezeigt werden konnte, dass sekundäre, *sacB*-inaktivierende Mutationen sehr leicht akkumulieren können (Muyrers *et al.*, 2000). Auch das Fusarsäure/*tetAR*-System ist nicht leicht zu handhaben, da die Selektionseffizienz eher gering und nicht stringent genug ist (Podolsky *et al.*, 1996). Andere Gegenselektionssysteme sind wiederum nicht universell einsetzbar. So kann z.B. das Streptomycin/*rpsL*-System bei *P. putida* aufgrund seines ArpABC-Efflux-Systems, das ihm eine natürliche Resistenz gegen Streptomycin verleiht (Kieboom und de Bont, 2001), nicht verwendet werden. Auch das für *P. putida* KT2440 entwickelte I-*Sce*I-System (Martínez-García und de Lorenzo, 2011) bringt Probleme mit sich, da zum einen das Helferplasmid mit dem Gen für die Endonuklease kuriert werden muss, und zum anderen eine strenge Regulation der I-*Sce*I-Expression gewährleistet werden muss, um die Zellen nicht vor der Rekombination abzutöten.

2.1.1 Etablierung eines 5-FU/*upp*-Gegenselektionssystems für *P. putida*

Für die genetische Manipulation von *P. putida* KT2440 wurde auf der Grundlage der toxischen Wirkung des Antimetaboliten 5-FU durch die UPRTase ein neues und effizientes Gegenselektionssystem etabliert. Die folgenden Ergebnisse (Kapitel 2.1.1 und 2.1.2) wurden publiziert (siehe Graf und Altenbuchner, 2010; Kapitel 4.1) und werden hier zusammengefasst dargestellt und diskutiert.

In einem Titerverfahren wurde zunächst die Minimale-Hemm-Konzentration [MHK] von 5-FU für *P. putida* auf 10 µg ml^{-1} bestimmt. Um das System in *P. putida* KT2440 anwenden zu können, musste zunächst das *upp* Gen (PP_0746) im Genom von *P. putida* KT2440 über homologe Rekombination deletiert werden. Die Deletion des *upp* Gens im erhaltenen 5-FU-resistenten Stamm *P. putida* ΔUPP4 wurde nach Isolierung der chromosomalen DNA über PCR bestätigt. Dieser Stamm zeigt auch bei der doppelten $MHK_{5\text{-}FU}$ von 20 µg ml^{-1} noch ein normales Wachstum. Für die späteren Selektionen wurde daher die doppelte MHK verwendet, um das Auftreten von falsch-positiven Klonen zu minimieren. Eine höhere Dosis wurde nicht getestet, da 5-FU in Dimethylsulfoxid [DMSO] gelöst werden musste, welches ebenfalls wachs-tumshemmende Effekte zeigen kann (Basch und Gadebusch, 1968), und damit zu Fehlinterpretationen führen könnte.

Um weitere Gene im Genom von *P. putida* ΔUPP4 zu deletieren bzw. um Gene zu integrieren, wurde der Integrationsvektor pJOE6261.2 konstruiert (Abb.2-1A). Dieser Vektor wurde mit einer Ampicillin-Resistenz (*bla*) für die Selektion in *E. coli* ausgestattet. Daneben wurden das *upp* Gen (PP_0746) aus *P. putida* KT2440 zusammen mit seinem eigenen Promotor für die spätere Gegenselektion, sowie ein Kanamycin-Resistenzgen (*neo*) für den ersten Selektionsschritt in *P. putida* integriert. Ein Polylinker diente als Insertionsstelle für die homologen Regionen. Um ein Gen zu deletieren und polare Effekte zu vermeiden, wurden jeweils ca. 1 kb der stromauf- und stromabwärts liegenden Sequenzen dieses Gens über PCR so amplifiziert, dass Start- und Stoppcodon des zu deletierenden Gens erhalten blieben (Abb. 2-1B). Durch eine 3-Fragment-Ligation wurden anschließend die homologen Regionen über den Polylinker in den Integrationsvektor pJOE6261.2 gebracht.

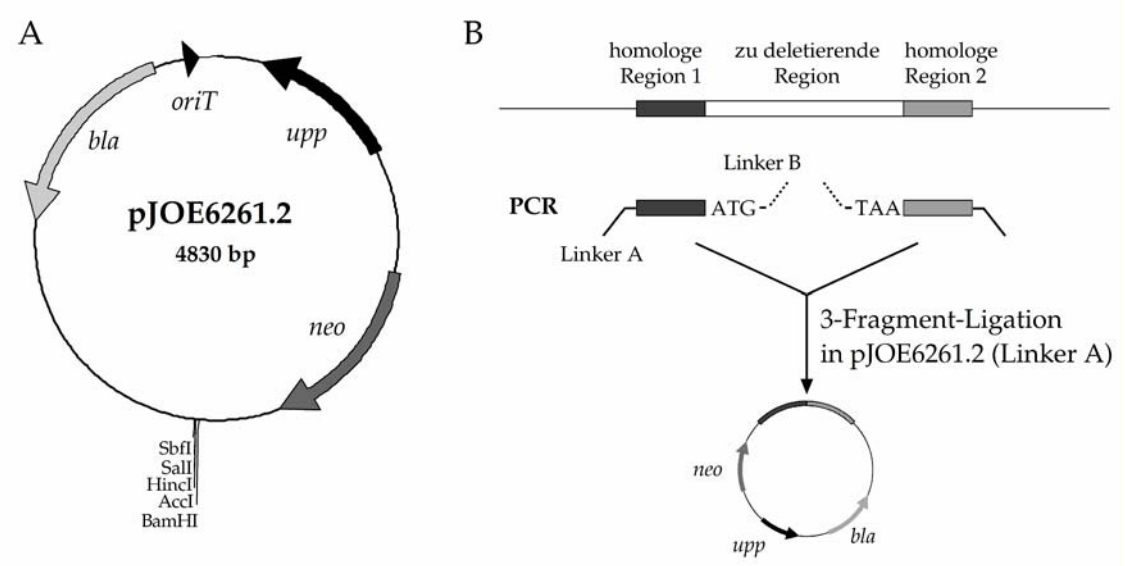


Abbildung 2-1: (**A**) Physikalische Karte des Klonierungsvektors pJOE6261.2. Neben den Resistenzgenen gegen Ampicillin (*bla*) und Kanamycin (*neo*) und dem Gen für die UPRTase (*upp*) ist die Polylinker-Region eingezeichnet, in welche die homologen Regionen eingefügt wurden. (**B**) Vorgehensweise bei der Konstruktion der Integrationsvektoren: Nach PCR-Amplifikation der stromauf- und stromabwärts liegenden Sequenzen (jeweils ca. 1 kb) erfolgt bei der 3-Fragment-Ligation die Verknüpfung der beiden PCR-Fragmente über den Linker B (6 bp-Restriktionsschnittstelle), und die Insertion in pJOE6261.2 über den Linker A (SbfI, SalI, HincII, AccI oder BamHI).

Die Deletion der chromosomalen Region verlief in zwei Schritten (Abb. 2-2): Im ersten Schritt erfolgte die Integration des in *P. putida* nicht replikationsfähigen Vektors über einfache homologe Rekombination, was zu Kanamycin-resistenten und 5-FU-sensitiven Zellen führte. Dazu wurden die konstruierten Integrationsvektoren in elektrokompetente, 5-FU-resistente *P. putida* Zellen gebracht. Die Transformationseffizienz betrug im Mittel etwa 25 cfu pro µg Plasmid-DNA bei Verwendung von etwa 5×10^9 elektrokompetenten Zellen. Im zweiten Schritt wurde einer dieser Transformanten unter nicht-selektiven Bedingungen inkubiert, wobei über ein weiteres Rekombinationsereignis der Vektor wieder aus dem Genom entfernt wurde und aufgrund seiner Replikationsunfähigkeit verloren ging. Nach Gegenselektion auf Wachstum auf 5-FU-haltigem Nährmedium, führte dies wieder zu Kanamycin-sensitiven und 5-FU-resistenten Klonen. Mit einer theoretisch 50%igen Wahrscheinlichkeit sollte dabei entweder der ursprüngliche Zustand wiederhergestellt oder das Gen deletiert worden sein. Die Modifikationen wurden über eine Kolonie-PCR überprüft.

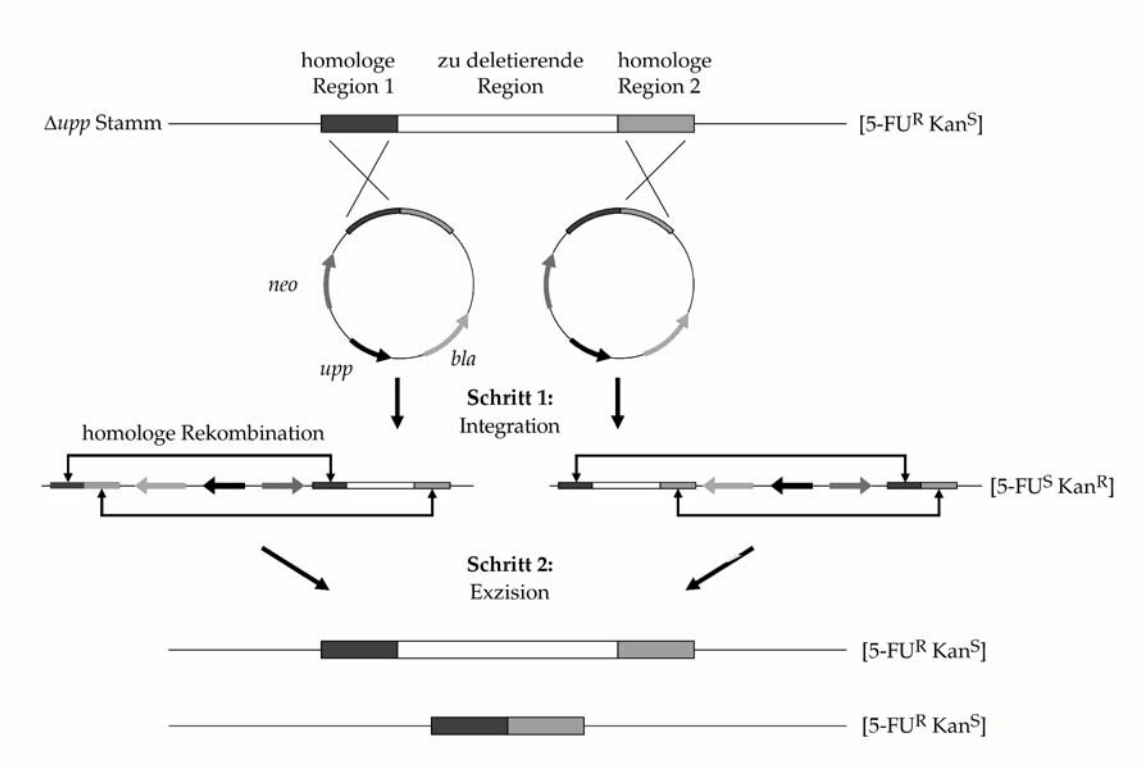


Abbildung 2-2: Schematische Darstellung des entwickelten 5-FU/*upp*-Gegenselektionssystems für *P. Putida* (verändert nach Graf und Altenbuchner, 2011). Die Integrationsvektoren enthalten neben den homologen Regionen (jeweils ca. 1 kb), Resistenzgene gegen Kanamycin (*neo*) und Ampicillin (*bla*), sowie das *upp* Gen für die Gegenselektion. Im ersten Schritt integriert der Vektor über homologe Rekombination in das Genom eines 5-FU-resistenten *P. putida* Stamms (Δ*upp*). Der resultierende Stamm ist sensitiv gegen 5-FU und resistent gegen Kanamycin. Unter nicht selektiven Bedingungen erfolgt über homologe Rekombination im zweiten Schritt die Exzision des Vektors, wobei entweder der Ausgangszustand wiederhergestellt oder die gewünschte Region deletiert wird. Die Selektion der Stämme mit möglicher Deletion erfolgt durch Wachstum auf 5-FU-haltigem Medium.

2.1.2 Anwendung des neuen Gegenselektionssystems

Um die Leistungsfähigkeit des neuen Systems zu testen, wurden hinsichtlich der Konstruktion eines zukünftigen Produktionsstamms Gene und Gencluster unterschiedlicher Länge deletiert:

- Zunächst wurde das ca. 26 kb lange Gen für das Oberflächenadhäsionsprotein LapA (PP_0168) in 40% der getesteten Klone deletiert. LapA stellt neben LapF einen essentiellen Faktor für die Biofilmbildung dar (Reva *et al.*, 2006). Mithilfe eines modifizierten Biofilm-Assays (Merritt *et al.*, 2005) konnte gezeigt werden,

dass sich die Zellen durch die Deletion von *lapA* nicht mehr als Biofilm an Oberflächen anheften können.

- Des Weiteren wurde ein ca. 65 kb langes Gencluster, welches die für die Flagellen-Biosynthese verantwortlichen Gene PP_4333 - PP_4396 enthält, in 20% der getesteten Klone deletiert. Da kein Elektronenmikroskop zur Verfügung stand, wurden die Abwesenheit von Flagellen und die damit verloren gegangene Motilität der Zellen indirekt über einen Schwärmagartest nachgewiesen.
- Damit die Zellen aufgenommene DNA nicht so schnell abbauen können, wurden die für Endonukleasen codierenden, 693 bp bzw. 966 bp langen Gene PP_2451 (*endA-1*) und PP_3375 (*endA-2*) in 10 bzw. 30% der getesteten Klone deletiert. Die Deletion der beiden Endonukleasen hatte jedoch im Vergleich zum Wildtyp (*P. putida* KT2440) keinen signifikanten Einfluss auf die Transformationseffizienz.

Tatsächlich konnte der theoretische Maximalwert von 50% richtigen Klonen bei keiner der durchgeführten Modifikationen erzielt werden. Die Deletionswahrscheinlichkeit lag nur zwischen 10 und 40%. Eine Korrelation mit der Länge der zu deletierenden Region konnte dabei nicht festgestellt werden. Zu den Gründen für den Ausbeuteverlust, die bereits auch schon durch Fabret *et al*. (2002) für die Anwendung des Systems bei *B. subtilis* erörtert wurden, zählen eine mögliche Inaktivierung des *upp* Gens durch Mutation oder Suppressormutationen. Diese könnten der Zelle zu einer Resistenz gegen 5-FU verhelfen, indem z.B. dessen Aufnahme in die Zelle verhindert wird. Außerdem denkbar wären gesteigerte Expressionsraten der Gene für die Dihydropyrimidin-Dehydrogenase (PP_4038), die 5-FU in 5,6-Dihydrouracil umwandelt und damit unschädlich macht, sowie für die Thymidin-Kinase (PP_1414), die Thymidin verwertet und es in dTMP umwandelt.

Dennoch steht mit dem 5-FU/*upp*-Gegenselektionssystem eine sehr effiziente Methode für die genetische Manipulation von *P. putida* zur Verfügung, die neben *B. subtilis* auch schon für *Enterococcus faecalis, Lactobacillus acidophilus* und *Desulfovibrio vulgaris* erfolgreich adaptiert wurde (Fabret *et al.*, 2002; Goh *et al.*, 2009; Keller *et al.*, 2009; Kristich *et al.*, 2005). Mit diesem System konnten sowohl kleine Gene als auch große Gencluster einfach, schnell und markerlos deletiert werden. Integrationen und

Punktmutationen sind mit diesem System ebenfalls möglich. Der entscheidende Vorteil dieses Systems liegt vor allem darin, dass es beliebig oft und sukzessive angewendet werden kann, ohne Spuren, wie z. B. Sequenzen für eine ortsspezifische Rekombinase, zu hinterlassen, die später zu Problemen führen könnten. Außerdem wird auf den Einsatz von Helferplasmiden verzichtet, die vor der nächsten Deletion erst kuriert werden müssten. Einziger Nachteil ist die anfängliche Deletion des chromosomalen *upp* Gens, die in jedem *P. putida* Stamm, der modifiziert werden soll, zuvor durchgeführt werden muss. Eine alternative Verwendung von 5-Fluorcytosin würde gegenüber dem 5-FU/*upp*-Gegenselektionssystem keinen Vorteil bringen, da *P. putida* KT2440 ein Gen für die Cytosin-Deaminase (*codA*, PP_3189) besitzt (Nelson *et al.*, 2002), welches ebenfalls erst deletiert werden müsste.

2.2 Biotransformation von Ferulasäure zu Vanillin mit ruhenden *P. putida* Zellen

P. putida ist aufgrund seines ausgeprägten Aromatenstoffwechsels prädestiniert für die industrielle Produktion von Vanillin aus Ferulasäure. Im Rahmen des BMBF-Forschungsprojekts wurde daher das Ziel einer möglichst effizienten Biotransformation mit ruhenden *P. putida* Zellen verfolgt, indem (i) der Vanillin-Abbau gestoppt und (ii) die Umsetzungsrate maximiert werden sollte (Abb. 2-3).

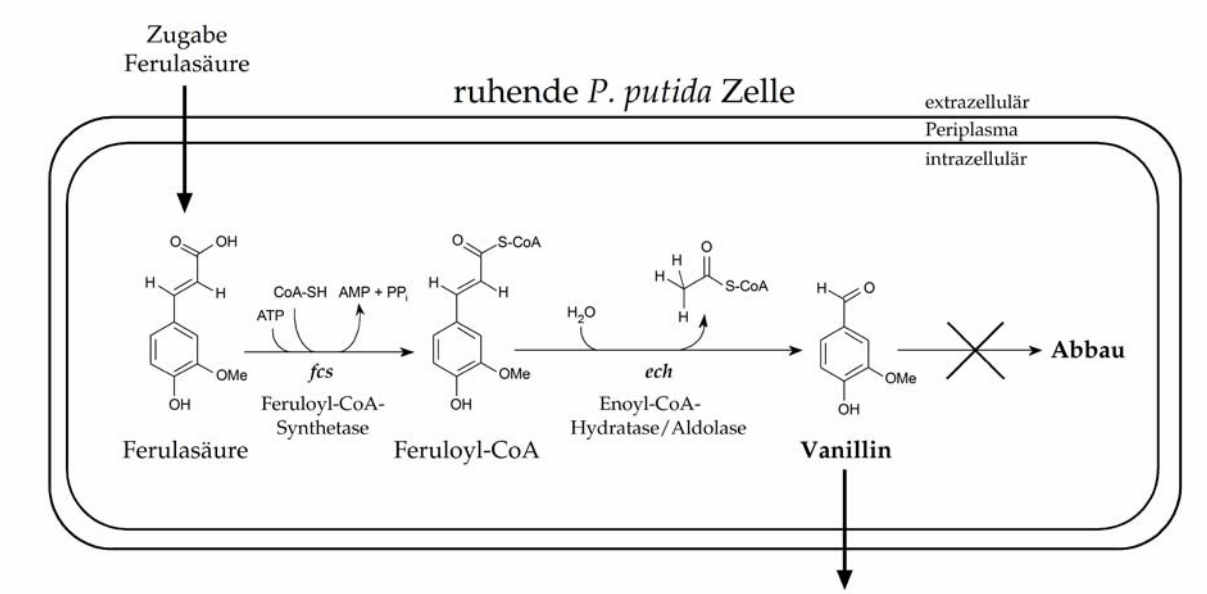


Abbildung 2-3: Schematische Darstellung des Biotransformationsprozesses von Ferulasäure zu Vanillin mit ruhenden *P. putida* Zellen. Der Transport von Ferulasäure in die Zelle bzw. von Vanillin aus der Zelle erfolgt passiv durch einfache bzw. erleichterte Diffusion (dicke Pfeile). Die Umsetzung erfolgt intrazellulär mithilfe der überexprimierten Feruloyl-CoA-Synthetase [Fcs] und Enoyl-CoA-Hydratase/Aldolase [Ech].

Um die Umsetzungsrate von Ferulasäure zu Vanillin zu verbessern, ist der Level der intrazellulären Enzyme Feruloyl-CoA-Synthetase [Fcs] und Enoyl-CoA-Hydratase/Aldolase [Ech] entscheidend. Eine Überexpression der jeweiligen Gene kann man dabei entweder chromosomal oder episomal über den Einsatz von Expressionsvektoren erreichen. Der Einsatz von Vektoren bringt zwar den Vorteil einer hohen Gendosis mit sich, birgt allerdings das Risiko von Ausbeuteverlusten aufgrund

möglicher Instabilitäten des Vektors. Um eine hohe Stabilität des Vektors zu gewährleisten, müssten als Selektionsdruck z.B. Antibiotika verwendet werden. Diese sollten bei der Produktion von einem Zusatzstoff für die Nahrungsmittel- und Kosmetikindustrie, wie Vanillin, allerdings nicht zum Einsatz kommen. In diesem Teil der Arbeit wurde daher ein plasmidfreier Ansatz verfolgt. Dabei wurde *P. putida* KT2440 mithilfe des entwickelten 5-FU/*upp*-Gegenselektionssystems genetisch so modifiziert, dass mit ruhenden Zellen die bis dato höchste Ausbeute an Vanillin aus Ferulasäure mit einem *Pseudomonas* Stamm produziert werden konnte. Die folgenden Ergebnisse (Kapitel 2.2.1 – 2.2.5) wurden publiziert (siehe Graf und Altenbuchner, 2014; Kapitel 4.2) und werden hier zusammengefasst dargestellt und diskutiert.

2.2.1 Konstruktion eines Vanillin negativen Stamms

Da bei der Biotransformation von Ferulasäure zu Vanillin ruhende Zellen zum Einsatz kommen sollten, die in der späteren, großindustriellen Anwendungsphase evtl. immobilisiert werden, wurde zunächst die Biofilmbildung durch Deletion der *lapABC* Gene (PP_0166 - PP_0168) mithilfe des 5-FU/*upp*-Gegenselektionssystems verhindert (GN23). Da *P. putida* KT2440 auf Vanillin als alleiniger Kohlenstoffquelle wachsen kann (Overhage *et al.*, 1999b; Plaggenborg *et al.*, 2003), wurde im nächsten Schritt, um den Abbau von Vanillin zu Vanillinsäure zu verhindern, das Gen *vdh* (PP_3357) für die Vanillin-Dehydrogenase in GN23 deletiert (GN235). Im Gegensatz zu den *P. fluorescens* Stämmen AN103 und BF13 (Di Gioia *et al.*, 2010; Martínez-Cuesta *et al.*, 2005) reichte die Deletion von *vdh* hier nicht aus, um den Abbau von Vanillin zu stoppen. Der erhaltene Stamm GN235 war immer noch in der Lage mit Vanillin als alleiniger Kohlenstoff- und Energiequelle zu wachsen. Dieses Verhalten zeigten auch *Pseudomonas* sp. HR199Δ*vdh* sowie *P. putida* KT2440*vdh*ΩKm (Overhage *et al.*, 1999a; Plaggenborg *et al.*, 2003).

Bisher konnte noch nicht herausgefunden werden, welche weiteren Gene im Abbau von Vanillin eine Rolle spielen. Aus diesem Grund wurde mit GN235 eine Transposonmutagenese durchgeführt. Der Vergleich von ca. 20.000 Klonen, die auf Glucose aber nicht mehr auf Vanillin wachsen konnten, brachte nur 2 Klone hervor,

bei denen jeweils die Inaktivierung des *modA* Gens (PP_3828) durch das Transposon mini-Tn*5495* (Onaca *et al.*, 2007) zu diesem Phänotyp führte. ModA ist ein periplasmatisches Molybdat-Bindeprotein und Teil des Molybdat-ABC-Transporters ModABC, der für die Molybdän-Toleranz und -Homöostase verantwortlich ist (Cánovas *et al.*, 2003; Nelson *et al.*, 2002). Die Deletion des gesamten, mutmaßlichen Operons inklusive der Gene *modABC* (PP_3827 – PP_3832) in GN235 führte zum Stamm GN276 mit identischem Phänotyp zur Transposonmutante GN275. In *Pseudomonas* wurden Molybdat-Ionen als Cofaktoren von Oxidoreduktasen identifiziert (Blaschke *et al.*, 1991; Frunzke *et al.*, 1993; Koenig und Andreesen, 1990). Da bei der Transposonmutagenese nur die *modA* Mutante gefunden wurde, ist es möglich oder sogar wahrscheinlich, dass in *P. putida* KT2440 mehrere, bisher unbekannte Molybdat-abhängige Oxidoreduktasen existieren, die Vanillin als Substrat erkennen und umsetzen können. Bisher konnten diese Enzyme jedoch nicht identifiziert werden.

2.2.2 Biotransformation mit *P. putida* GN276

Mit *P. putida* GN276 wurde nun ein Stamm erhalten, der Vanillin nicht mehr als Kohlenstoffquelle verwerten kann und bei Zugabe von Ferulasäure Vanillin akkumuliert. Die Umsetzung von Ferulasäure zu Vanillin erfolgte mit 5×10^9 ruhenden Zellen. Diese wurden zuvor in LB-Medium angezogen und für 6 h mit Ferulasäure induziert, um die Expression der Gene *ech* und *fcs* für die Umsetzung zu induzieren. Die Zellen wurden in Natriumphosphatpuffer pH 7,2 aufgenommen und mit 10 mM Ferulasäure bei 30 °C inkubiert. Die Δ*vdh* Mutante GN235 zeigte während der gesamten Umsetzung keine Akkumulation von Vanillin. Mit der Δ*vdh* Δ*modABC* Doppelmutante GN276 konnten nach 18 h Umsetzung 5,2 mM Vanillin gebildet werden. Allerdings entstanden dabei auch die Nebenprodukte Vanillylalkohol und Vanillinsäure mit Konzentrationen von 1,5 bzw. 0,3 mM. Die Ferulasäure konnte mit diesem Stamm und unter den gewählten Bedingungen somit nicht vollständig umgesetzt werden.

2.2.3 Stammoptimierung zur Steigerung der Ausbeute und Produktivität

Um die Ausbeute und Produktivität zu verbessern, wurde unter Zuhilfenahme des 5-FU/*upp*-Gegenselektionssystems ein starker, IPTG-induzierbarer *tac*-Promotor vor die Gene *ech* und *fcs* integriert (GN299). Für die Regulation wurde zusätzlich das Gen $lacI^q$ für den Lac-Repressor chromosomal integriert. Damit war es möglich die Expression der Gene *ech* und *fcs* über IPTG und nicht mehr über Ferulasäure zu induzieren. Mit *P. putida* GN299 konnten bereits nach 5 h ungefähr 83% der Ferulasäure umgesetzt werden. Da der ursprüngliche Promotor vermutlich schwächer als der integrierte *tac*-Promotor war, führten die nun höheren intrazellulären Konzentrationen von Ech und Fcs zu einer deutlichen Steigerung der Umsatzrate. In einem vergleichbaren System mit einem *P. fluorescens* Stamm, mit dem bisher die höchsten Ausbeuten bei der Biotransformation von Ferulasäure zu Vanillin erzielt wurden, wurden *ech* und *fcs* auf einem Vektor mit niedriger Kopienzahl überexprimiert und die Umsetzung in einem Bioreaktor durchgeführt (Di Gioia *et al.*, 2010). Dabei wurden nach 5 bzw. 24 h Umsetzung molare Ausbeuten von 63 bzw. 84% erzielt. Damit weist dieses System zwar eine ähnlich hohe Ausbeute, jedoch eine wesentlich schlechtere Produktivität auf als das neue System mit *P. putida* GN299. Allerdings konnten weder die Variation von Induktionszeit, Induktor- und Zellmenge die Ausbeute und Produktivität mit GN299 weiter steigern.

2.2.4 Minimierung der Nebenproduktbildung

Wie GN276, zeigt auch GN299 die Bildung der Nebenprodukte Vanillylalkohol und Vanillinsäure in ähnlich hohen Konzentrationen. Interessanterweise wurde eine Bildung von Vanillylalkohol bei *P. fluorescens* nicht beobachtet (Di Gioia *et al.*, 2010). Da diese Zellen im Bioreaktor und nicht im Schüttelkolben angezogen wurden, kann ein möglicher Einfluss der Wachstumsbedingungen auf die Nebenproduktbildung nicht ausgeschlossen werden. Eine chemische Oxidation durch Luftsauerstoff von Vanillin zu Vanillinsäure konnte bei *P. fluorescens* durch Variation der Begasungsrate ausgeschlossen werden, wonach es sich also um eine enzymatisch katalysierte Reaktion handeln muss (Di Gioia *et al.*, 2010).

Um die Bildung dieser Nebenprodukte zu senken und damit die molare Ausbeute noch weiter zu steigern, wurden weitere Deletionen durchgeführt: Beispielsweise wurde der von Overhage *et al*. (1999b) postulierte, alternative Stoffwechselweg von Ferula- zu Vanillinsäure (Abb. 1-2) durch Deletion der Gene PP_3354 (Acyl-CoA-Dehydrogenase) und PP_3355 (*aat*, β-Ketothiolase) in GN299 inaktiviert. Dies führte jedoch zu geringeren Umsetzungsraten und Ausbeuten. Da diese beiden Gene zum gleichen Operon wie *ech* und *fcs* gehören (Nelson *et al*., 2002), wird vermutet, dass durch die Deletion evtl. die Stabilität der mRNA negativ beeinträchtigt wurde und damit weniger Ech und Fcs für die Umsetzung zur Verfügung standen.

Anhand von Proteomanalysen, die im Rahmen des BMBF-Forschungsprojekts ebenfalls durchgeführt wurden, konnten zwei Aldehyd-Dehydrogenasen identifiziert werden (PP_2680 und PP_0545), deren Expression bei Wachstum von *P. putida* KT2440 auf Vanillin hochreguliert wird (Simon, Pfannstiel und Huber, unveröffentlichte Daten, Manuskript in Bearbeitung). Die Gene für diese beiden Aldehyd-Dehydrogenasen sowie das Gen für die Benzaldehyd-Dehydrogenase (PP_1948), von der vermutet wird, dass sie Vanillin ebenfalls als Substrat erkennen kann, wurden nacheinander deletiert. Durch die Deletion dieser drei Gene wurde ein Stamm erhalten (GN442), der aus 10 mM Ferulasäure in nur 3 h bis zu 8,6 mM Vanillin produziert. Allerdings entstehen auch bei diesem Stamm geringe Mengen an Vanillylalkohol (0,8 mM) und Vanillinsäure (0,1 mM). Diese Ausbeuteverluste sind jedoch verhältnismäßig gering, so dass mit GN442 die höchste Produktivität erzielt werden konnte, die bisher mit einem *Pseudomonas* Stamm beschrieben wurde.

Weder die Transposonmutagenese noch die Proteomanalyse gaben Aufschluss über die Existenz einer bisher unbekannten Dehydrogenase, die Vanillin zu Vanillylalkohol bzw. Vanillinsäure umsetzen kann. Es ist anzunehmen, dass mehrere Enzyme daran beteiligt und daher schwer zu identifizieren sind.

Es konnte beobachtet werden, dass die Nebenproduktbildung mit zunehmender Umsetzungsdauer ansteigt, so dass die Reduktion von Vanillin zu Vanillylalkohol ein Detoxifikationsmechanismus sein könnte, der auch schon bei rekombinanten *E. coli* Stämmen beobachtet wurde (Overhage *et al*., 2003). Die Toxizität von Vanillin in hohen Konzentrationen wurde auch in dieser Arbeit nachgewiesen. Sowohl beim

Wachstum von *P. putida* Zellen auf Vanillin als auch bei den Umsetzungen wurde ein maximaler Grenzwert von ca. 13,5 mM festgestellt. Höhere Mengen Vanillin führen zur Wachstumshemmung sowie zur vermehrten Bildung von Vanillylalkohol und Vanillinsäure. Außerdem wird die Umsetzung von Ferulasäure gehemmt. Diese Produktinhibierung wurde auch bei rekombinanten *E. coli* Stämmen beobachtet (Overhage *et al.*, 2003), ist jedoch bei *P. putida* teilweise reversibel. Nach Entfernung des Vanillins durch Aufnahme der Zellen in neuem Puffer, konnten wieder neue Umsetzungen gestartet werden, allerdings mit einer deutlich geringeren Ausbeute von ca. 50%. Eine kontinuierliche Extraktion des Vanillins direkt bei der Umsetzung durch beispielsweise adsorbierendes Säulenmaterial könnte demnach zu noch höheren Ausbeuten führen, so wie es bereits zuvor mit anderen Systemen erfolgreich getestet wurde (Hua *et al.*, 2007; Lee *et al.*, 2009; Yoon *et al.*, 2007).

Ein weiterer Ansatz, die Reduktion von Vanillin zu Vanillylalkohol zu verhindern, war die intrazelluläre Konzentration der Reduktionsequivalente NADH + H^+ bzw. $FADH_2$ zu reduzieren, die die Bildung dieser Nebenprodukte begünstigen können. Sie werden v.a. im Citratzyklus gebildet, der auch bei der Regeneration des Cofaktors CoA, der für die Reaktion der Feruloyl-CoA-Synthetase benötigt wird, eine Rolle spielt. Um die Regenerationsrate von CoA aus Acetyl-CoA zu erhöhen, wurde mit rekombinanten *E. coli* Stämmen ein Ansatz entwickelt, der auf der Deletion der Isocitrat-Dehydrogenase und der Aktivierung des Glyoxylatzyklus beruht (Lee *et al.*, 2009). Dieses System hat außerdem den Vorteil, dass durch die Inaktivierung der Isocitrat-Dehydrogenase auch die Bildung der Reduktionsequivalente durch den Citratzyklus verhindert wird. Aus diesem Grund wurde versucht, die beiden Gene PP_4011 und PP_4012, die für eine Isocitrat-Dehydrogenase codieren, in *P. putida* zu deletieren. Allerdings schlug die Inaktivierung dieser Gene sowohl über Deletion durch das 5-FU/*upp*-Gegenselektionssystem als auch über einfache Disruption wiederholt fehl. Offensichtlich scheint die Isocitrat-Dehydrogenase essentiell für *P. putida* zu sein.

2.2.5 Übersicht der konstruierten *P. putida* Stämme

Tabelle 2-1: Liste der konstruierten *P. putida* Stämme (KT2440-Derivate) für die „Biotransformation von Ferulasäure zu Vanillin mit ruhenden *P. putida* Zellen". PP_0166-0168 = Oberflächenadhäsionsprotein; PP_0545 = Aldehyd-Dehydrogenase; PP_1948 = Benzaldehyd-Dehydrogenase; PP_2680 = Aldehyd-Dehydrogenase; PP_3354 = Acyl-CoA-Dehydrogenase; PP_3355 = β-Ketothiolase; PP_3827-3832 = Molybdat-ABC-Transporter.

Stamm	Relevanter Genotyp
ΔUPP4	Δ*upp*
GN23	Δ*upp* ΔPP_0166-0168
GN235	Δ*upp* ΔPP_0166-0168 Δ*vdh*
GN275	Δ*upp* ΔPP_0166-0168 Δ*vdh modA*::mini-Tn*5495*
GN276	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832
GN299	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 *lacI*^{q}-P_{tac}-*ech-fcs*
GN347	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_3354-3355 *lacI*^{q}-P_{tac}-*ech-fcs*
GN440	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 Δ*PP_2680 lacI*^{q}-P_{tac}-*ech-fcs*
GN441	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 *lacI*^{q}-P_{tac}-*ech-fcs*
GN442	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 ΔPP_1948 *lacI*^{q}-P_{tac}-*ech-fcs*

2.3 *De novo* Biosynthese von Vanillin mit *P. putida*

In der Arbeitsgruppe von Prof. Dr. G. Sprenger am Institut für Mikrobiologie der Universität Stuttgart sollte im Rahmen einer z.Z. noch laufenden Doktorarbeit von Silvia Lorenz ein System für die *de novo* Biosynthese von Vanillin mit *P. putida* etabliert werden. Hierfür wurden *P. putida* Stämme konstruiert und für weiterführende Experimente an S. Lorenz übergeben (Eine Publikation mit dem Thema der Kapitel 2.3.1 + 2.3.2 ist geplant).

2.3.1 *De novo* Biosynthese von Vanillin aus Glucose

Geplant war zunächst ein Stoffwechselweg, bei dem Vanillin aus Glucose über die Zwischenstufe Chorismat hergestellt werden sollte (Abb. 2-4). Die Umsetzung von Glucose über den Pentosephosphatweg und den Aromatenstoffwechsel zum 3,4-trans-Cyclohexadiendiol [3,4-trans-CHD] wurde für die Produktion von L-Phenylalanin mit *E. coli* beschrieben (Sprenger, 2007). Für die Umsetzung von Chorismat zum 3,4-trans-CHD und weiter zu Protocatechuat werden für *P. putida* Enzyme zur Abspaltung von Pyruvat und zur anschließenden Aromatisierung benötigt. Für den ersten Schritt wurden die Isochorismatasen EntB aus *E. coli* sowie PhzD aus dem Phenazin Biosynthese-Weg von *P. aeruginosa* als mögliche Kandidaten in Betracht gezogen. Für die Aromatisierung sollte anschließend die 2,3-Dihydro-2,3-Dihydroxybenzoatdehydrogenase EntA aus *E. coli* verwendet werden. Das entstandene Protocatechuat sollte durch Methylierung der 3'-OH-Gruppe mithilfe von S-Adenosylmethionin-abhängigen Catechol-*O*-Methyltransferasen weiter zu Vanillat umgesetzt werden. Hierbei sollten die humane Catechol-*O*-Methyltransferase sowie eine Variante aus dem Cyanobacterium *Synechocystis* sp. Stamm PCC 6803 [SynOMT] getestet werden (Kopycki *et al.*, 2008). Die Reduktion der Carboxylgruppe des Vanillats zur Aldehydgruppe stellt den letzten Schritt bei der Synthese von Vanillin dar. Da Vanillin mithilfe von *Nocardia* sp. NRRL 5646 Zellen aus Vanillinsäure hergestellt werden konnte (Li und Rosazza, 2000), wurden die Aromatische Carboxylat-Reduktase [ACAR] sowie die Phosphopantetheinyl-Transferase [PPTase] aus diesem Stamm gewählt (Lambalot *et al.*, 1996; Venkitasubramanian *et al.*, 2007).

Die PPTase wird benötigt, da sie den Phosphopantheteinyl-Rest des Coenzyms A auf das inaktive ACAR-Apoenzym transferiert, und dieses damit in das aktive ACAR-Holoenzym umwandelt. Für die Akkumulierung des gewünschten Produkts sollte außerdem der Abbau von Vanillin in *P. putida* verhindert werden. Die hierfür benötigten genetischen Modifikationen wurden im Rahmen dieser Arbeit mithilfe des entwickelten 5-FU/*upp*-Gegenselektionssystems für *P. putida* durchgeführt.

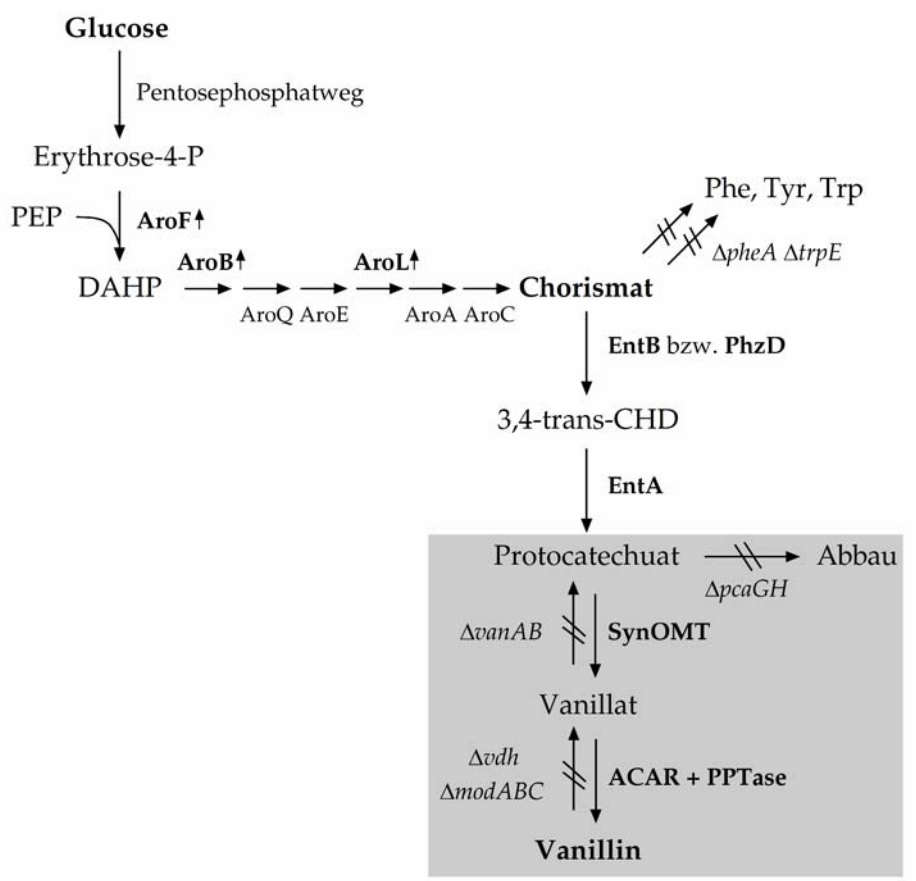


Abbildung 2-4: Geplanter Stoffwechselweg für die *de novo* Biosynthese von Vanillin aus Glucose über Chorismat bzw. aus Protocatechuat (grau hinterlegt) in *P. putida* KT2440**.** Die heterologen Enzymfunktionen AroF, AroB, AroL, EntA und EntB aus *E. coli*, PhzD aus *P. aeruginosa*, SynOMT aus *Synechocystis* sp., sowie ACAR (Aromatische Carboxylat-Reduktase) und PPTase (Phosphopantetheinyl-Transferase) aus *Nocardia* sp. NRRL 5646 sollten heterolog exprimiert und die Gene für die Abbauwege bzw. gegenläufige Reaktionen deletiert werden (durchgestrichene Pfeile). PEP = Phosphoenolpyruvat, DAHP = 3-Desoxy-D-arabinoheptulosonat-7-phosphat.

Das erste Ziel war die Entwicklung eines *P. putida* Stamms, der Chorismat akkumuliert und nicht weiter zu den aromatischen Aminosäuren Phenylalanin, Tryptophan und Tyrosin abbaut. Aus diesem Grund sollten die Gene *pheA* (PP_1769) und *trpE* (PP_0417) deletiert werden. Außerdem sollte der Vanillinabbau durch Deletion der Gene für die Vanillin-Dehydrogenase (PP_3357) und des Molybdat-ABC-Transportsystems (PP_3827-3832) verhindert werden. Des Weiteren war eine Steigerung des Stoffflusses zu Chorismat durch chromosomale Integration und Überexpression der *E. coli* Gene für die DAHP-Synthase^{Tyr} (*aroF*), 3-Dehydroquinat-Synthase (*aroB*) und Shikimatkinase II (*aroL*) geplant. Hierbei wurden zwei Ansätze verfolgt: Zum einen die Integration von *aroFBL* unter gemeinsamer Kontrolle des starken Promotors P_{tac} in die Region des Genclusters für die Chemotaxis und Flagellen-Synthese (PP_4333-4396, *cheW* bis *flgN*), wobei diese deletiert werden sollte. Zum anderen sollten in diese Region anstatt *aroFBL* nur *aroFB* integriert werden, während *aroL* separat anstelle der Gene für die Vanillat-*O*-Demethylase (PP_3736-3737, *vanAB*) integriert werden sollte - jeweils unter Kontrolle des Promotors P_{tac}.

2.3.2 *De novo* Biosynthese von Vanillin aus Protocatechuat

Ein anderer Ansatz verfolgte das Ziel, Vanillin durch Zugabe von Protocatechuat in *P. putida* zu produzieren (Abb. 2-4; graue Box). Protocatechuat sollte über die heterolog exprimierten Gene der Enzyme SynOMT und ACAR / PPTase zu Vanillat und weiter zu Vanillin umgesetzt werden. Hierfür sollte zunächst der Abbau von Protocatechuat zu 3-Carboxy-cis,cis-Muconsäure durch die Protocatechuat-3,4-Dioxygenase durch Deletion der Gene *pcaGH (*PP_4655-4656) gestoppt werden. Die weiteren Deletionen sollten Schritt für Schritt erfolgen: Damit das, durch die SynOMT, gebildete Vanillat nicht wieder demethyliert wird, sollte zunächst die Aktivität der Vanillat-*O*-Demethylase durch Deletion der Gene *vanAB* (PP_3736-3737) unterbunden werden. Die nächste Reaktion, die Umsetzung von Vanillat zu Vanillin durch die ACAR und PPTase, sollte durch die Deletionen von *vdh* (PP_3357) und *modABC* (PP_3827-3832), sowie des Gens für die Benzaldehyd-Dehydrogenase (PP_1948) nicht mehr in die umgekehrte Richtung ablaufen können.

2.3.3 Übersicht der konstruierten *P. putida* Stämme

Tabelle 2-2: Liste der konstruierten *P. putida* Stämme (KT2440-Derivate) für die „*De novo* Biosynthese von Vanillin aus Glucose bzw. Protocatechuat". PP_0166-0168 = Oberflächenadhäsionsprotein; PP_1948 = Benzaldehyd-Dehydrogenase; PP_3827-3832 = Molybdat-ABC-Transporter.

Stamm	Relevanter Genotyp
Ansatz über Glucose	
ΔUPP4	Δ*upp*
GN23	Δ*upp* ΔPP_0166-0168
GN88	Δ*upp* ΔPP_0166-0168 Δ*pheA*
GN91	Δ*upp* ΔPP_0166-0168 Δ*trpE*
GN118	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA*
GN243	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh*
GN295	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh* ΔPP_3827-3832
GN317	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh* ΔPP_3827-3832 Δ*cheW-flgN*::*aroFBL*
GN334	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh* ΔPP_3827-3832 Δ*cheW-flgN*::*aroFB*
GN348	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh* ΔPP_3827-3832 Δ*cheW-flgN*::*aroFB* Δ*pcaGH*
GN360	Δ*upp* ΔPP_0166-0168 Δ*trpE* Δ*pheA* Δ*vdh* ΔPP_3827-3832 Δ*cheW-flgN*::*aroFB* Δ*pcaGH* Δ*vanAB*::*aroL*
Ansatz über Protocatechuat	
ΔUPP4	Δ*upp*
GN396	Δ*upp* Δ*pcaGH*
GN399	Δ*upp* Δ*pcaGH* Δ*vanAB*
GN400	Δ*upp* Δ*pcaGH* Δ*vanAB* Δ*vdh*
GN401	Δ*upp* Δ*pcaGH* Δ*vanAB* Δ*vdh* ΔPP_3827-3832
GN426	Δ*upp* Δ*pcaGH* Δ*vanAB* Δ*vdh* ΔPP_3827-3832 ΔPP_1948

2.4 Charakterisierung eines neuen, MEK-induzierbaren Expressionssystems für *P. putida* KT2440 und *E. coli* JM109

Bisher wurden nur wenige Expressionssysteme für die heterologe Genexpression in *Pseudomonas* sp. entwickelt. Das Pm/XylS-Expressionssystem des TOL Plasmids aus *P. putida* wird in den meisten Fällen eingesetzt und wird mittlerweile durch die Firma Vectron Biosolutions AS (Tronsheim, Norwegen) kommerziell für *P. fluorescens* vermarktet[2]. Im Rahmen des BMBF-Projekts sollte daher ein neues Expressionssystem für *P. putida* KT2440 entwickelt werden. Die folgenden Ergebnisse (Kapitel 2.4.1 - 2.4.4) basieren auf einer Publikation (siehe Graf und Altenbuchner, 2013; Kapitel 4.3) und werden hier kurz dargestellt und diskutiert.

2.4.1 Etablierung eines MEK-induzierbaren Expressionssystems

Als geeigneter und vielversprechender Ansatz stellte sich ein auf dem Abbau von Methylethylketon [MEK] beruhendes System aus *P. veronii* MEK700 heraus (Onaca *et al.*, 2007). Dieses Expressionssystem sollte in *E. coli* und *P. putida* charakterisiert und getestet werden. Für die Charakterisierung wurde ein pBBR1MCS2-basierter Vektor verwendet, der in beiden Spezies replizieren kann. Als Reporter wurde die β-Galactosidase, codiert durch *lacZ*, verwendet. Die *lacZ*-Expression sollte unter Kontrolle des Promoters P_{mekA} des *mekAB* Operons unter verschiedenen Bedingungen untersucht werden. Um die Größe des Vektors weiterhin gering zu halten, wurden neben einem gemeinsamen Replicon für *E. coli* und *P. putida* auch ein gemeinsamer Resistenzmarker verwendet. Hierfür wurde das Gen für die Aminoglycosid-3-*N*-Acetyltransferase AAC(3)-IV aus *E. coli* gewählt, welches zu einer Resistenz gegen das Aminoglycosid-Antibiotikum Apramycin führt (Magnet und Blanchard, 2005). Mit dem erhaltenen Vektor (pNG217.1) konnte nach Induktion mit MEK jedoch keine β-Galactosidase-Aktivität festgestellt werden. Erst nachdem das Gen für den Regulator MekR unter Kontrolle seines eigenen Promotors in entgegengesetzter Orientierung zur P_{mekA}-*lacZ*-Expressionskassette in den Vektor integriert wurde

[2] http://www.vectronbiosolutions.com

(pNG247.1), zeigte sich eine hohe β-Galactosidase-Aktivität. MekR, der aufgrund seiner Struktur in die AraC/XylS-Familie von Transkriptionsaktivatoren eingeordnet werden kann, wird demnach für die Expression benötigt, was bereits von Onaca *et al*. (2007) angenommen wurde.

Durch Variation der Induktormenge und Induktionszeit (stündlich und über Nacht) wurden die optimalen Bedingungen für das Expressionssystem in *E. coli* und *P. putida* bestimmt. Für die Induktion von 5 ml Kultur wurden 20, 40 oder 60 µl MEK zugegeben. Dies entspricht Ausgangskonzentrationen von 45, 90 bzw. 135 mM MEK. Die genauen Grenzen bzw. Molaritäten für die Toleranz gegen MEK konnten aufgrund seiner hohen Flüchtigkeit allerdings nicht definiert werden, da die tatsächliche Konzentration von MEK im Kulturmedium trotz luftdicht verschlossener Inkubationsgefäße nach Induktion vermutlich deutlich niedriger liegt. Es zeigte sich jedoch, dass MEK in hoher Menge (60 µl / 135 mM) zu geringeren spezifischen Wachstumsraten führt. Dabei zeigt sich *E. coli* sensitiver gegen MEK als *P. putida*, welcher eine höhere Toleranz aufweist. Allgemein zeigen *Pseudomonas* Spezies eine höhere Toleranz gegen organische Lösungsmittel als andere Mikroorganismen, da sie Transporter verwenden, die organische Lösungsmittel aktiv aus der Zelle transportieren, und Veränderungen in der Membranzusammensetzung und -durchlässigkeit aufweisen (Ramos *et al.*, 2002; Sardessai und Bhosle, 2002 + 2004; Sikkema *et al.*, 1995). Ein weiterer, entscheidender Vorteil ist die Bildung von Biofilmen (Halan *et al.*, 2011), bei denen die extrazelluläre Matrix zur Toleranz beitragen kann. Aber auch Zellen ohne das für die Biofilmbildung essentielle Oberflächenadhäsionsprotein LapA, zeigen durch eine vermehrte Ausscheidung von Exopolysacchariden Kompensationseffekte, um Lösungsmittel besser tolerieren zu können (Martínez-Gil *et al.*, 2013). Die höchsten β-Galactosidase-Aktivitäten zeigten beide Spezies nach 4 h bei Zugabe von 40 µl (90 mM) MEK, mit 12.000 Miller Units [M.U.] bei *P. putida* KT2440 und 22.000 M.U. bei *E. coli* JM109.

Weitere Ketone mit unterschiedlich langer Kohlenwasserstoffkette wurden getestet, um das Spektrum möglicher Effektoren für MekR zu analysieren. Dazu wurden Aceton, 2-/3-Pentanon sowie 2-/3-Hexanon untersucht. Diese erwiesen sich jedoch hinsichtlich der geringeren β-Galactosidase-Aktivitäten und z.T. höheren Toxizität,

die sich in einer geringen spezifischen Wachstumsrate bemerkbar machte, im Vergleich zu MEK als schlechtere Induktoren. Da MEK in Analogie zu anderen Beispielen von Mitgliedern der AraC/XylS-Familie vermutlich allosterisch an die N-terminale Domäne von MekR bindet (Gallegos *et al.*, 1997), führt eine Verkürzung oder Verlängerung der Kohlenwasserstoffkette des Ketons zu einer schwächeren Bindung an MekR, der somit schwerer in seine aktive Form übergehen kann.

2.4.2 Charakterisierung der Promotorregion P_{mekA}

Der Transkriptionsstart der Promotorregion P_{mekA} wurde über Primer Extension Experimente identifiziert. Dabei zeigten sich sowohl bei *P. putida* als auch *E. coli* zwei Banden und damit zwei mögliche Startpunkte für die Transkription. Mutations- und Deletionsanalysen ließen vermuten, dass es sich bei der zweiten, weiter gelaufenen Bande um ein falsches Signal und nicht um einen tatsächlichen Transkriptionsstart handelt. Diese zweite Bande kann durch eine mögliche Bildung einer Stamm-Schleife-Struktur am 5'-Ende der *mekAB*-mRNA erklärt werden, da Reverse Transkriptasen an solchen Strukturen stagnieren und somit zu einem falschen Signal führen können (Olsen *et al.*, 1994). Solche Stamm-Schleife-Strukturen am 5'-Ende der mRNA sind in Prokaryoten häufig anzutreffen, da sie zur Stabilisierung und damit zur Erhöhung der Halbwertszeit beitragen (Grunberg-Manago, 1999; Varani, 1995). Demnach ist davon auszugehen, dass auch die *mekAB*-mRNA eine hohe Halbwertszeit besitzt und damit auch zu den hohen β-Galactosidase-Aktivitäten beiträgt.

Ausgehend vom Transkriptionsstart wurden die mutmaßlichen Bindestellen für die RNA-Polymerase abgeleitet. Dabei konnte allerdings keine typische -35 Region gefunden werden, da diese durch die in dieser Arbeit indirekt identifizierte Operatorsequenz verdeckt wird und damit auf einen Promotor der Klasse II hindeutet (Browning und Busby, 2004).

Da MekR nicht in löslicher und damit aktiver Form produziert werden konnte, musste die Bindestelle über Deletions- und Mutationsanalysen gefunden werden. Dabei wurde in der P_{mekA}-Region eine sich im Abstand von 6 bp direkt wiederholende Sequenz $CACCN_5CTTCAA$ identifiziert, die dem Erkennungsmuster

anderer Regulatoren der AraC/XylS-Familie ähnlich ist. Tatsächlich führte jedwede Veränderung dieser Sequenz zu einer mehr oder weniger starken Verringerung der β-Galactosidase-Aktivität, während eine Verkürzung der Promotorregion bis unmittelbar vor diese Sequenz keinen negativen Einfluss hatte. Eine solche Sequenz wurde in der Promotorregion von *mekR* nicht identifiziert, was zu der Annahme führte, dass MekR vermutlich konstitutiv exprimiert wird.

2.4.3 Untersuchungen zur Katabolitrepression

Das MekR/P_{mekA}-Expressionssystem lässt sich in Vollmedium (LB) mit MEK induzieren. Dies zeigt, dass keine Katabolitrepression [CCR] durch Aminosäuren stattfindet, die bei *Pseudomonas* die primäre Kohlenstoffquelle darstellen (Collier *et al.*, 1996; Rojo, 2010) und im Trypton-Anteil des LB-Mediums vorhanden sind. Allerdings wurde eine CCR durch Glucose beobachtet, durch die die β-Galactosidase-Aktivität unter MEK-induzierten Bedingungen in LB-Medium bei Zugabe von 0,2% (w/v) Glucose um ca. 30% reduziert wird. Da die Cytochrom-*o*-Ubiquinol-Oxidase [Cyo] hauptsächlich die Expression von Genen für Porine und Transporter reguliert (Morales *et al.*, 2006), und MEK vermutlich passiv durch einfache Diffusion in die Zelle aufgenommen wird, wurde in dieser Arbeit nur der Einfluss von Crc und PtsN als mögliche Regulatoren untersucht.

Die Inaktivierung von *crc* konnte die CCR durch Glucose nicht aufheben. Auch konnten für den Translationsrepressor Crc keine mRNA-Bindestellen mit der Konsensussequenz AA(C/U)AA(C/U)AA durch Sequenzanalyse der P_{mekR} und P_{mekA} Regionen identifiziert werden. Eine Crc-abhängige Repression durch Aminosäuren konnte ebenfalls ausgeschlossen werden, da (i) das MekR/P_{mekA}-Expressionssystem in LB-Medium induzierbar war, und (ii) keine Erhöhung des Expressionsniveaus in der Δ*crc* Mutante nach Induktion mit MEK festgestellt werden konnte.

Auch die Inaktivierung von *ptsN* führte zu keiner Veränderung im Expressionsverhalten bei Zugabe von Glucose. Bei der CCR durch Glucose wurde PtsN in *Pseudomonas* zwar als globaler Regulator identifiziert, stellt jedoch nicht den entscheidenden Schlüsselfaktor dar (Cases *et al.*, 2001; Rojo, 2010). Dieser Schlüssel-

faktor - sofern er existiert - konnte bisher noch nicht identifiziert werden. Somit kann auch im Falle des MekR/P_{mekA}-Expressionssystems über die CCR durch Glucose nur spekuliert werden. Cyo wurde zwar bisher nicht mit einer CCR durch Glucose in Verbindung gebracht, ein möglicher Einfluss kann aber nicht komplett ausgeschlossen werden. Eventuell interagieren auch mehrere verschiedene Regulationssysteme, wie beispielsweise beim Alkan- oder Toluolabbau in *P. putida*, bei denen Crc und Cyo bzw. Crc und PtsN gemeinsam agieren (Rojo, 2010). Die Verwendung von Doppelmutanten könnte darüber Aufschluss geben, ob Crc und PtsN evtl. gemeinsam für die CCR durch Glucose beim MekR/P_{mekA}-Expressionssystem verantwortlich sind und der Wegfall des einen Regulators durch den anderen kompensiert werden kann.

2.4.4 Vergleich mit etablierten Expressionssystemen für *P. putida*

Im Vergleich zu etablierten Expressionssystemen wie XylS/Pm und AraC/$araP_{BAD}$ zeigt das neue MekR/P_{mekA}-System sowohl in *E. coli* als auch in *P. putida* eine extrem niedrige Basalexpression, die bei beiden Spezies nur maximal 0,1% des induzierten Niveaus entspricht. Dadurch sind nach Induktion Erhöhungen des Expressionsniveaus um bis zu drei Größenordnungen möglich. Auf der anderen Seite liegt der Vorteil des XylS/Pm-Systems in einer höheren Gesamtaktivität, und der des AraC/$araP_{BAD}$-Systems im deutlich besser verträglichen Induktor Arabinose. Sowohl MEK als auch m-Toluylsäure führen bei zunehmender Dosis zu Wachstumshemmungen. Insgesamt stellt das neue System jedoch aufgrund der relativ hohen Expressionsraten und der extrem niedrigen Basalexpression eine geeignete Alternative für die Produktion von insbesondere toxischen Proteinen in *E. coli* und *P. putida* dar.

2.5 Erforschung des Ethylenglycol-Stoffwechsels in *P. putida*

In den Arbeitsgruppen von Prof. Dr. B. Hauer am Institut für Technische Biochemie der Universität Stuttgart sowie von Prof. Dr. A. Huber am Institut für Physiologie der Universität Hohenheim sollten Wege zur Synthese des industriell bedeutsamen Ausgangsstoffs Glyoxylsäure aus Ethylenglycol mit *P. putida* KT2440 und JM37 gefunden werden. Primäres Ziel war es, den Ethylenglycol-Stoffwechsel zu untersuchen und anhand der erhobenen Daten einen Stamm für die Produktion der Glyoxylsäure zu entwickeln. Hierfür wurden *P. putida* Stämme konstruiert und zur weiteren Bearbeitung an die Arbeitsgruppen übergeben. Die Ergebnisse wurden publiziert (siehe Mückschel *et al.*, 2012; Kapitel 4.4) und werden in diesem Kapitel kurz zusammengefasst.

Nach Analyse der Wachstumseigenschaften der beiden *P. putida* Stämme KT2440 und JM37 wurden über Proteom-Analysen mehrere Proteine identifiziert, deren Expression bei Anwesenheit von Ethylenglycol und Glyoxylsäure hochreguliert wird (Mückschel *et al.*, 2012). Dazu gehörten die Isocitrat-Lyase (*aceA*), die Glyoxylat-Carboligase (*gcl*), die Malat-Synthase G (*glcB*) und die 2-Methyl-isocitrat-Lyase (*prpB*), die in den beiden Stämmen unterschiedlich induziert werden und beim Abbau von Glyoxylsäure eine mögliche Rolle spielen. Mithilfe des 5-FU/*upp*-Gegenselektionssystems wurden u.a. die Gene für diese Enzyme im Rahmen dieser Arbeit in KT2440 deletiert (s. Tabelle 2-3) und die Stämme weitergegeben, so dass die Auswirkung auf den Ethylenglycol-Stoffwechsel untersucht werden konnte. Tatsächlich konnte durch die gleichzeitige Deletion von *aceA*, *gcl*, *glcB* und *prpB* ein *P. putida* Stamm (GN259) konstruiert werden, der Glyoxylsäure nach Umsetzung mit Ethylenglycol akkumulieren kann (Mückschel *et al.*, 2012).

Durch Deletion der Gene PP_2674 und PP_2679, die für periplasmatische, Pyrrolochinolinchinon [PQQ]-abhängige Alkohol-Dehydrogenasen codieren und in Gegenwart von Ethylenglycol um ein vielfaches hochreguliert waren, konnte gezeigt werden, dass diese beiden Enzyme in den ersten Schritten des Ethylenglycol-Stoffwechsels eine entscheidende Rolle spielen. Bei der Umsetzung von Ethylen-

glycol zu Glyoxylsäure ist jedoch nur die Doppelmutante (*P. putida* GN127) stark beeinträchtigt (Mückschel *et al.*, 2012).

Anhand der konstruierten Mutanten (s. Tabelle 2-3) und der damit gewonnen Daten konnten für die untersuchten *P. putida* Stämme KT2440 und JM37 mögliche Stoffwechselwege postuliert werden (s. Mückschel *et al.* (2012), Fig. 4). Für weiterführende Arbeiten zu diesem Thema wurden weitere Stämme konstruiert, bei denen die Gene PP_2680 und PP_0545, die für zwei Aldehyd-Dehydrogenasen codieren, deletiert wurden (s. Tabelle 2-3).

Tabelle 2-3: Liste der konstruierten *P. putida* Stämme (KT2440-Derivate) für das Teilprojekt „Wege zur Synthese von Glyoxylsäure aus Ethylenglycol". PP_0166-0168 = Oberflächenadhäsionsprotein; PP_0545 = Aldehyd-Dehydrogenase; PP_2674 = Alkohol-Dehydrogenase; PP_2679 = Alkohol-Dehydrogenase; PP_2680 = Aldehyd-Dehydrogenase.

Stamm	Relevanter Genotyp
ΔUPP4	Δ*upp*
GN104	Δ*upp* ΔPP_2679
GN116	Δ*upp* ΔPP_2674
GN127	Δ*upp* ΔPP_2674 ΔPP_2679
GN133	Δ*upp* Δ*prp*
GN140	Δ*upp* Δ*prp* Δ*gcl*
GN187	Δ*upp* Δ*prp* Δ*gcl* Δ*glcB*
GN259	Δ*upp* Δ*prpB* Δ*gcl* Δ*glcB* Δ*aceA*
GN284	Δ*upp* ΔPP_2680
GN332	Δ*upp* ΔPP_2674 ΔPP_2679 ΔPP_2680
GN343	Δ*upp* ΔPP_0545
GN344	Δ*upp* ΔPP_2674 ΔPP_2679 ΔPP_0545
GN345	Δ*upp* ΔPP_2680 ΔPP_0545
GN346	Δ*upp* ΔPP_2674 ΔPP_2679 ΔPP_2680 ΔPP_0545

3 Literaturverzeichnis

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4 Publikationen

Übersichtsverzeichnis der Publikationen

4.1 Development of a Method for Markerless Gene Deletion in *Pseudomonas putida*

Nadja Graf and Josef Altenbuchner

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4.2 Genetic engineering of *Pseudomonas putida* KT2440 for rapid and high yield production of vanillin from ferulic acid

Nadja Graf and Josef Altenbuchner

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4.3 Functional characterization and application of a tightly regulated MekR/ P_{mekA} expression system in *Escherichia coli* and *Pseudomonas putida*

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4.4 Ethylene glycol metabolism by *Pseudomonas putida*

Björn Mückschel, Oliver Simon, Janosch Klebensberger, Nadja Graf, Bettina Rosche, Josef Altenbuchner, Jens Pfannstiel, Armin Huber and Bernhard Hauer

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4.1 Development of a Method for Markerless Gene Deletion in *Pseudomonas putida*

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Abstract

We developed a negative counterselection system for *Pseudomonas putida* based on the uracil phosphoribosyl-transferase (UPRTase) and the sensitivity against the antimetabolite 5-fluorouracil (5-FU). We constructed a *P. putida* strain that is resistant to 5-FU and constructed vectors for the deletion of the surface adhesion protein gene, the flagellum biosynthesis operon and two endonuclease genes. The genes were efficiently disrupted and left a markerless chromosomal in-frame deletion.

Introduction

Since the genome of the rod shaped Gram-negative bacterium *Pseudomonas putida* KT2440 has been completely sequenced (GenBank accession number: AE015451) (10), the study of gene functions is more and more focused. Usually genes are simply replaced by antibiotic resistance markers (15) to see what phenotypic effects result. The generation of a strain with multiple deletions, however, cannot be achieved with this method, because the availability of resistance markers is limited. Therefore other gene disruption methods based on homologous recombination were developed. By flanking the resistance cassette with recognition sites for site-specific recombinases, such as Flp/FRT and Cre/*loxP* (5, 8), the cassette can pricisely be removed and used again for the next deletion step. However, scars are left in the chromosome after each

deletion step in form of FRT or *loxP* sites, respectively. This may cause severe problems because the recognition sites in the chromosome can become recombined, which would then lead to large chromosomal deletions or inversions. Another disadvantage is the elaborate selection for positive clones that have dropped out the resistance cassette. To solve this problem, counterselectable marker systems have been developed. The most powerful are the fusaric acid (*tetAR*), streptomycin (*rpsL*) and the sucrose (*sacB*) sensitivity systems (13). Recently, a novel counterselection system has been approved first for *Bacillus subtilis* (3) and subsequently for several other microorganisms (4, 6, 7). This system is based on *upp*, encoding for the uracil phosphoribosyl-transferase (UPRTase). This enzyme (EC 2.4.2.9) belongs to the pyrimidine salvage pathway and creates UMP from uracil and phosphoribosyl-pyrophosphate (11). The toxic antimetabolite 5-fluorouracil (5-FU) becomes also converted by UPRTase into 5-fluoro-UMP. After metabolization into 5-fluoro-dUMP it acts as a suicide inhibitor of the thymidylate synthase, subsequently resulting in cell death. Therefore, microorganisms with UPRTase activities are sensitive to 5-FU. In *P. putida* KT2440 an *upp* gene (PP_0746) has been identified, as well (10).

In this study, we describe a gene deletion procedure based on the *upp* gene as a new counterselectable marker in *P. putida*. We deleted the *upp* gene to get a 5-FU resistant strain. With that strain we were able to delete further genes efficiently with regard to create a strain applicable in industrial productions.

Materials and Methods

Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *P. putida* strains and *E. coli* JM109 (16) were grown at 30°C in LB medium (2), supplemented with kanamycin (Kan; 50 µg ml^{-1}), or 5-FU (20 µg ml^{-1}). 5-FU was purchased from Sigma-Aldrich Corporation (Taufkirchen, Germany) and prepared as a stock solution of 100 mg ml^{-1} in DMSO. *P. putida* strains were also grown in M9 minimal medium (48 mM $Na_2HPO_4 \times 7\ H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), supplemented with 0.2 % glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 6 µM thiamine hydrochloride.

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, Δ(lac-proAB), F' [traD36 proAB+ lacI^q lacZΔM15]*	(16)
P. putida		
KT2440	wild type	ATCC 47054
ΔUPP4	Δ*upp*	This study
GN24	Δ*upp* ΔPP_0168	This study
GN109	Δ*upp* ΔPP_0168 ΔPP_2451	This study
GN112	Δ*upp* ΔPP_0168 Δ PP_4333-4396	This study
GN125	Δ*upp* ΔPP_0168 ΔPP_2451 ΔPP_3375	This study
Plasmids		
pIC20HE	cloning vector for blue-white-screening	(1)
pNEO	cloning vector with kanamycin resistance gene	GenBank U13862
pJOE6186.1	pIC20HE with a kanamycin resistance gene cloned into BamHI / ClaI sites	This study
pJOE6227.1	pJOE6186.1 with the up- and downstream regions of *upp* cloned into BamHI site	This study
pJOE6261.2	pJOE6186.1 with a copy of *upp* from *P. putida* KT2440 cloned into NdeI / NheI sites	This study
pJOE6348.1	pJOE6261.2 with the up- and downstream regions of PP_0168 cloned into BamHI site	This study
pNG90.7	pJOE6261.2 with the upstream region of PP_4333 and the downstream region of PP_4396 cloned into BamHI site	This study
pNG100.1	pJOE6261.2 with the up- and downstream regions of PP_3375 cloned into BamHI site	This study
pNG105.2	pJOE6261.2 with the up- and downstream regions of PP_2451 cloned into SalI site	This study

Results and Discussion

Plasmid and strain construction for *upp*-based counterselection

Standard recombinant DNA techniques and transformation methods were used as described by Sambrook et al. (14). The oligonucleotide primers used in this study are shown in the supplemental material (Table S1). The usage of the *upp* counterselection system (Fig. 1) for *P. putida* requires a strain resistant to 5-FU. The MIC was determined as 10 µg ml^{-1} 5-FU for *P. putida* KT2440, but 20 µg ml^{-1} were chosen for selection. That reduced the appearance of false positive clones without affecting the

growth rate. The plasmid used to delete the *upp* gene in *P. putida* KT2440 was constructed by starting with plasmid pIC20HE (1). That vector was cut with BamHI / ClaI and the kanamycin resistance gene (*neo*), which was cut out from the plasmid pNEO (GenBank U13862), was inserted by ligation, obtaining pJOE6186.1.

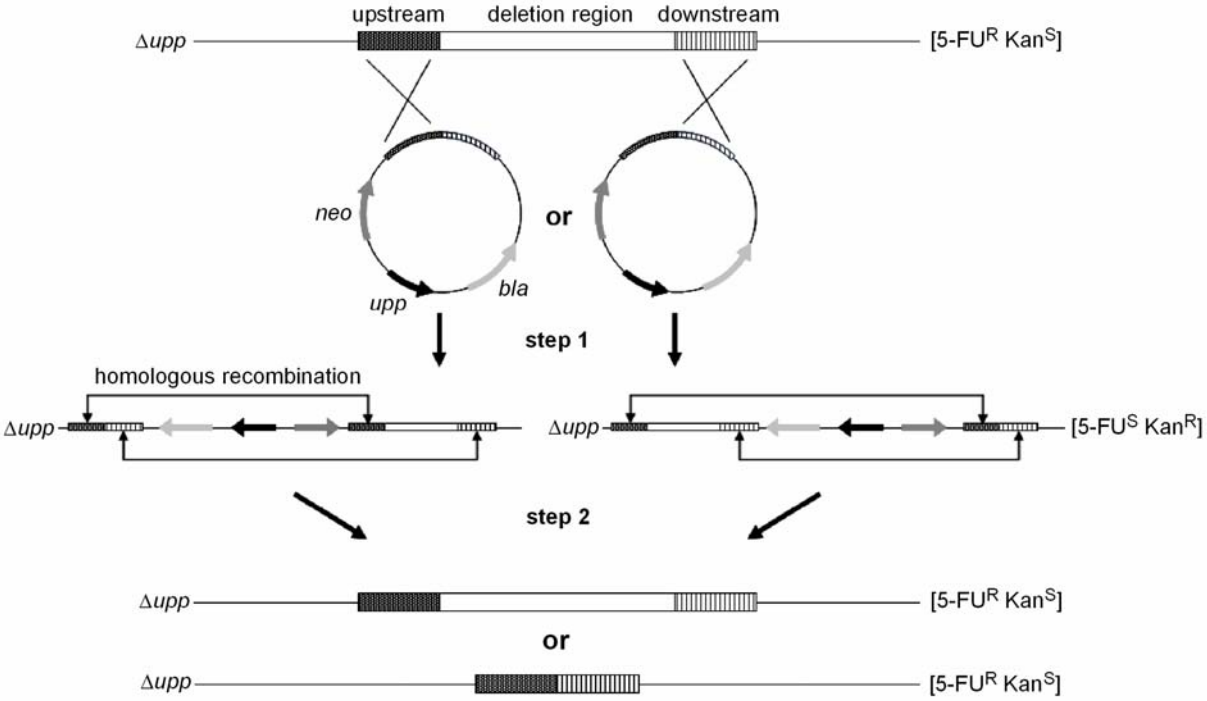

Figure 1: Schematic presentation of the *upp* counterselection system for *P. putida*. The integration vectors for deletion of a chromosomal region (white box) contain the up- and downstream regions (each about 1 kb; shaded boxes), kanamycin and ampicillin resistance genes (*neo* and *bla*) and the *upp* gene. In the first step, integration occurs via a single crossover recombination event either in the up- or downstream region. The resulting strain is sensitive to 5-FU and resistant to kanamycin. In the second step another recombination event takes place, in which either the original state is restored or the region of interest is deleted. These strains are now resistant to 5-FU and sensitive to kanamycin. When other genes are inserted between the up- and downstream regions of the integration vectors this method can also be used for gene integration or gene replacement in the *P. putida* chromosome.

For chromosomal deletion of *upp* the up- and downstream regions were PCR amplified, using oligonucleotides s5380 / s5381 and s5382 / s5383, respectively. Chromosomal DNA from *P. putida* KT2440 was used as a template. The fragments, which included homologous regions of 792 bp and 883 bp, respectively, were cut with BamHI and HindIII. Integration occured via 3-fragment ligation into BamHI cut pJOE6168.1 creating pJOE6227.1

P. putida KT2440 was transformed with pJOE6227.1 by electroporation. Since the plasmid cannot be autonomously replicated, it has to integrate via single crossover into the chromosome. Selection occured on LB_{Kan}. Positive clones should be Kan^R and $5\text{-}FU^S$. Therefore, clones were checked on M9 minimal plates containing 20 µg ml^{-1} 5-FU and 0.2 % glucose. One of the Kan^R $5\text{-}FU^S$ clones was incubated in LB for 24 h at 30°C. During this time the plasmid should be excised again through homologous recombination and get lost. Thereby, either the original genotype will be reestablished or the *upp* gene will be lost. Afterwards, different dilutions (10^{-3}, 10^{-5}) were plated on $M9_{Glc\ +\ 5\text{-}FU}$. After incubation for 48 h at 30°C, up to 250 colony forming units (cfu) were gained. Thereof 50 clones were checked on LB_{Kan} and on $M9_{Glc\ +\ 5\text{-}FU}$. Of the clones, which were $5\text{-}FU^R$ and Kan^S, 3 were checked by colony PCR: Cells were picked from M9 minimal plates and resuspended in 100 µl of double distilled water. After treatment for 10 min at 99 °C cells were pelletized (5 min, 16.1 g, room temperature). From the supernatant 10 µl were used as template for the PCR, using s5381 / s5383 as primers. The resulting positive strain was designated ΔUPP4. The counterselection system can only be used in *P. putida*, if 5-FU sensitivity can be restored by reintroducing the *upp* gene. Therefore, *upp* was PCR amplified using oligonucleotides s5378 / s5379 and chromosomal DNA from *P. putida* KT2440 as template. The fragment was integrated between the restriction sites NdeI and NheI of vector pJOE6168.1, creating pJOE6261.2. In the BamHI restriction site of that vector the flanking regions of target genes can now be easily integrated.

Deletion of selected chromosomal genes

To demonstrate the potential of the new developed *upp* counterselection system for *P. putida* (Fig. 1) and with regard to construct an eligible production strain, different genes were selected and deleted: PP_0168 (*lapA*), coding for the surface adhesion protein, which is an important stress response element and responsible for the formation of biofilms (12); PP_4333-4396, coding for flagellum biosynthesis; PP_2451 and PP_3375, coding for endonucleases. Table 2 is summing up the constructions of the different strains. Generally, construction began with PCR amplification of the up- and downstream regions (each about 1 kb), including the start and stop codon of the

target gene. The PCR fragments were cut and cloned into pJOE6261.2 according to Table 2. Then, *P. putida* with deleted *upp* was transformed with the constructed integration vectors by electroporation. Selection steps occured as described in the section above. The transformation efficiencies were about 25 cfu μg^{-1} DNA using 5×10^9 electroporated cells. In each case 10 putative positive clones were checked by colony PCR, whereas the reverse primers were located in the downstream region and the forward primers once in the upstream region (positive control) and once in the region to be deleted (negative control). As further controls, the initial strain and the strain with the integrated vector were used. Theoretically, half of the clones should have lost the gene, while the other half should have restored the original sequence. For reasons, already discussed by Fabret et al. (3), the maximum ratio of 50 % could not be achieved (see Table 2). Deletion of PP_0168 resulted in strains no longer capable to form biofilms, which was verified by a modified version of a biofilm assay described by Merritt et al. (9) as detailed in the supplemental material. Deletion of the flagellum biosynthesis operon led to strains with a lack of motility, which was observed microscopically and onto motility agar plates (supplemental material; Fig. S2). Deletion of the endonucleases, however, had no significant effect on transformation efficiencies.

TABLE 2. Overview of the strain constructions.

Deleted genes / regions	PP_0168 (*lapA*)	PP_4333-4396 (flagellum biosyn.)	PP_2451 (*endA-1*)	PP_3375 (*endA-2*)
region length	26049 bp	64722 bp	693 bp	966 bp
upstream primers (length of homology)	s5278 / s5279 (976 bp)	s5840 / s5841 (944 bp)	s5943 / s5944 (864 bp)	s5947 / s5948 (950 bp)
downstream primers (length of homology)	s5280 / s5281 (1031 bp)	s5842 / s5843 (1047 bp)	s5945 / s5946 (1051 bp)	s5949 / s5950 (1052 bp)
cloned via	BamHI / HindIII	BamHI / EcoRI	SalI / HindIII	BamHI / HindIII
integration vector	pJOE6348.1	pNG90.7	pNG105.2	pNG100.1
target strain	ΔUPP4	GN24	GN24	GN109
colony PCR primers	s5761 / s5762 s5764 / s5762	s6257 / s6258 s6368 / s6258	s6261 / s6262 s6370 / s6262	s6263 / s6264 s6371 / s6264
deletion ratio	40 %	20 %	10 %	30 %
resulting strain	GN24	GN112	GN109	GN125

Conclusion

The *upp* gene has been approved for *B. subtilis, E. faecalis, L. acidophilus* and *D. vulgaris* yet as an efficient negative selection marker for gene deletion, integration and replacement (3, 4, 6, 7). Here we demonstrate that it can also be an efficient genetic tool for *P. putida.*

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Supplemental Material

TABLE S1. Oligonucleotide primers used in this study.

Primer	Sequence (5′ → 3′)[a]
s5278	AAA AAA **GGA TCC** CGC TCT CTT CAT CCT TGT G
s5279	AAA AAA **AAG CTT** CAT TGG ACT CTC CGT GTG A
s5280	AAA AAA **AAG CTT** TGA CAG ACC ACC GGG GC
s5281	AAA AAA **GGA TCC** CAT CAC CCA GGC CGA TGT
s5378	GGG **CAT ATG** GTC AGG CGT CCT TTT GCT T
s5379	GG**G CTA GC**A TTT TGG AAA AGC GGG CAA AG
s5380	AAA AAA **AAG CTT** TGA CCA TGC AGG ACG GCT TC
s5381	AAA AAA **GGA TCC** GGT GCA GGC CAG GCT TTT T
s5382	AAA AAA **AAG CTT** ATA GGG GAG GGC TCC GAA A
s5383	AAA AAA **GGA TCC** GGC GGA ATG TAG GTG CTT G
s5761	CAG GCC GGC TCA CCC TGG CA
s5762	GGT AGA CAG CAG CAT AGG CAA AG
s5764	TCC AAC TTC TTC AAC AGC ACC ATG
s5840	AAA AAA **GGA TCC** CAC CCG ATG GGC TGC TG
s5841	AAA AAA **AAG CTT** TGA GTT GAT CCT AGA GGT TG
s5842	AAA AAA **AAG CTT** TGA TTT TTC TAT CAA GGC ACA
s5843	AAA AAA **GGA TCC** TGG ACC CGC GGG TGG AT
s5943	CAT CAT C**GT CGA C**GA TAC C
s5944	AAA AAA **AAG CTT** TGA TCC TCA GGC CAG CGT
s5945	AAA AAA **AAG CTT** CAT GCG CAG TCA ATC TTC C
s5946	AAA AAA **GTC GAC** TGG CCG TAG CCA GCG AC
s5947	AAA AAA **GGA TCC** GGT CGG TAC GCG GGT TC
s5948	AAA AAA **AAG CTT** CAT GGG GAA AAC ATA TTT CAG
s5949	AAA AAA **AAG CTT** TGA CCG TTT CAA AGG CTG C
s5950	AAA AAA **GGA TCC** GCG CGA TAG CTC GCG TC
s6257	CAG CGG GTA CTG AAC GAG CCT GG
s6261	GGC TTG TGT CAG GCG CCG TTC AC
s6262	AAG AGC TGC AGC GGA TCT TCT GGG AG
s6263	GGT TGA GCA TTA CCC GCT TTT ACC CCC
s6264	TGC GCG TGC TGT ATC ACA GCC AGC
s6258	GAT GAT GTC GAC CAG CTG CGA TA
s6368	CTG ACT TCA CGG ACG CCA GAA A
s6370	GGC ATC ACC TTC CTC GCC TTG A
s6371	TGC ACC ACC AAG GTC GAT TTC G

[a] Restriction sites are indicated in boldface.

Biofilm assay. To check biofilm formation, a modified version of a method described by Merritt et al. (2). The overnight cultures of KT2440 and GN24 (with deletion of *lapA*, coding for the surface adhesion protein) were diluted 1:100 in LB_0 and 200 µl of each dilution were inoculated in each of 32 wells in a polystyrene microtiter plate. The plates were covered and incubated at 30 °C for 20 hours. After that, the cell cultures were removed by shaking the plate and the wells were washed 2 times

with water. To stain the attached cells, 125 µl of a 0.1 % (w/v) crystal violet solution was added and incubated 10 minutes at room temperature.

Afterwards the crystal violet solution was removed. The wells were washed 3 times with water and dried at air. The stained cells were solubilized in 200 µl 95 % ethanol for 15 minutes at room temperature. The solution was diluted 1:10 with 95 % ethanol and transferred to a semi-micro-cuvette and the absorbance was determined at 595 nm.

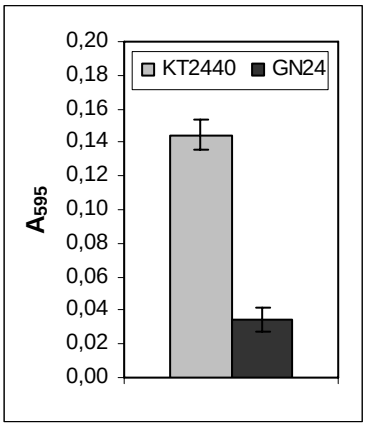

Figure S1: Biofilm formation on polystyrene by KT2440 (wild type) and GN24 (Δ*lapA*) in LB_0 medium. The attached cells were stained with 0.1 % crystal violet after incubation for 20 hours in a microtiter plate. The stained cells wer solubilized in 95 % ethanol and the absorbance measured at 595 nm.

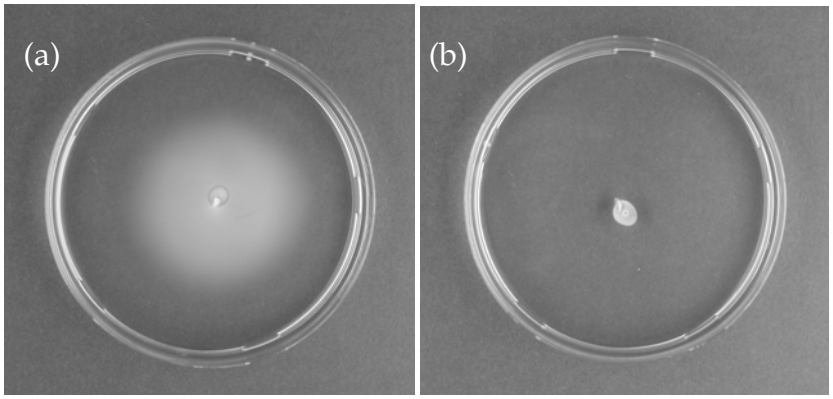

Figure S2: Motility assay of (a) KT2440 (wild type) and (b) GN112 (with deletion of the flagellum biosynthesis operon). 10 µl of an overnight culture in LB_0 was inoculated in a motility agar plate (1 % tryptone, 0.5 % NaCl, 0.25 % bacto agar) (1) and incubated at 30 °C for 16 hours.

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4.2 Genetic engineering of *Pseudomonas putida* KT2440 for rapid and high yield production of vanillin from ferulic acid

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Abstract

Vanillin is one of the most important flavoring agents used today. That is why many efforts have been made on biotechnological production from natural abundant substrates. In this work, the non-pathogenic *Pseudomonas putida* strain KT2440 was genetically optimized to convert ferulic acid to vanillin. Deletion of the vanillin dehydrogenase gene (*vdh*) was not sufficiant to prevent vanillin degradation. Additional inactivation of a molybdate transporter, identified by transposon mutagenesis, led to a strain incapable to grow on vanillin as sole carbon source. The bioconversion was optimized by enhanced chromosomal expression of the structural genes for feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*) by introduction of the strong *tac* promoter system. Further genetic engineering led to high initial conversion rates and molar vanillin yields up to 86% within just 3 h accompanied with very low by-product levels. To our knowledge, this represents the highest productivity and molar vanillin yield gained with a *Pseudomonas* strain so far. Together with its high tolerance for ferulic acid the developed, plasmid-free *P. putida* strain represents a promising candidate for the biotechnological production of vanillin.

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde), the organoleptic compound of the vanilla flavor, is one of the quantitative most widely used flavoring agents worldwide. Its demand has long exceeded the supply by the botanical source *Vanilla planifolia*. At present, most of the vanillin is synthesized chemically from guaiacol, which originates from fossile raw materials, and lignin, a component in waste material from the wood pulp industry (Ramachandra Rao and Ravishankar 2000). However, the demand for this "nature-identical" vanillin, which is mostly used in the food and beverage industry, is shifted towards the "natural" vanillin due to a rising health- and nutrition-consciousness of the costumers. Thus, biotechnological production of "natural" vanillin becomes more and more important (reviewed by Krings and Berger 1998; Priefert et al. 2001).

Efforts have been made to produce vanillin by *in vitro* cultured *Vanilla planifolia* cells (Davidonis and Knorr 1991). A *de novo* synthesis was also implemented using genetically modified yeast strains (Hansen et al. 2009). The main focus, however, was put on the biotransformation using isolated enzymes or different prokaryotic microorganisms as whole cell biocatalysts (Havkin-Frenkel and Belanger 2008; Berger 2009).

Besides lignin and phenolic stilbenes, like eugenol, the biotransformation of ferulic acid to vanillin is the most intensively studied method to produce "natural" vanillin (reviewed by Rosazza et al. 1995; Priefert et al. 2001). The precursor ferulic acid (3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid), a hydroxycinnamic acid, is a highly abundant substance since it is a constituent of many plant cell walls (Ishikawa et al. 1963; Escott-Watson and Marais 1992; Ishii 1997; Oosterveld et al. 2000). Many different microorganisms have been evaluated for the production of vanillin from ferulic acid comprising recombinant strains of *E. coli, Pseudomonas* ssp., *Rhodococcus* ssp., *Bacillus subtilis*, *Aspergillus niger, Pycnoporus cinnabarinus*, *Amycolatopsis* ssp. and *Streptomyces* ssp. (Lesage-Meessen et al. 1996; Okeke and Venturi 1999; Muheim and Lerch 1999; Achterholt et al. 2000; Overhage et al. 2003; Peng et al. 2003; Plaggenborg et al. 2006; Yoon et al. 2007; Barghini et al. 2007; Hua et al. 2007; Di Gioia et al. 2010; Tilay et al. 2010; Fleige et al. 2013). However, in most cases vanillin yields were low

and biotransformation reactions slow. The low yields can mostly be ascribed to the high toxicity of vanillin (Krings and Berger 1998). Enhanced vanillin production with adsorbent resins improved the vanillin levels up to 19.2 g l^{-1}, but the molar yield of about 43% was rather low (Hua et al. 2007). Other drawbacks were inefficient heterologous gene expression and plasmid instabilities. A focus was also set on prevention of further degradation of vanillin to vanillyl alcohol or vanillic acid (Stentelaire et al. 1997; Bonnin et al. 1999; Oddou et al. 1999; Civolani et al. 2000; Overhage et al. 2000).

Bacteria from the genus *Pseudomonas* show a broad metabolic versatility as they can use a wide range of aromatic molecules as sole carbon sources (Clarke 1982). The ferulic acid catabolism in *Pseudomonas* sp. strain HR199, *P. fluorescens* BF13 and *P. putida* KT2440 occures via a coenzyme A-dependent, non-β-oxidative pathway as depicted in Figure 1 (Narbad and Gasson 1998; Gasson et al. 1998; Overhage et al. 1999b; Plaggenborg et al. 2003; Calisti et al. 2008). First, ferulic acid becomes activated to feruloyl-CoA catalyzed by feruloyl-CoA synthetase (EC 6.2.1.34; encoded by *fcs*). Second, the CoA thioester is hydrated and cleaved to vanillin and acetyl-CoA catalyzed by enoyl-CoA hydratase/aldolase (EC 4.2.1.101; encoded by *ech*). The vanillin dehydrogenase (EC 1.2.1.67; encoded by *vdh*), oxidizes vanillin to vanillic acid which is further catabolized to protocatechuic acid by vanillate-*O*-demethylase (EC 1.14.13.82; encoded by *vanAB*). Overhage et al. (1999b) also proposed a second route over 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA and vanillyl-CoA catalyzed by enzymes encoded by PP_3355 (*aat*) and probably PP_3354.

A recent study has used a metabolic engineered strain of *P. fluorescens* for the production of vanillin from ferulic acid (Di Gioia et al. 2010). By deletion of the gene *vdh* for the vanillin dehydrogenase and by overexpression of the structural genes *fcs* and *ech* on a low-copy vector the authors were able to produce up to 8.41 mM vanillin from 10 mM ferulic acid which was the highest final titer of vanillin produced with a *Pseudomonas* strain so far.

In this study, we used the non-pathogenic, fully sequenced *Pseudomonas putida* strain KT2440 (ATCC 47054) (Nelson et al. 2002), a plasmid-free derivative of the biosafety strain *P. putida* mt-2 (Kojima et al. 1967; Williams and Murray 1974; Nakazawa 2002).

We describe a highly efficient way for the biotransformation of ferulic acid to vanillin using plasmid-free, resting *P. putida* mutant cells. Genetic manipulation of *P. putida* KT2440 using the *upp* counterselection system (Graf and Altenbuchner 2011) led to cells which were able to rapidly convert ferulic acid to vanillin accompanied with molar yields up to 86%, high productivities and only little by-product formation.

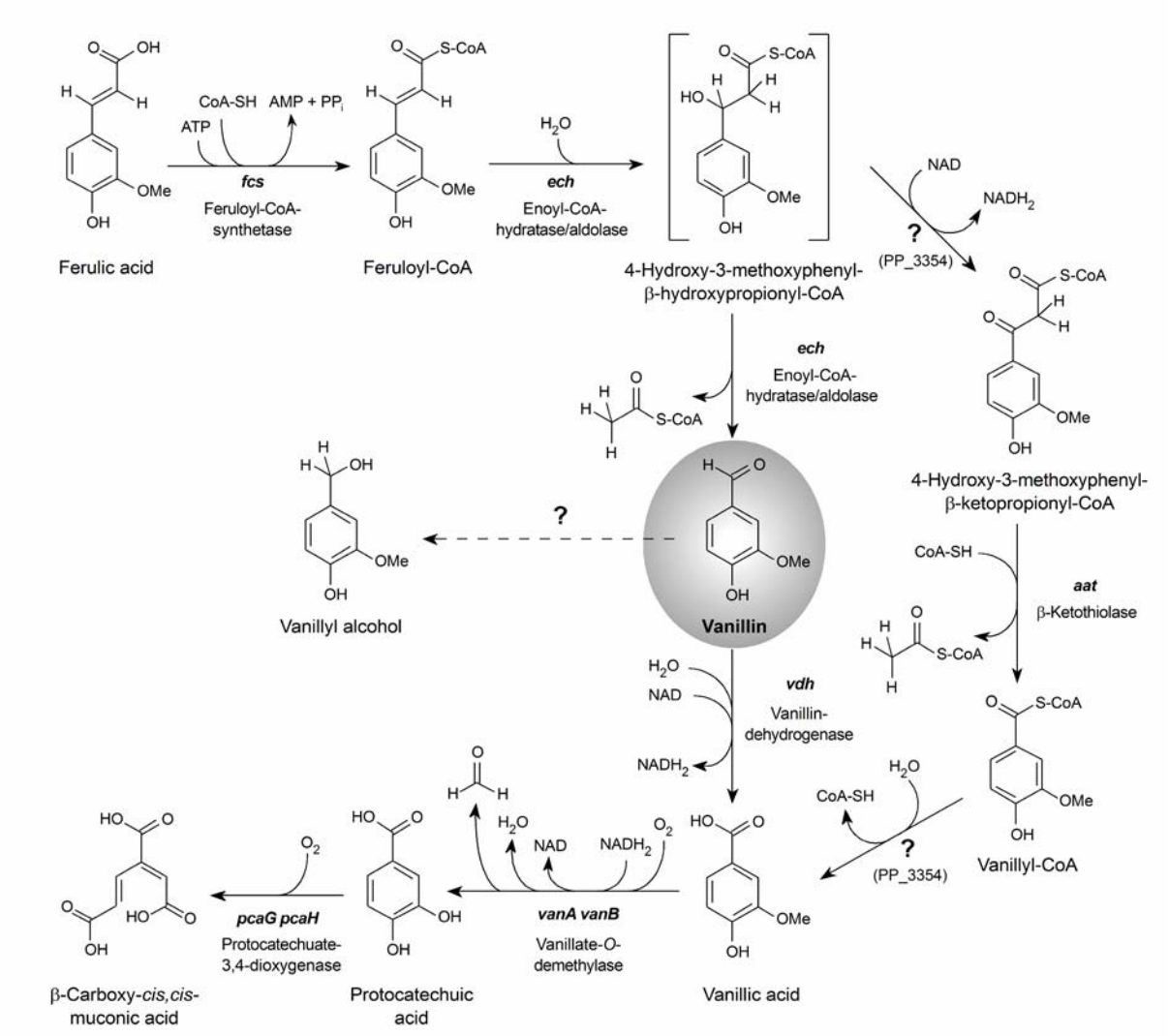

Fig 1 Proposed route for the catabolism of ferulic acid over vanillin in *Pseudomonas* strains. The alternative route from 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA to vanillic acid is shown on the right (proposed by Overhage et al. 1999b). The reduction of vanillin to vanillyl alcohol is depicted by a dashed arrow. Question marks symbolize reactions catalyzed by unknown enzymes

Materials and Methods

Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *P. putida* strains were grown at 30 °C and *E. coli* JM109 (Yanisch-Perron et al. 1985) at 37 °C in LB medium (Bertani 1951). For selection of plasmids 50 µg ml^{-1} kanamycin (Kan) was added. During the deletion procedure and for the tolerance tests M9 minimal medium was used for growth of *P. putida* strains (48 mM $Na_2HPO_4 \times 7\ H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), supplemented with 0.2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 6 µM thiamine hydrochloride and 20 µg ml^{-1} 5-fluorouracil (5-FU; prepared as a stock solution of 100 mg ml^{-1} in dimethyl sulfoxide [DMSO]).

Chemicals and other materials

Chemicals used in this study were of analytical grade and purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Sigma-Aldrich Corporation (Taufkirchen, Germany) and Merck KGaA (Darmstadt, Germany). In particular, 5-FU, ferulic acid, vanillin, vanillic acid and vanillyl alcohol were purchased from Sigma-Aldrich. Synthetic DNA oligonucleotides (Table 2) were purchased from Eurofins MWG Operon GmbH (Ebersberg, Germany). Restriction enzymes and DNA modifying enzymes were purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany), New England Biolabs GmbH (Frankfurt am Main, Germany) and Fermentas GmbH (part of Thermo Fisher Scientific, St. Leon-Rot, Germany). PCRs were run with High Fidelity PCR Enzyme Mix from Fermentas GmbH on a TPersonal Thermocycler from Biometra GmbH (Göttingen, Gemany).

Vector construction and genetic manipulation of P. *putida* strains

Cloning steps were performed with *E. coli* JM109 (Yanisch-Perron et al. 1985) using standard recombinant DNA techniques (Sambrook et al. 1989). Transformation of *E. coli* with plasmid DNA occured via the TSS (Transformation and Storage Solution) method (Chung et al. 1989). *P. putida* strains were transformed with plasmid DNA via electroporation (Sambrook et al. 1989). Construction of the plasmids and strains is summarized in Table 3.

For chromosomal deletions and integrations in *P. putida* KT2440 the *upp* / 5-FU counterselection system was used as described previously (Graf and Altenbuchner 2011). First, the up- and downstream regions including the start and stop codons of the target gene were PCR amplified using chromosomal DNA of *P. putida* KT2440 (GenBank accession number AE015451) as template. These fragments were cloned via 3-fragment ligation into pJOE6261.2. The resulting integration vector was then used for electroporation of *P. putida* ΔUPP4 or other *upp* deleted strains. One of the Kan^r $5\text{-}FU^s$ clones obtained, was incubated in LB medium for 24 h at 30 °C under shaking conditions (200 rpm). Afterwards, different dilutions were plated on minimal plates containing 20 µg ml^{-1} 5-FU and 0.2% glucose. Ten $5\text{-}FU^r$ and Kan^s clones were checked by colony PCR, using oligonucleotides binding to the up- and downstream sequences of the gene to be deleted.

Construction of the *lacI*^{q}-P_{tac} integration vector pNG283.5 started with the PCR amplification of *ech* using oligonucleotide primers s6936 / s6937 and chromosomal DNA of *P. putida* KT2440 as template. The purified PCR fragment (897 bps) was cloned via *Nde*I / *Bam*HI into pJOE5304.1 resulting in pNG281.1, a vector with a *lacI*^{q}-P_{tac}-*ech* cassette. Next, this cassette was PCR amplified with s6936 / s6965 (fragment A; 2376 bps). Also, the upstream region of *ech* was PCR amplified with s6938 / s6939 (fragment B; 952 bps). Fragment A and B were cut with *Bam*HI / *Mfe*I and *Eco*RI / *Bam*HI, respectively, and cloned via 3-fragment ligation into *Bam*HI cut pJOE6261.2, giving pNG283.5.

Mating and transposon mutagenesis

Overnight cultures of *E. coli* S17.1/pCro2a (contains mini-Tn*5495*) (Onaca et al. 2007) and *P. putida* GN235 grown in LB with and without kanamycin, respectively, were mixed equally (200 µl each) and 100 µl of that mixture was dropped onto a LB agar plate without antibiotics. After incubation for 24 h at 30 °C grown cells were scraped off the plate with 3 ml LB liquid medium. In each case 100 µl of a 10^{-2} dilution (giving about 50-100 colonies) were plated on a total of 50 LB agar plates containing 50 µM kanamycin and 10 µM nalidixid acid (Nal) (for counterselection of the *E. coli* donor). The plates were then incubated for 40 h at 30 °C. From each plate the colonies were replica plated on M9 minimal agar plates, one with 0.2% (w/v) glucose and the other one with 0.1% (w/v) vanillin. Incubation occured overnight at 30 °C. Colonies which were grown on M9 plates with glucose but not on M9 plates with vanillin were toothpicked on a LB agar plate with 50 µM kanamycin and 10 µM nalidixid acid, on a M9 agar plate with 0.1% vanillin and on a M9 agar plate with 0.1% vanillic acid and incubated overnight at 30 °C. The chromosomal DNA from clones which had grown on $LB_{Kan/Nal}$ and on M9 with vanillic acid but not on M9 with vanillin was isolated (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) and digested with restriction enzymes *Bsr*GI, *Eco*RI and *Sal*I, respectively. The chromosomal fragments were purified (NucleoSpin Extract II Kit, Macherey-Nagel, Düren, Germany), ligated overnight at 4 °C, precipitated with isopropanol for 2 h on ice, washed with ethanol and resuspended in 10 µl H_2O (bidest.). *E. coli* JM109 was transformed with the ligated chromosomal fragments. Selection occured on LB agar plates containing 50 µM kanamycin. Plasmids were isolated from kanamycin resistant clones and checked by restriction enzyme digestion. After sequencing of the plasmids with primers s4052 and s4037 (Onaca et al. 2007) (GATC Biotech, Constance, Germany) the obtained sequences were finally subjected to a BLAST search.

Table 1 Bacterial strains and plasmids used in this study

Strain / plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, Δ(lac-proAB), F' [traD36 proAB+ lacI^q lacZΔM15]*	Yanisch-Perron et al. (1985)
S17.1	*recA pro hsdR* RP4-2-*Tc::Mu-Km::*Tn7	Simon et al. (1983)
P. putida		
KT2440	wild type	ATCC 47054
ΔUPP4	Δ*upp*	Graf and Altenbuchner (2011)
GN23	Δ*upp* ΔPP_0166-0168	This study
GN235	Δ*upp* ΔPP_0166-0168 Δ*vdh*	This study
GN275	Δ*upp* ΔPP_0166-0168 Δ*vdh modA::*mini-Tn*5495*	This study
GN276	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832	This study
GN299	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN347	Δ*upp* 0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_3354 ΔPP_3355 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN440	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 Δ*PP_2680 lacI^q-P$_{tac}$-ech-fcs*	This study
GN441	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 *lacI^q-P$_{tac}$-ech-fcs*	This study
GN442	Δ*upp* ΔPP_0166-0168 Δ*vdh* ΔPP_3827-3832 ΔPP_2680 ΔPP_0545 ΔPP_1948 *lacI^q-P$_{tac}$-ech-fcs*	This study
Plasmids		
pCro2a	mini-Tn*5495* delivery vector	Onaca et al. (2007)
pJOE5304.1	expression vector with *lacI^q*-P$_{tac}$-*eGFP*	laboratory stock
pJOE6261.2	pIC20HE (Altenbuchner et al. 1992) backbone with a kanamycin resistance gene and a copy of *upp* from *P. putida* KT2440	Graf and Altenbuchner (2011)
pNG53.1	pJOE6261.2 with the upstream region of PP_0166 and downstream region of PP_0168 cloned into *Bam*HI site	This study
pNG173.1	pJOE6261.2 with the up- and downstream regions of *vdh* cloned into *Bam*HI site	This study
pNG260.4	pJOE6261.2 with the upstream region of PP_3827 and downstream region of *PP_3832* cloned into *Bam*HI site	This study
pNG276.1	pJOE6261.2 with the up- and downstream regions of PP_2680 cloned into *Sal*I site	This study
pNG281.1	pJOE5304.1 derivative with *lacI^q*-P$_{tac}$-*ech*	This study
pNG283.5	pJOE6261.2 with 900bp of *ech*, *P$_{tac}$*, *lacI^q* and the downstream region of *ech* cloned into *Bam*HI site	This study
pNG338.1	pJOE6261.2 with the up- and downstream regions of PP_0545 cloned into *Bam*HI site	This study
pNG340.2	pJOE6261.2 with the upstream region of PP_3354 and downstream region of *aat* cloned into *Bam*HI site	This study
pNG412.1	pJOE6261.2 with the up- and downstream regions of PP_1948 cloned into *Bam*HI site	This study

Table 2 Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)[a]
s6007	AAAAAA**GGATCC**TAAAGCAATGGCGAAACCC
s6008	AAAAAA**AAGCTT**ACCCAGTACGCCAACAGCCT
s6009	AAAAAA**AAGCTT**GACAGTGCCGGCAAGCCA
s6010	AAAAAA**GGATCC**GTGGTCTGTCAGCTGTCCTT
s6534	AAAAAA**GGATCC**TAAAGCACGATGCCGAGG
s6535	AAAAAA**GAATTC**TAGACCTCCGGCAAGATGA
s6536	AAAAAA**GAATTC**CATGCTCATTCCTCTTGTTG
s6537	AAAAAA**GGATCC**TTATGCGATTCGGCTAGAGA
s6882	AAAAAA**GGATCCC**CCGCGCTTGTCGATATCC
s6883	AAAAAA**CCATGG**CATGCGATTCTCCTTGCGT
s6884	AAAAAA**CCATGG**TGAGCGTCACCCGAGGG
s6885	AAAAAA**GGATCC**GGTCAGTCAGCCTGTTGAT
s6927	AAAAAA**GTCCAG**ACAAGGACGGCGGCAAGG
s6928	AAAAAA**TGTACA**CATGCTGAGCCTCTGCGG
s6930	AAAAAA**GTCCAG**AGTAGTCGATACCCTGGGC
s6936	AAAAAA**GGATCC**CTCTTGTTGTCGTTATAGAGA
s6937	AAAAAA**CATATG**AGCAAATACGAAGGCCG
s6938	AAAAAA**GAATTC**GGTTCTGCACTCTTGTTGTT
s6939	AAAAAA**GGATCC**TGGCCATTATCTGGCTCAG
s6946	AAAAAA**TGTACA**CTGGTGAGCTACGACATCAA
s6965	AAAAAA**CAATTG**TCACTGCCCGCTTTCCAGT
s7343	AAAAAA**GGATCC**ACGGCAGGAAGCTGCTGG
s7344	AAAAAA**GAATTC**TAGACCGCGTCGCCTTCTT
s7345	AAAAAA**GAATTC**CATGGTGTGTCTCCTTGGTA
s7346	AAAAAA**GGATCC**CGCGATACGTCGGGGCG
s7390	AAAAAA**GGATCC**TTGACGTGCATCCGGTCAC
s7391	AAAAAA**GAATTC**CATTCATTGCCGAATCGTTCT
s7392	AAAAAA**GAATTC**CATCTGGACGATGGCCGTG
s7393	AAAAAA**GGATCC**GCGCTACGCGAGGTGTTC
s7982	AAAAAA**GGATCC**TTCCATGCTCAGGACCCTAT
s7983	AAAAAA**CAATTG**CATGCACTTTTGATTAATCGATT
s7984	AAAAAA**CAATTG**TGATTCGGGTGCGAGCTGT
s7985	AAAAAA**GGATCC**CGCCGGACAGCATGAGCA

[a] Restriction sites are indicated in boldface.

Table 3 Overview of the strain constructions

Gene / region	PP_0166-PP_0168 (*lapABC*)	PP_3357 (*vdh*)	PP_3827-PP_3832 (*modABC*)	P_{tac}-*lacI*^{q}	PP_3354 + PP_3355	PP_2680	PP_0545	PP_1948
Deletion / integration region (bp)[a]	190765-219759	3796527-3797987	4352876-4358100	between 3798896 and 3798897	3791583-3794516	3068912-3070390	631921-633435	2203324-2204796
Primers upstream region (fragment length)	s6007 / s6008 (808bp)	s6534 / s6535 (936bp)	s6882 / s6883 (955bp)	s6937 / s6936 (897bp)	s7390 / s7391 (782bp)	s6927 / s6928 (950bp)	s7343 / s7344 (876bp)	s7982 / s7983 (823bp)
Primers downstream region (fragment length)	s6009 / s6010 (791bp)	s6536 / s6537 (1053bp)	s6884 / s6885 (1072bp)	s6938 / s6939 (952bp)	s7392/ s7393 (923bp)	s6946 / s6930 (953bp)	s7345 / s7346 (820bp)	s7984 / s7985 (829bp)
Cloned via	*Bam*HI / *Hin*dIII	*Bam*HI / *Eco*RI	*Bam*HI / *Nco*I	*Bam*HI / *Eco*RI / *Mfe*I	*Bam*HI / *Eco*RI	*Sal*I / *Bsr*GI	*Bam*HI / *Eco*RI	*Bam*HI / *Mfe*I
Integration vector	pNG53.1	pNG173.1	pNG260.4	pNG283.5	pNG340.2	pNG276.1	pNG338.1	pNG412.1
***P. putida* target strain**	ΔUPP4	GN23	GN235	GN276	GN299	GN299	GN440	GN441
Resulting strain	GN23	GN235	GN276	GN299	GN347	GN440	GN441	GN442

[a] bp numbers are derived from the sequenced *P. putida* KT2440 genome (GenBank accession number AE015451)

Bioconversion assay of ferulic acid to vanillin

Overnight cultures of *P. putida* strains were diluted 1:50 in fresh LB medium and grown for 2 h at 30 °C in shaking flasks (200 rpm). Induction of the ferulic acid metabolic genes occured by addition of 5 mM ferulic acid or 5 mM IPTG depending on the strain. After further growth for 6 h at 30 °C under shaking conditions 25 × 10^9 cells were harvested by centrifugation (10 min, 3,500 g, room temperature), washed and resuspended with 5 ml of 50 mM sodium phosphate buffer (pH 7.2). A total of 10 mM of ferulic acid (1 M stock solution in DMSO) was added to the cell suspension. The bioconversion was conducted in long glass culture tubes at 30 °C under shaking conditions (200 rpm). Samples of 200 µl were taken after 1, 2, 3, 4, 5 and 18 h conversion time. After a centrifugation step (10 min, 16,000 g, room temperature) to pellet the cells 100 µl of the supernatant was collected and stored at -70 °C until analysis through HPLC.

Analytical methods

Samples from the bioconversion assay were diluted 1:10 with 0.2% acetic acid prior to HPLC application. Ferulic acid, vanillin, vanillic acid and vanillyl alcohol were quantified with a Merck-Hitachi HPLC system (Merck, Darmstadt, Germany) equipped with a RP Purospher®-Star RP-18e column (250 mm x 4.6 mm, 5 µm), a LiChroCART® guard column (4 mm x 4 mm, 5 µm), an L7612 degasser, an L6200A gradient pump, a D6000A interface module, an L4200 UV‑visible detector, a Rheodyne injection valve 7125 with a 100-µl sample loop, and D7000 HPLC System Manager software. For measurements a modified procedure was used as described previously (Sinha et al. 2007): Methanol, acetonitril and 0.2% acetic acid (3 : 3 : 14) were used as the mobile phase. The flow rate was 1 ml min^{-1} and the absorbance was measured at 231 nm for 20 min. Solutions of ferulic acid, vanillin, vanillic acid and vanillyl alcohol with seven different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1 mM) were used for calibration.

Results

Construction and characterization of a *P. putida* KT2440 mutant unable to grow on vanillin as sole carbon source

As reported previously (Overhage et al. 1999b; Plaggenborg et al. 2003), *P. putida* KT2440 is able to grow on ferulic acid as sole carbon source. Ferulic acid is metabolized in a few steps to vanillin, catalized by feruloyl-CoA-synthetase (PP_3356, *fcs*) and enoyl-CoA-hydratase/aldolase (PP_3358, *ech*). Vanillin in turn gets further degraded to vanillic acid by the vanillin dehydrogenase (PP_3357, *vdh*). The last step has to be prevented, if vanillin accumulation is desired. The chromosomal organization of these genes in *P. putida* KT2440 and other strains constructed in this study is shown in Figure 2.

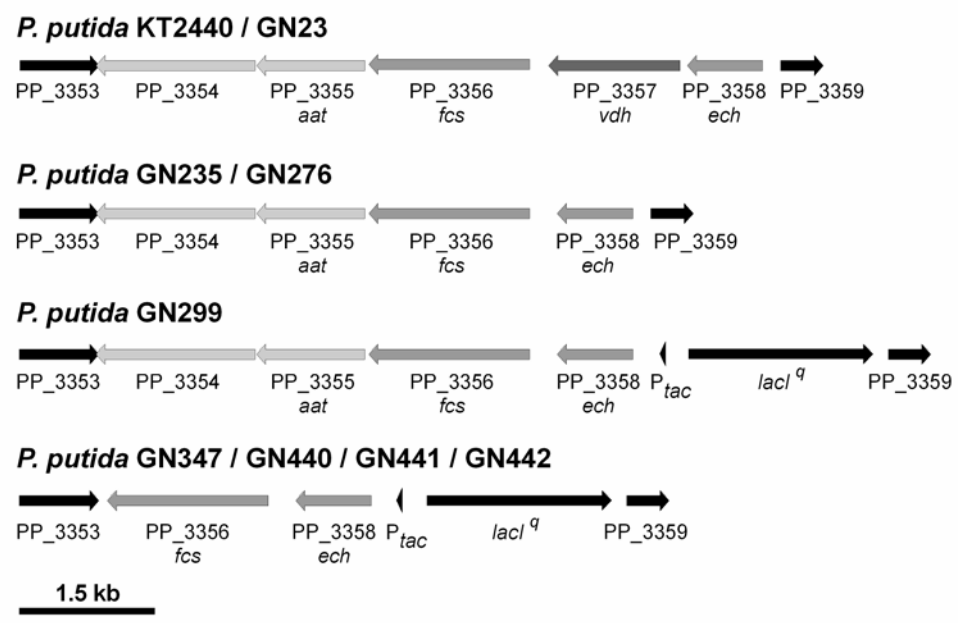

Fig 2 Organization of the structural genes of the enoyl-CoA hydratase/aldolase (*ech*), feruloyl-CoA synthetase (*fcs*), and vanillin dehydrogenase (*vdh*), β-ketothiolase (*aat*) and acetyl-CoA dehydrogenase (PP_3354) in the *P. putida* mutant strains used in this study. The integration site of the *tac* promoter region including the *lac* operator (P_{tac}) and the gene for the *lac* repressor ($lacI^q$) is depicted

With respect to industrial applications, we constructed *P. putida* strain GN23 with a deletion in the *lapABC* operon including the gene for the surface adhesion protein (PP_0168, *lapA*) using the previously described *upp* counterselection method (Graf and Altenbuchner 2011). The surface adhesion protein is responsible and essential for

the formation of biofilms as previously shown for another *P. putida* KT2440 Δ*lapA* mutant strain (Graf and Altenbuchner 2011).

In a second step, the chromosomal *vdh* gene of *P. putida* GN23 was deleted leaving just the start and stop codon of *vdh* (Fig. 2). The resulting strain, designated as GN235, was still able to grow on ferulic acid as sole carbon source demonstrating functional expression of *fcs*. GN235 also retained the ability to grow on vanillin and vanillic acid (Fig. 3).

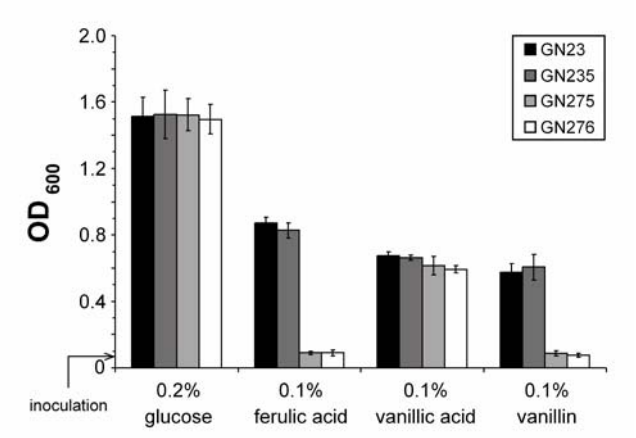

Fig 3 Growth of *P. putida* mutant strains GN23, GN235, GN275 and GN276 in M9 minimal medium with different carbon sources. Strains were inoculated with 0.05 OD_{600} as indicated by an arrow. Growth was documented by measuring the OD_{600}. The OD_{600} after 24 h at 30 °C is presented to show the ability of the strains to grow on glucose, ferulic acid, vanillic acid and vanillin, respectively, as sole carbon source

Using transposon mutagenesis of GN235, we found a mutant (GN275) which was unable to grow on ferulic acid or vanillin as sole carbon sources. However, growth on vanillic acid was retained (Fig. 3). Identification of the gene disrupted by the transposon revealed *modA* (PP_3828), which encodes a periplasmic molybdate-binding protein, which is part of a molybdate ABC transporter. The whole operon including *modABC* (PP_3827 – PP_3832) was markerlessly deleted with the *upp* counterselection method resulting in strain GN276. The phenotype of this strain was the same as the transposon mutant (Fig. 3).

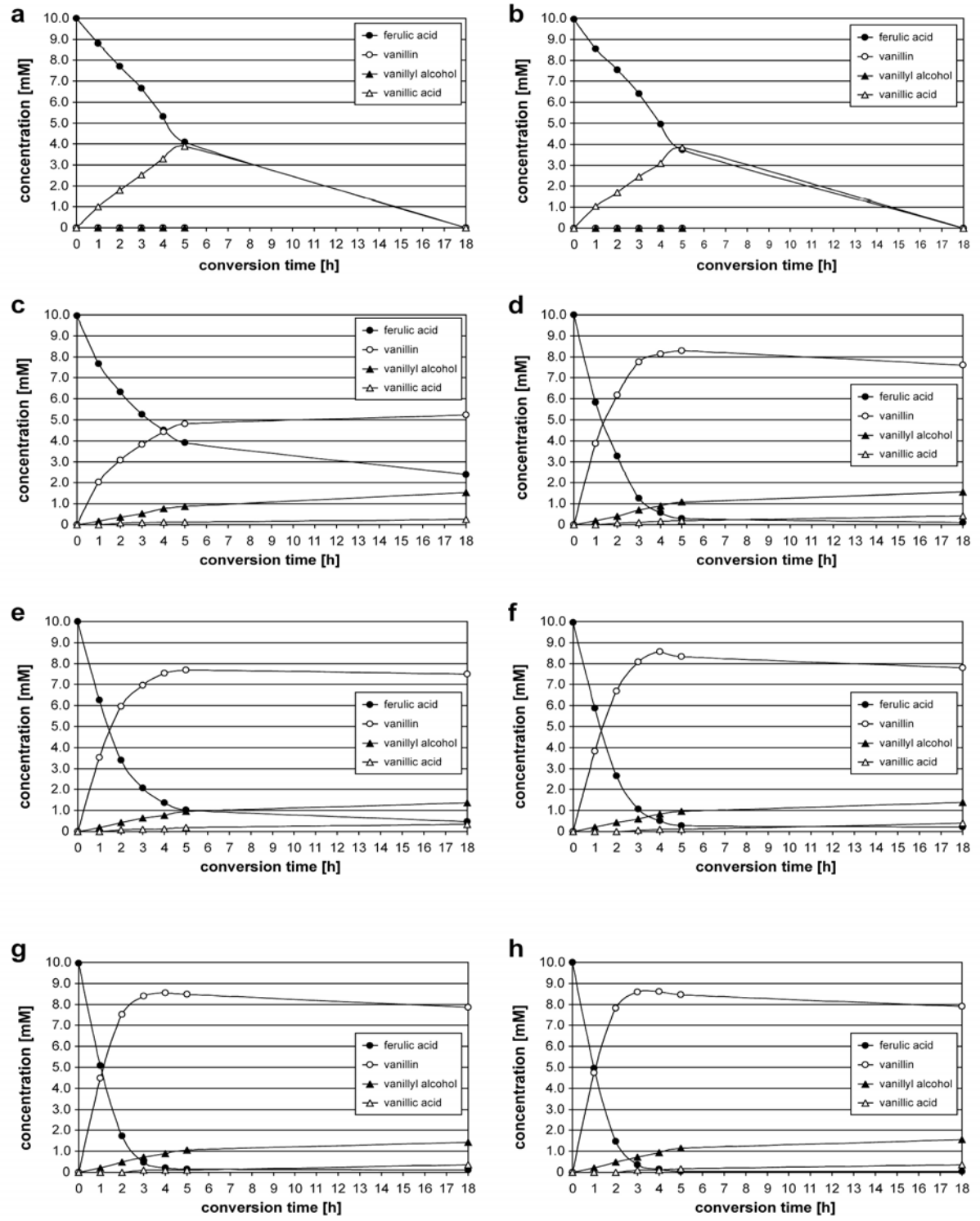

Fig 4 Bioconversion assays of ferulic acid to vanillin. Metabolic genes *ech* and *fcs* were induced for 6 h with 5 mM inducer before bioconversion of ferulic acid to vanillin was started with 5 x 10^9 resting cells ml^{-1} of *P. putida* strains (a) GN23, (b) GN235, (c) GN276, (d) GN299, (e) GN347, (f) GN440, (g) GN441 and (h) GN442. Concentrations of ferulic acid (black circles), vanillin (white circles), vanillyl alcohol (black triangle) and vanillic acid (white triangle) were measured by HPLC and plotted over the conversion time. The figure shows the mean values of at least three independently repeated assays. The standard deviation was less than 10%

Bioconversion assays of strains GN23, GN235 and GN276

Resting cells of strains GN23 and GN235 were used for bioconversion assays. 10 mM of ferulic acid were added to the resting cells and the concentrations of ferulic acid, vanillin, vanillyl alcohol and vanillic acid were measured by HPLC taking samples at regular intervals. The assay was stopped after 18 h conversion time. Both strains, GN23 and GN235, showed a rapid conversion of ferulic acid accompanied with a temporary accumulation of vanillic acid in the first 5 h (Fig. 4a,b). Furthermore, accumulation of vanillin, vanillyl alcohol and vanillic acid could not be observed in either of them.

A bioconversion assay of ferulic acid with GN276 (Fig. 4c) showed a decreased conversion rate of ferulic acid. Whereas with GN23 all of the applied ferulic acid (10 mM) was converted after 18 h, 2.4 mM could still be measured using GN276. In contrast to GN23 and GN235, GN276 accumulated 4.8 mM vanillin after 5 h conversion time. Vanillin concentration slighlty increased to 5.2 mM after further 13 h conversion. At the end of the conversion (18 h) also vanillyl alcohol and vanillic acid were accumulated up to 1.5 mM and 0.3 mM, respectively. To improve the ferulic acid conversion rate further steps were necessary.

Increase of chromosomal *ech-fcs* expression leads to high conversion rates and high vanillin molar yields

Feruloyl-CoA-synthetase (*fcs*) and enoyl-CoA-hydratase/aldolase (*ech*) catalyze the conversion of ferulic acid to vanillin. We assumed that the conversion rate of ferulic acid should be directly proportional to the number of these two metabolic enzymes in the cell, if the required cofactors, ATP and CoA-SH, are available in excess or regenerated. Using the *upp* counterselection system, the strong *tac* promoter (P_{tac}) and $lacI^q$ were integrated immidiately upstream of *ech* and *fcs* in the chromosome of GN276 in order to control the expression of these two genes (Fig. 2).

The resulting strain was designated GN299. After induction of *ech* and *fcs* expression with IPTG, bioconversion assays were conducted with this strain. After 5 h, nearly all of the 10 mM ferulic acid were converted to 1.1 mM vanillyl alcohol, 0.2 mM vanillic acid and 8.3 mM of vanillin, corresponding to a molar yield of 83% (Fig. 4d). After

18 h conversion, the vanillin concentration slightly decreased to 7.6 mM accompanied with an increase of vanillyl alcohol and vanillic acid to 1.6 mM and 0.4 mM, respectively.

Optimization of the bioconversion assay revealed a treshold for vanillin

Resting cells of *P. putida* GN299 were used for bioconversion experiments. Several parameters were varied aiming a high and reproducible product yield combined with a high initial conversion rate.

First, we analyzed the influence of the inducer concentration (1 and 5 mM IPTG) and of the induction time (2, 4, and 6 h) for the expression of the metabolic enzymes needed for the bioconversion of ferulic acid to vanillin (Fig. 5 + 6a). We found that conversion of ferulic acid was much slower using less than 5 mM IPTG (Fig. 5). However, the vanillin yields after 18 h conversion time were similar (not shown). Regarding the influence of the induction time (Fig. 6a), the highest conversion rates and vanillin yields were found after 6 h induction of the metabolic enzymes.

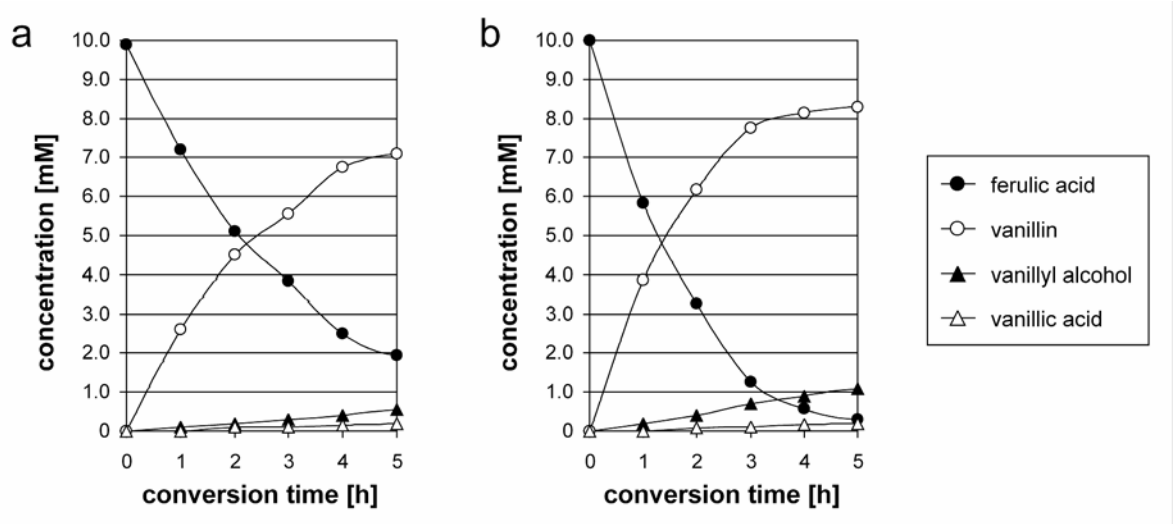

Fig 5 Influence of the amount of inducer on the bioconversion of ferulic acid to vanillin. Cells of *P. putida* GN299 were induced for 6 h with (a) 1 mM IPGT and (b) 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5 x 10^9 resting cells ml^{-1}. Concentrations of ferulic acid (black circles), vanillin (white circles), vanillyl alcohol (black triangle) and vanillic acid (white triangle) were measured by HPLC and plotted over the conversion time. The figure shows the mean values of at least three independently repeated assays. The standard deviation was less than 10%

Furthermore, the amount of resting cells was also varied (5, 10, and 20 × 10^9 cells ml^{-1}). The best results were aimed with the lowest concentration of 5 × 10^9 cells ml^{-1} (Fig. 6b). Higher cell concentrations led to raised levels of vanillyl alcohol and vanillic acid accompanied with a decrease in the vanillin molar yield after prolonged conversion (18 h).

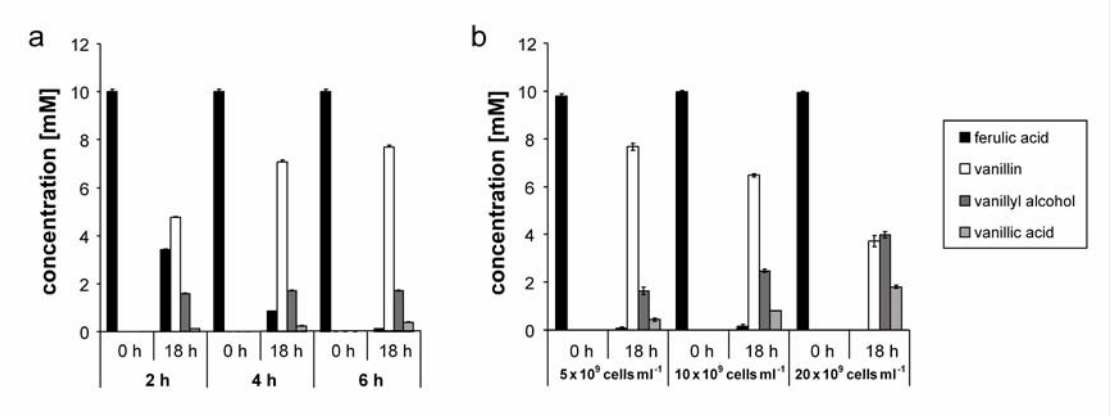

Fig 6 Influence of the (a) induction time and (b) amount of resting cells of *P. putida* GN299 on the bioconversion of ferulic acid to vanillin. (a) Cells were induced for 2, 4, and 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5 x 10^9 resting cells ml^{-1}. (b) Cells were induced for 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with varying amounts of resting cells (5, 10, and 20 x 10^9 cells ml^{-1}). Concentrations of ferulic acid (black bars), vanillin (white bars), vanillyl alcohol (dark gray bars) and vanillic acid (light gray bars) were measured by HPLC and shown at the beginning (0 h) and at the end (18 h) of the bioconversion assay. The figure shows the mean values of at least three independently repeated assays. The standard deviation is represented by error bars

In this study, we found that there is a threshold for vanillin production. Induced and resting cells were incubated with increasing amounts of ferulic acid in the bioconversion broth (10, 20, 30, and 40 mM). Using up to 30 mM ferulic acid, strains did not produce more than 13.5 mM vanillin (Fig. 7a). With 40 mM ferulic acid, no vanillin was produced at all. The best yields were achieved using 10 mM ferulic acid for the bioconversion assay. Growth kinetics of *P. putida* KT2440 mutant strains in buffered M9 minimal medium (pH 7.0) with glucose and increasing amounts of ferulic acid (0 – 50 mM) showed no influence of the ferulic acid concentration (Fig. 8a).

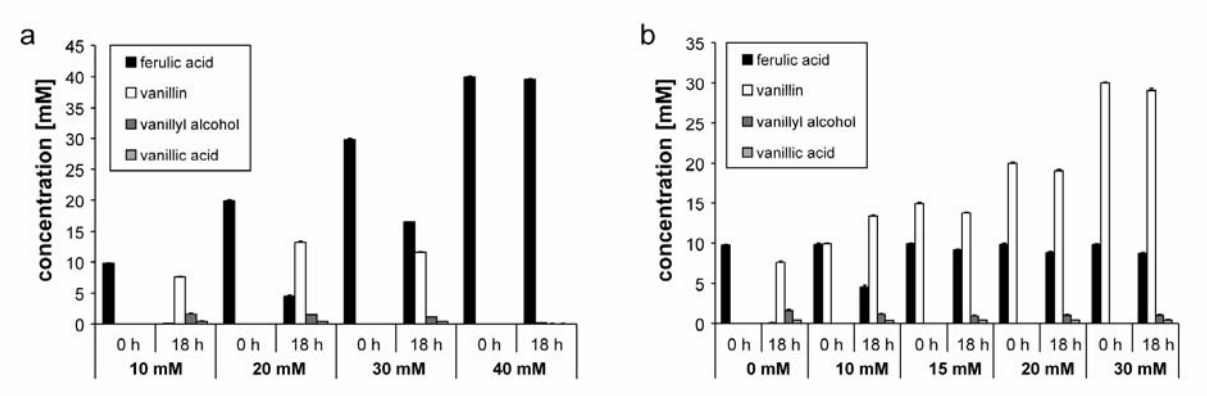

Fig 7 Influence of (a) ferulic acid and (b) vanillin concentration on the bioconversion. Metabolic genes *ech* and *fcs* were induced for 6 h with 5 mM IPTG before bioconversion of ferulic acid to vanillin was started with 5 x 10^9 resting cells ml^{-1} of *P. putida* GN299. (a) Increasing concentrations of ferulic acid (10, 20, 30, and 40 mM) were used for the conversion to vanillin. (b) Increasing concentrations of vanillin (0, 10, 15, 20, and 30 mM) were added at the beginning of the bioconversion assay with 10 mM ferulic acid. Concentrations of ferulic acid (black bars), vanillin (white bars), vanillyl alcohol (dark gray bars) and vanillic acid (light gray bars) were measured by HPLC and shown at the beginning (0 h) and at the end (18 h) of the bioconversion assay. The figure shows the mean values of at least three independent assays. The standard deviation is represented by error bars

The vanillin threshold effect was confirmed by incubation of resting cells with 10 mM ferulic acid and additional increasing amounts of vanillin (10, 15, 20 and 30 mM). The cells incubated with additional 10 mM vanillin produced only further 3.2 mM vanillin after 18 h (Fig. 7b). On the other hand, higher amounts of vanillin resulted in a slight decrease of vanillin concentration and an increase of vanillyl alcohol and vanillic acid concentrations. Growth kinetics of *P. putida* KT2440 mutant strains in M9 minimal medium with glucose and increasing amounts of vanillin (0 – 25 mM) showed significant influence of the vanillin concentration (Fig. 8b). With up to 12.5 mM vanillin the strains showed moderate growth. With over 15 mM vanillin, growth was strongly impaired.

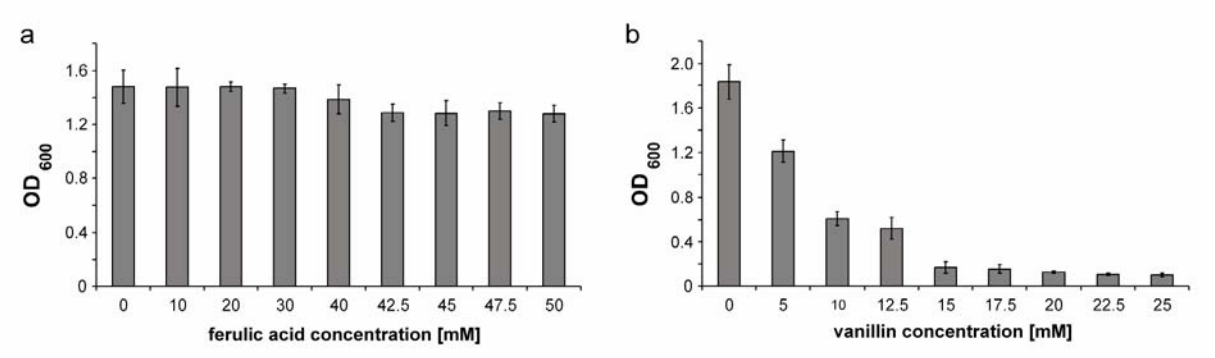

Fig 8 Tolerance of *P. putida* mutant strain GN299 towards different (a) ferulic acid and (b) vanillin concentrations in M9 minimal medium. After inoculation with 0.1 OD_{600} in M9 minimal medium with 0.4% glucose and increasing concentrations of (a) ferulic acid and (b) vanillin, growth was documented by measuring the OD_{600}. The OD_{600} after 24 h at 30 °C is presented to show the tolerance of GN299 towards different concentrations of ferulic acid and vanillin, respectively. The figure shows the mean values of at least three independent assays. The standard deviation is represented by error bars

Deletion of further genes and consequences on vanillin production and by-product formation

The bioconversion assays conducted with GN299 showed formation of vanillyl alcohol and vanillic acid which inevitably reduce the product yield. Therefore, the effect of the inactivation of several genes potentially involved in the ferulic acid metabolism was analyzed. The genes chosen for this analytical approach were PP_3354 (acyl-CoA dehydrogenase) and PP_3355 (β-ketothiolase), PP_2680 and PP_0545 (aldehyde dehydrogenases), and PP_1948 (benzaldehyd dehydrogenase).

First, a second pathway from ferulic to vanillic acid (Fig. 1) proposed by Overhage et al. (1999b) was interrupted in strain GN299 by combined deletion of PP_3354 and PP_3355 coding for an acyl-CoA dehydrogenase and a β-ketothiolase (*aat*) as depicted in Figure 2. The resulting strain GN347 was used for bioconversion assays (Fig. 4e). After 5 h 9 mM ferulic acid were converted to 7.7 mM vanillin, 1 mM vanillyl alcohol and 0.2 mM vanillic acid. After 18 h further 0.5 mM ferulic acid were converted. The vanillin concentration decreased to 7.5 mM, whereas vanillyl alcohol and vanillic acid slightly increased to 1.4 mM and 0.3 mM, respectively. Compared to GN299 the conversion rate and vanillin yield within the first 5 h decreased.

Since deletion of *vdh* could not prevent degradation of vanillin to vanillic acid, other aldehyd dehydrogenases may catalyze this reaction. From a proteomics approach it could be shown that two aldehyde dehydrogenases (encoded by PP_2680 and PP_0545) and the benzaldehyde dehydrogenase (PP_1948) were upregulated in *P. putida* KT2440 growing on vanillin (Simon, Pfannstiel and Huber, unpublished, manuscript in preparation). Sequential inactivation of PP_2680 and PP_0545 in GN299 by markerless deletion resulted in strains GN440 and GN441, respectively. The benzaldehyde dehydrogenase may also accept vanillin as a substrate, since it is a derivative of benzaldehyde. Therefore, the corresponding gene (PP_1948) was deleted in GN441, resulting in strain GN442. The mutant strains GN440, GN441 and GN442 were used in bioconversion assays (Fig. 4f–h). The strains showed very similar results. After 4 h about 9.5 mM ferulic acid were converted to 8.6 mM vanillin with resting cells of GN440 and GN441. The measured vanillyl alcohol and vanillic acid concentrations were about 0.8 and 0.1 mM, respectively. Strain GN442 showed the same results, however, already after 3 h conversion. After 18 h conversion, nearly all of the ferulic acid was converted with all three strains. Again, vanillin concentration decreased to 7.9 mM, whereas vanillyl alcohol and vanillic acid increased to about 1.5 and 0.4 mM, respectively.

Discussion

In contrast to *P. fluorescens* strains AN103 and BF13 (Martinez-Cuesta et al. 2005; Di Gioia et al. 2010), our findings with *P. putida* GN235 confirm that simple inactivation of *vdh* is not sufficient to prevent vanillin degradation. This was reported previously with a *vdh* knockout mutant of *Pseudomonas* sp. HR199 strain and a *Pseudomonas* KT2440*vdh*ΩKm mutant (Overhage et al. 1999a; Plaggenborg et al. 2003). KT2440*vdh*ΩKm and GN235 were still able to grow on vanillin as sole carbon source. The main difference, however, was that *P. putida* GN235 was also able to grow on ferulic acid, due to a functional expression of the adjacent genes of *vdh*, namely *ech* and *fcs*. The clean deletion of *vdh* sustained expression of *ech* and *fcs*, which was most probably not the case in the KT2440*vdh*ΩKm mutant. A random transposon mutagenesis conducted with GN235 revealed a mutant with a transposon in the gene

locus of *modA*, which encodes for a periplasmic molybdate-binding protein. Molybdate ions are known to play a role as cofactors in oxidoreductases in *Pseudomonas* species (Koenig and Andreesen 1990; Blaschke et al. 1991; Frunzke et al. 1993). Since the Δ*modABC* strain GN276 was not able to grow on vanillin as sole carbon source, it can be assumed that unknown molybdate depending oxidoreductases may accept vanillin as a substrate complementing the *vdh* inactivation. The inhibition of the molybdate uptake could inactivate these enzymes and vanillin is not oxidized to vanillic acid, which is further degraded. Since ferulic acid was not completely converted with GN276, further improvements were necessary.

A concurrent expression of the structural genes *ech* and *fcs* on a low-copy plasmid in a *vdh* negative *P. fluorescens* strain led to a vanillin molar yield of 63% within 5 h using resting cells from shaken flask experiments and up to 84% within 24 h using resting cells from a stirred tank reactor (Di Gioia et al. 2010). To circumvent possible problems of plasmid instabilities and usage of antibiotics, the strong *tac* promoter was introduced into the chromosome of *P. putida* GN276 to control expression of *ech* and *fcs* (GN299). This improved the vanillin molar yield up to 83% within just 5 h. We assume that raising the expression rate of *ech* and *fcs* through induction with IPTG led to higher concentrations of the encoded metabolic enzymes than using the original promoter system. In contrast to ferulic acid, the inducer IPTG gets not metabolized and the expression rates can stay on a high level. Lowering the amount of inducer led to a decrease in product yield and productivity, which can be explained by lower enzyme concentrations. We also checked the effect of induction time, showing that less than 6 h resulted in lower product yields, probably due to lower enzyme levels. Longer induction times were also checked, but did not improve the product yields (data not shown). Raising the cell concentration in the assay led to higher levels of the by-products vanillic acid and vanillyl alcohol and did not accelerate the conversion time. We assume that higher cell densities are accompanied with higher levels of reduction equivalents which in turn may favor the formation of vanillyl alcohol.

Further improvements of the conversion process showed that raising the concentration of ferulic acid in the bioconversion broth results in a reduction of the vanillin molar yield, if higher concentrations than 10 mM of ferulic acid are used. A ferulic acid concentration of 40 mM even inhibited any conversion to vanillin. A toxic effect of ferulic acid, however, could be excluded, as growth experiments with increasing amounts of ferulic acid with up to 50 mM have shown.

On the other hand, *P. putida* GN299 showed a vanillin threshold of about 13.5 mM in the bioconversion assays. Raising the vanillin concentration above this threshold led to formation of more vanillyl alcohol and vanillic acid and inhibited conversion of ferulic acid. Such a product inhibition was also observed with recombinant *E. coli* strains converting ferulic acid to vanillin (Overhage et al. 2003). The toxic character of vanillin was confirmed with growth experiments of *P. putida* GN299 in the presence of increasing vanillin concentrations, where only up to 12.5 mM vanillin were tolerated. In contrast to *P. fluorescens* BF13, however, which showed a 98% reduction of the molar yield by increasing the ferulic acid concentration from 5 to 12.5 mM (Di Gioia et al. 2010), *P. putida* did not show such sensible reductions. Indeed, resting cells of *P. putida* GN442 could be reused after conversion for 18 h. Distracting vanillin by resuspending cells in new buffer with 10 mM ferulic acid and further incubation at 30 °C for 18 h resulted in production of 5 mM vanillin (4,5 mM ferulic acid, 0,5 mM vanillyl alcohol, 0 mM vanillic acid). Therefore, immediate distraction of the toxic product vanillin by adsorbent resins would allow *P. putida* cells to convert more ferulic acid as it could be shown previously for other systems (Yoon et al. 2007; Hua et al. 2007; Lee et al. 2009).

Inactivation of the alternative pathway from ferulic to vanillic acid proposed by Overhage et al. (1999b) by deletion of PP_3355 (*aat*) and PP_3354 in GN299 had no positive effect on formation of the unwanted formation of the by-products vanillyl alcohol and vanillic acid. It was observed that the conversion rate even decreased. A possible explanation for this behavior could be a shorter half-life of the mRNA provoked by the deletion of the two genes and therefore a diminished level of the metabolic enzymes. However, inactivation of the upregulated aldehyde dehydrogenases, encoded by PP_2680 and PP_0545, and the benzaldehyde

dehydrogenase (PP_1948) in GN299 led to higher initial conversion rates and high molar yields. The results with this strain (GN442) represent the highest productivity of a *Pseudomonas* strain in bioconversion of ferulic acid to vanillin found in the literature so far. However, the deletions had no significant effect on formation of the by-products compared to GN299. In stirred tank reactor experiments it could previously be shown that raising the dissolved oxygen concentrations did not result in formation of more vanillic acid excluding a chemical oxidation process (Di Gioia et al. 2010). It was proposed that other broad substrate specificity dehydrogenases may act in *Pseudomonas* strains that have to be determined yet (Overhage et al. 1999b).

All our bioconversion experiments showed that prolonged bioconversion times up to 18 h reduced the vanillin molar yield due to formation of the by-products. Therefore, a fast and complete as possible conversion of ferulic acid to vanillin is desirable in the first few hours. The reduction of vanillin to vanillyl alcohol seems to represent a detoxification mechanism like it was also observed with recombinant *E. coli* cells converting ferulic acid to vanillin (Overhage et al. 2003). Another approach to reduce the formation of vanillyl alcohol was to lower the amount of $NADH_2$ by deletion of the genes PP_4011 and PP_4012, encoding the isocitrate dehydrogenase, like it was proposed for recombinant *E. coli* (Lee et al. 2009). Unfortunately, deletion by the *upp* counterselection system as well as simple gene disruptions failed.

Further investigations will focus on finding the dehydrogenases responsible for formation of vanillyl alcohol. Another aspect of the established and promising bioconversion system with *P. putida* GN442 is the use of adsorbent resins to reduce the toxicity of the product vanillin and upscaling, in order to proove the versatility in industrial processes. It would also be interesting to know, if resting cells of *P. putida* strains grown in a stirred tank reactor do not show any formation of vanillyl alcohol, like it was stated for the *P. fluorescens* BF13 system (Di Gioia et al. 2010).

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4.3 Functional characterization and application of a tightly regulated MekR/P_{mekA} expression system in *Escherichia coli* and *Pseudomonas putida*

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Abstract

A methyl ethyl ketone (MEK)-inducible system based on the broad-host-range plasmid pBBR1MCS2 and on the P_{mekA} promoter region of the MEK degradation operon of *Pseudomonas veronii* MEK700 was characterized in *Escherichia coli* JM109 and *Pseudomonas putida* KT2440. For validation, β-galactosidase (*lacZ*) was used as a reporter. The novel system, which is positively regulated by MekR, a member of the AraC/XylS family of regulators, was shown to be subject to carbon catabolite repression by glucose, which however, could not be attributed to the single action of the global regulators Crc and PtsN. An advantage is its extremely tight regulation accompanied with a three magnitudes fold increase of gene expression after treatment with MEK. The transcriptional start site of P_{mekA} was identified by primer extension, thereby revealing a potential stem-loop structure at the 5'-end of the mRNA. Since MekR was highly insoluble, its putative binding site was identified through sequence analysis. The operator seems to be composed of a 15-bps tandem repeat ($CACCN_5CTTCAA$) separated by a 6-bps spacer region, which resembles known binding patterns of other members of the AraC/XylS family. Subsequent mutational modifications of the putative operator region confirmed its importance for transcriptional activation. As the -35 promoter element seems to be overlapped by the putative operator, a class II activation mechanism is assumed.

Introduction

Methyl ethyl ketone (MEK), also known as 2-butanone, is industrially produced by the dehydrogenation of 2-butanol at large scale and is used as an effective and common solvent in many industrial applications. The strain *Pseudomonas veronii* MEK700, which was isolated from a biotrickling filter cleaning waste air loaded with MEK, is able to grow on MEK as the sole carbon source (Onaca et al. 2007). The essential genes for MEK degradation were isolated and found to be organized in an operon (GenBank accession number DQ855566). It consists of two structural genes *mekA* and *mekB*, whose products, a Baeyer-Villiger-monooxygenase and a homoserine acetyltransferase-like esterase, have been studied in detail (Onaca et al. 2007; Völker et al. 2008). The *mekR* gene, coding for the *mekAB* operon transcriptional activator MekR (GenBank accession number ABI15713), is located immediately downstream of the *mekAB* operon (Onaca et al. 2007).

MekR has a length of 296 amino acids (aa). Its C-terminal domain exhibits high similarities to the transcriptional regulators of the AraC/XylS family. Members of this constantly expanding family can be found in many bacteria. They control genes that are involved in metabolism, stress response, and virulence (Gallegos et al. 1997; Martin and Rosner 2001; Egan 2002; Ibarra et al. 2008). Nearly 2,000 proteins were identified to belong to this family so far by means of experimental analysis and *in silico* predictions, but only a small fraction has been approved for biotechnological applications (Tobes and Ramos 2002; Ibarra et al. 2008; Schüller et al. 2012). In general, most members are comprised of 250 to 300 aa and consist of two domains, a nonconserved N-terminal domain which seems to be involved in effector recognition and dimerization, and a conserved C-terminal domain with a significant 100-aa stretch, that forms two helix-turn-helix DNA-binding motifs (Gallegos et al. 1997). Typical DNA-binding sites of these regulators are arranged as two bipartite DNA-binding motifs, proposing that one monomer of the dimeric regulator binds to one repeat (Gallegos et al. 1997; Domínguez -Cuevas et al. 2008). For example, the activation of the *P. putida* TOL plasmid pWW0 *meta*-operon promoter Pm by XylS, requires a tandem of 15-bp direct repeats, which is separated by 6 bps and overlaps with the -35 region (Kaldalu et al. 1996). The proteins of the AraC/XylS family have a

tendency to become insoluble upon purification, making them unamenable to biochemical analysis (Egan and Schleif 1994; Jair et al. 1995; Michan et al. 1995; Munson and Scott 2000; Timmes et al. 2004). Only a few proteins could be purified in soluble form and crystallized in order to determine their three-dimensional structure, e.g. MarA and the Rob protein of *E. coli* (Rhee et al. 1998; Kwon et al. 2000).

A characteristic feature of pseudomonads is their broad metabolic versatility, meaning that they can use a wide range of compounds to grow on. However, there seems to be a sequential hierarchy where organic acids like amino acids or succinate are the most preferred compounds, followed by carbohydrates like glucose and hydrocarbons as the less preferred compounds (reviewed by Collier et al. 1996; Rojo 2010). The regulatory process for selection of the preferred substrates is called carbon catabolite repression (CCR). Although CCR is not well studied in pseudomonads, some of the main regulatory factors have been found. These are the translational repressor protein Crc and the nitrogen phosphotransferase system (PTS^{Ntr}), which is composed of PtsP, PtsO and PtsN (Rojo 2010). A well studied example of CCR on promoters regulated by members of the AraC/XylS-family is the XylS/Pm expression system of the *P. putida* pWW0 plasmid, which was shown to be subject to a Crc-dependent CCR exerted by amino acids when cells are grown in a complete medium (Moreno et al. 2010). Another example is the XylR/Pu expression system of the same plasmid, which is subject to a PTS^{Ntr}-dependent CCR exerted by glucose and succinate (Duetz et al. 1994; Holtel et al. 1994; Duetz et al. 1996; del Castillo and Ramos 2007).

In this study, we analyzed the promoter P_{mekA} of the *mekAB* operon of *P. veronii* MEK700. The transcriptional start site of P_{mekA} was identified by primer extension experiments, and the putative binding sites of the extremely insoluble transcriptional activator MekR were localized by mutational and deletion analysis. In addition to MEK, a range of other cognate compounds were tested for their induction capabilities. The influence of Crc and PTS^{Ntr} on CCR of the novel system was also investigated using deletion mutants. Finally, the novel system was compared to established systems based on regulators of the AraC/XylS-family.

Materials and Methods

Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are summarized in Table S1 (Online Resource 1). *P. putida* strains were grown at 30 °C and *E. coli* JM109 (Yanisch-Perron et al. 1985) at 37 °C in LB medium (Bertani 1951). For selection 50 µg ml^{-1} apramycin (Am), 50 µg ml^{-1} kanamycin (Km) or 40 µg ml^{-1} gentamicin (Gm) were added. Induction of plasmid containing cells of *E. coli* JM109 and *P. putida* KT2440 started with growth in 5 ml LB medium in glass tubes with screw caps for 2 h at 37 °C (*E. coli*) or 2.5 h at 30 °C (*P. putida*) under shaking conditions (200 rpm). After addition of inducer, i.e. 40 µl MEK (or other ketones), 0.2% (w/v) arabinose or 2 mM m-toluic acid, respectively, depending on the used expression system, cells were incubated for another 4 h at the corresponding temperature. For CCR experiments glucose was added to a final concentration of 0.2% (w/v) additionally to the inducer.

Chemicals and other materials

Chemicals used in this study were of analytical grade and purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Sigma-Aldrich Corporation (Taufkirchen, Germany) and Merck KGaA (Darmstadt, Germany). Synthetic DNA oligonucleotides (Online Resource 1, Table S2) were purchased from Eurofins MWG Operon GmbH (Ebersberg, Germany). Restriction enzymes and DNA modifying enzymes were purchased from Roche Diagnostics Deutschland GmbH (Mannheim, Germany), New England Biolabs GmbH (Frankfurt am Main, Germany) and Thermo Fisher Scientific (St. Leon-Rot, Germany). PCRs were run with High Fidelity PCR Enzyme Mix from Thermo Fisher Scientific (St. Leon-Rot, Germany) on a TPersonal Thermocycler from Biometra GmbH (Goettingen, Germany).

Transformation with plasmid DNA

Transformation of *E. coli* with plasmid DNA occured via the TSS (Transformation and Storage Solution) method (Chung et al. 1989). *P. putida* strains were transformed with plasmid DNA via a modified TSS method: The main culture was inoculated with a 1:100 dilution of the overnight culture in LB medium and incubated for 2.5 h at 30 °C in shaking flasks till the optical density at 600 nm (OD_{600}) reached about 0.3. Cells were pelletized and resuspended in TSS medium to give a concentration of 7.5×10^9 cells ml^{-1}. A total of 200 ng plasmid DNA was added to 200 µl of the cell suspension. After incubation for 15 min on ice, cells were heat shocked for 45 sec at 42 °C. After the suspension was chilled on ice, 2 ml of LB medium was added. After incubation for 1 h at 30 °C, the suspension was centrifuged and the cell pellet plated on LB agar plates containing apramycin. Transformants were grown overnight at 30 °C.

Cloning of the MEK inducible promoter system

Cloning steps were performed with *E. coli* JM109 (Yanisch-Perron et al. 1985) using standard recombinant DNA techniques (Sambrook et al. 1989).

A shuttle vector for *E. coli* and *P. putida* with the MEK inducible promoter system for expression of the reporter gene *lacZ* was constructed. First, vector pJOE6533.4 was shortened by PCR amplification using primers s6430 / s6431. The fragment was circularized by PacI restriction sites, which were introduced by the primers, resulting in vector pNG176.6. Next, the reporter gene *lacZ* was introduced. After PCR amplification of *lacZ* using plasmid pSUN272.1-DNA as template and primers s6881 / s6433, the fragment was inserted via AgeI / BglII into AgeI / BamHI cut pNG176.6. By means of the used primers additional BsrGI and NdeI restriction sites were introduced. Using restriction sites AgeI / NdeI the *mekA* promoter sequence was inserted, which was previously PCR amplified using primers s6880 / s5678 and DNA of pJOE5332.10 as template, resulting in vector pNG217.1. Again, by means of the used primers additional BamHI and MfeI restriction sites were introduced. The *mekR* regulator gene together with its own promoter was PCR amplified using primers s5703 / s5704 and pJOE5332.10-DNA as template. The fragment was brought into pNG217.1 via BamHI / EcoRI and BamHI / MfeI, respectively. The resulting *lacZ*-expression and shuttle vector was designated pNG247.1 and sequenced using primers s5760, s6432, s6433, s6435, s6468, s6469, s6592, and s6593.

Construction of alternative expression vectors

Construction of a vector with the AraC/P_{BAD} expression system started with PCR amplification of the P_{BAD} promoter region and the adjacent regulatory gene *araC* using oligonucleotide primers s8032 / s8033 and plasmid pJOE6038.1 as template. The amplified fragment was restricted with BglII / NdeI and ligated into BamHI / NdeI restricted pNG247.1 giving pNG413.1. Analogously, for the vector construction with the XylS/Pm expression system, the Pm promoter region and the adjacent regulatory gene *xylS* were PCR amplified using oligonucleotide primers s8030 / s8079 and plasmid pJB659 as template. The amplified fragment was inserted via BamHI / NdeI into pNG247.1, resulting in expression vector pNG418.3.

Modification of the PmekA promoter region

The P_{mekA} promoter region was shortened by PCR amplification. The reverse primer was located in the *lacZ* gene of pNG247.1. The amplified fragments were cloned via BglII / EcoRV into pNG247.1. Sequencing occurred using primers s7472 and s6435. A fusion PCR method by overlap extension (Ho et al. 1989) was used for site-directed mutagenesis to introduce multiple-nucleotide substitutions using mutagenic primers as well as for deletions in the P_{mekA} promoter region using primers that omit this certain region. DNA of plasmid pNG247.1 was used as a template. The amplified DNA fragments, of which the 3′ and 5′ ends are to be fused, were mixed equally and fused by PCR using the flanking forward and reverse primers. The fusion products were inserted via NcoI / NdeI into pNG247.1. The

mutation was confirmed by DNA sequencing using primer s7475. The construction of the plasmids is summarized in detail in Table S1 (Online Resource 1).

Primer extension

Main cultures of *E. coli* JM109 and *P. putida* KT2440 carrying the P_{mekA} promoter containing plasmid pNG247.1 were induced as aforementioned. Cells were pelletized and the total RNA isolated with the QIAGEN RNeasy minikit (Hilden, Germany) according to the manufacturer's instructions. Oligonucleotide primer s5959 was designed for plasmid pNG247.1 and tagged with the fluorescent dye Cy5 at the 5'-end. For the primer extension experiment 10 µg of isolated RNA, primer s5959 and AMV-RT (Avian myeloblastosis virus reverse transcriptase from Roche, Mannheim, Germany) were used. The sequencing reactions were carried out using the dideoxy method (Sanger et al. 1977). More precisely, Cy5-labeled primer s5959 and 8 µg of plasmid pNG247.1-DNA were used for the sequencing reaction with the AutoRead™ Sequencing Kit (formerly Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. Separation of the fragments with the ALFexpress II DNA sequencer (formerly Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) was conducted according to the manufacturer's instructions on a 0.3-mm thick sequencing gel (ReproGel High Resolution, GE Healthcare Company, Munich, Germany).

Measurement of β-galactosidase activity

The β-galactosidase activity was determined as described previously (Miller 1972): Cells of *E. coli* JM109 and *P. putida* KT2440 with pNG247.1 or derivatives thereof, were in principal induced as aforementioned. For kinetic experiments different amounts of inducer were added and incubation times were varied. After measurement of the OD_{600}, 100 µl of cell culture were treated for 30 min at 37 °C with 700 µl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol) and 10 µl toluene. After 15 min evaporation of the toluene, the β-galactosidase reaction was started by adding 200 µl of 20 mM o-nitrophenyl-β-galactopyranoside (in Z buffer) at 22 °C. After the suspension turned yellowish, 500 µl of 1 M Na_2CO_3 was added to stop the reaction, for a maximum of 30 min. Miller Units (M.U.) were determined by measuring the OD at 420 and 550 nm.

Results

Construction of a reporter system for MEK inducible gene expression

Supplying the genes of the *mek* operon, *mekA*, *mekB*, and *mekR* of *P. veronii* MEK700 on the vector pJOE5332.10, enabled *P. putida* KT2440 to grow on MEK (Onaca et al. 2007). The necessity for the regulator gene *mekR* let us assume, that the *mekAB* operon is positively regulated. Therefore, P_{mekA} was fused to the reporter gene *lacZ* in order to determine basal activity and inducibility by MEK. This cassette was cloned into a

broad-host-range vector. To keep the vector size as small as possible, the replicon of pBBR1MCS (Kovach et al. 1994) and a common resistance marker for both *E. coli* and *P. putida* were used. The gene for the aminoglycoside 3-*N*-acetyltransferase AAC(3)-IV from *E. coli* was chosen, which causes resistance to a large number of aminoglycosides, including the antibiotic apramycin (Magnet and Blanchard 2005). After transformation of both *P. putida* KT2440 and *E. coli* JM109 with this expression vector (pNG217.1), almost no β-galactosidase activities (19 ± 3 Miller Units [M.U.] and 32 ± 4 M.U., respectively) could be measured after addition of MEK matching the corresponding basal activities. This confirmed the findings by Onaca et al. (2007) and thus, the gene for the transcriptional activator, *mekR*, including its own promoter, P_{mekR}, was additionally delivered in opposite direction of P_{mekA}-*lacZ*, resulting in the expression vector pNG247.1 (Fig. 1).

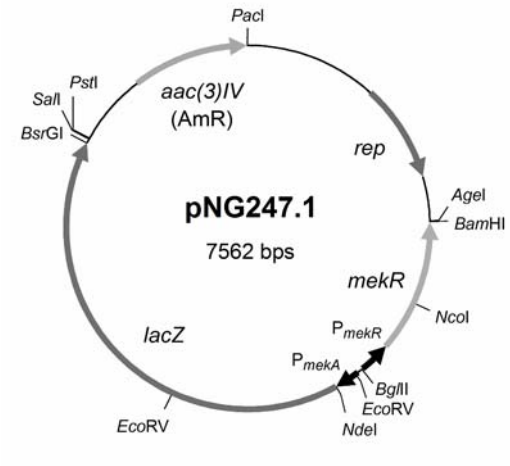

Fig. 1 Physical map of the medium copy number P_{mekA}-*lacZ* expression vector pNG247.1 for *E. coli* and *P. putida*. Restriction sites used for construction of pNG247.1 and its derivatives are shown

P. putida KT2440 as well as *E. coli* JM109 were transformed with pNG247.1, and β-galactosidase activities of the transformants were determined at certain time intervals in the absence and presence of increasing amounts of MEK (Fig. 2). Defined volumes of 20, 40, and 60 µl of MEK were added to 5 ml culture volume corresponding to initial concentrations of 45, 90, and 135 mM. However, due to the very high volatility of MEK we cannot say about the actual concentrations in the assay. After induction, culture tubes were closed airtight in order to avoid

evaporation of MEK. In both strains, the highest β-galactosidase activities were achieved with 40 µl MEK and 4 h induction time. Longer treatment with MEK or application of lower amounts led to lower β-galactosidase activities. In case of *E. coli*, a maximum of about 22,000 Miller Units [M.U.] was reached, whereas with *P. putida* about 12,000 M.U. were achieved. In general, *E. coli* showed higher activities as *P. putida* independently of the inducer amount and induction time. Remarkably, the basal acitivities were extremely low in either of the strains. The measured values were about 20 M.U. with *E. coli* and about 10 M.U. with *P. putida*.

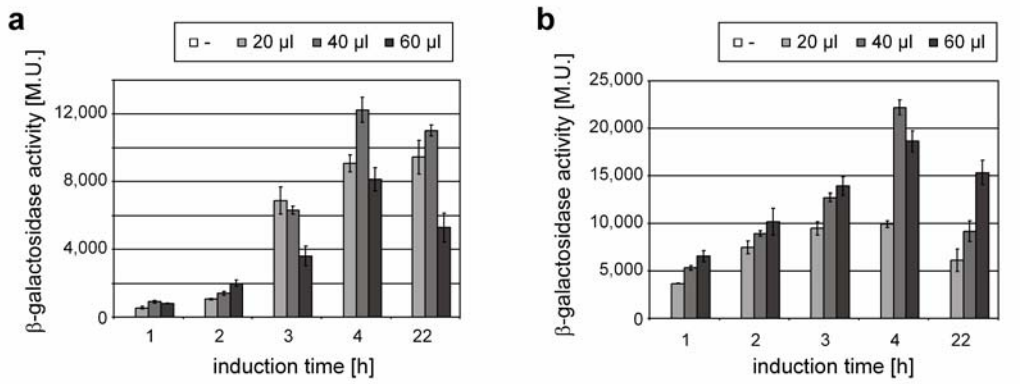

Fig. 2 β-galactosidase activity assays of [a] *P. putida* KT2440 / pNG247.1 and [b] *E.coli* JM109 / pNG247.1 using no (-) and increasing amounts of inducer MEK. Samples were taken at the indicated times after induction. The β-galactosidase activity is presented as the mean value of the determined Miller Units (M.U.) of at least three independent experiments. Error bars indicate the standard deviation

As MEK is an organic solvent which cannot be metabolized naturally by *P. putida* KT2440 and *E. coli* JM109, its effect on bacterial growth was also examined: With increasing MEK amounts, the specific growth rate µ of *P. putida* KT2440 decreased from 0.33, over 0.28 and 0.25 to 0.2 h^{-1} and of *E. coli* JM109 from 0.23, over 0.22 and 0.2 to 0.15 h^{-1}.

Determination of the transcription start site of P_{mekA}

In order to locate the promoter of *mekA* more precisely, primer extension experiments were performed to determine the transcription start site. Total RNA of *P. putida* KT2440 as well as *E. coli* JM109 carrying plasmid pNG247.1 was isolated after incubation of the cells in the presence and absence of MEK. Incubation of the cells under non-inducing conditions led to no detectable signals with *E. coli* (Fig. 3a) and only weak signals with *P. putida* (Fig. 3b). This behavior confirmed the tightly regulated expression from P_{mekA} as observed with the conducted β-galactosidase activity assays. Transcription from the *mekA* promoter was strongly increased in both microorganisms when cells were incubated with MEK, as shown by strong signals.

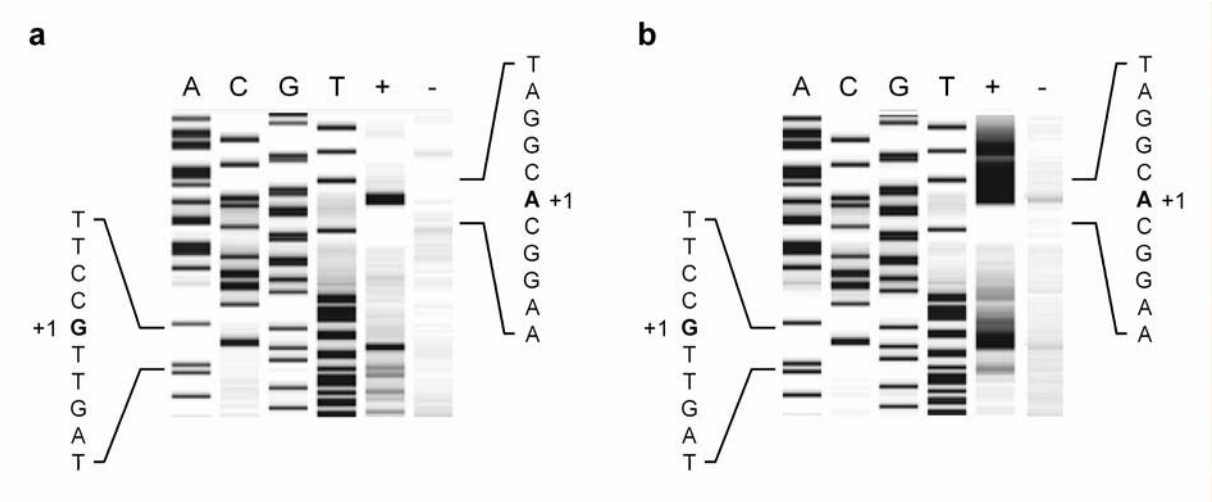

Fig. 3 Primer extension of P_{mekA} using RNA from induced and uninduced [a] *E. coli* JM109 / pNG247.1 and [b] *P. putida* KT2440 / pNG247.1 cells. A, C, G, and T represent the Sanger DNA sequencing reactions (dideoxynucleotide chain termination method) using pNG247.1 as a template. The (+) and (-) display the primer extension reaction of induced and uninduced sample, respectively

Interestingly, two potential transcription start sites were found with RNA isolated from both organisms. The upstream located transcription start (uTS) resulted in a stronger signal as the downstream one (dTS). The uTS was found to be a T residue, while the dTS was located 36 bps downstream of the uTS and found to be a C residue. Taking a closer look at the sequence between the two transcriptional start sites, an inverted repeat with a $GCCTTGAN_2TT$ motif was identified (Fig. 4). The repeats begin exactly with the uTS and end 1 bp downstream of the dTS, proposing a

stem-loop formation of the 5′ end of the mRNA. This sequence was further analyzed with the Vienna RNA Websuite tools, which give insights into RNA secondary structure energy landscapes and generate structural alignments (Mathews et al. 2004; Gruber et al. 2008). The predicted RNA fold is shown in Fig. 4. The free energy of the stem-loop formation was calculated to -17.8 kcal mol^{-1}.

We assume that the formation of this 5′-terminal stem-loop might lead to a premature stop of reverse transcription and thus to a false signal in the primer extension experiment.

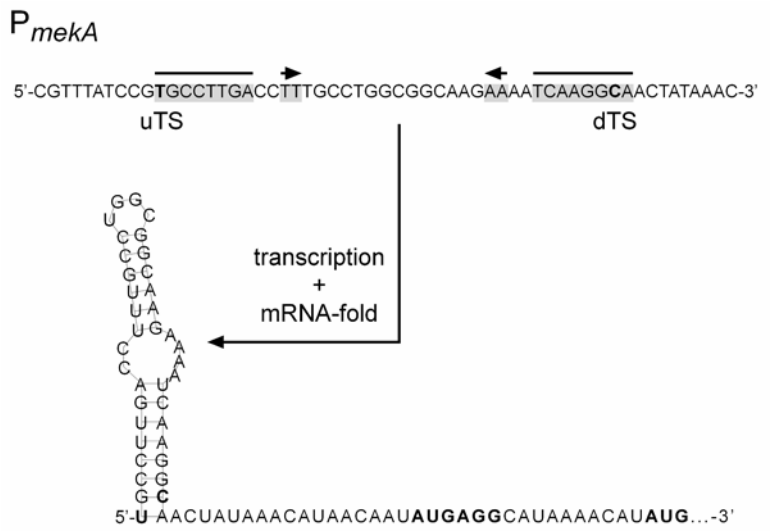

Fig. 4 Inverted repeat found between the two potential transcriptional start sites, uTS and dTS, and the proposed structure of the *mekAB* leader mRNA after transcription determined with the Vienna RNA Websuite tools (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; Mathews et al. 2004; Gruber et al. 2008). The two identified transcriptional start sites, the putative ribosomal binding site and the start codon are displayed in bold face

Mutational analysis of the *mekA* promoter region reveals the necessity of a tandem repeat for full transcriptional activity

On the search for typical promoter elements, an extended -10 element adjacent to a -10 hexamer (TGTTACCGT) upstream of the uTS could be deduced that resembled the house-keeping sigma factor (σ^{70}) binding site. Although the existence of the dTS can be challenged due to the proposed secondary structure of the 5′ end of the *mekA* mRNA, a -35 hexamer (TTGACC) resembling the σ^{70} binding site could be deduced

(Fig. 5a). As MekR belongs to the AraC/XylS familiy of transcriptional regulators, we scrutinized the upstream region for a typical binding site pattern. Indeed, a 15-bps tandem repeat consisting of two CACCN$_5$CTTCAA (with N = any nucleotide) motifs separated by a 6-bps spacer region was found, located from position -71 to -36 relative to the uTS (Fig. 5a). This region might represent the putative MekR binding site, and thus should be essential for transcriptional activation. No such motifs could be found in the P$_{mekR}$ region.

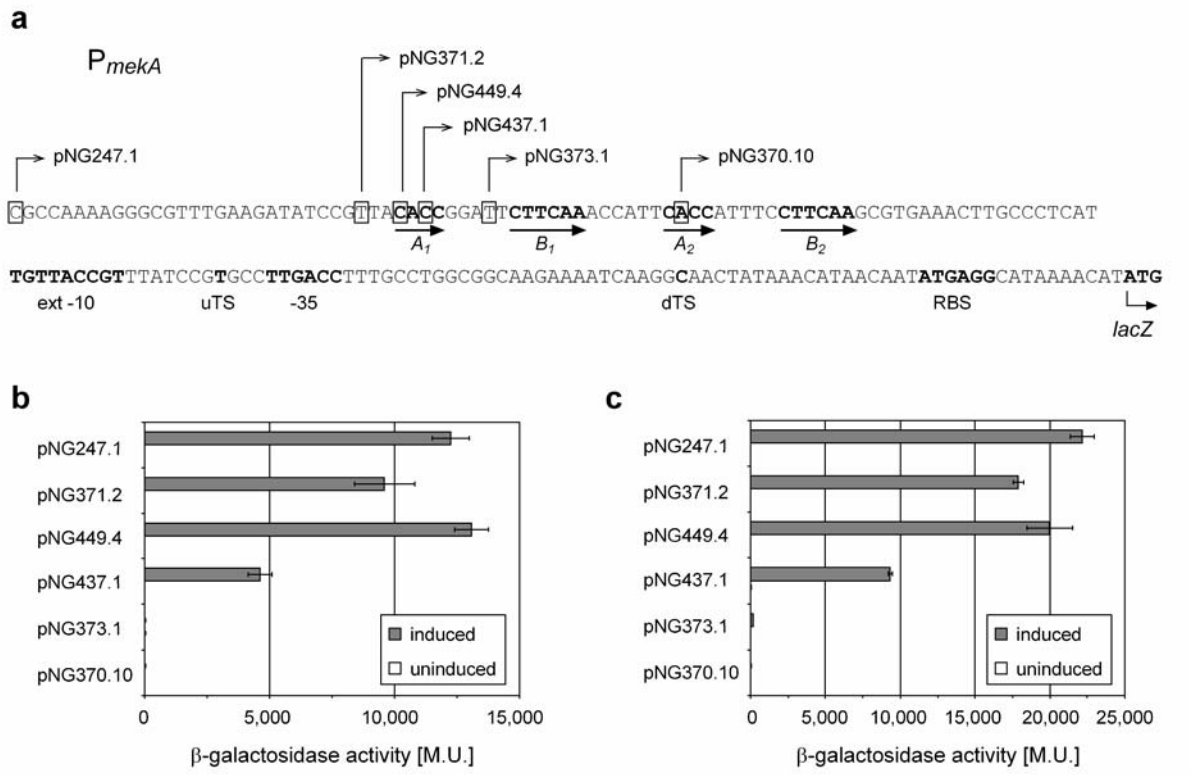

Fig. 5 Shortening of the 5′ end of P$_{mekA}$ region. [a] The identified direct repeats (boxes A_1, A_2, B_1 and B_2), the two observed transcriptional start sites (uTS and dTS) the deduced promoter elements, the putative ribosomal binding site (RBS) and the start codon of *lacZ* are written in bold letters. The start sites of P$_{mekA}$ in the used vectors are framed and indicated by arrows. The vectors were brought into [b] *P. putida* KT2440 and [c] *E. coli* JM109. β-galactosidase activities were measured in the presence and absence of MEK. The mean values of the determined Miller Units (M.U.) of three independent experiments are shown. Standard deviation is indicated by error bars

Therefore, deletion analysis was focused on the region with the tandem repeat. Stepwise shortening originated from the P_{mekA}-*lacZ* expression vector pNG247.1. After the modified vectors were brought into *E. coli* JM109 and *P. putida* KT2440, respectively, induction of the transformants with MEK and subsequent measurement of the β-galactosidase activities revealed the necessary region for transcription activation (Fig. 5b, c). The β-galactosidase activity was not significantly influenced by deletions not affecting the beginning (box A_1) of the tandem repeat (pNG371.2 and pNG449.4). However, further deletions affecting the tandem repeat led to strong decreases in β-galactosidase activity. Deletion of box A_1 (pNG373.1) and boxes A_1 and B_1 (pNG370.10) abolished β-galactosidase activity completely. Further deletions were not examined.

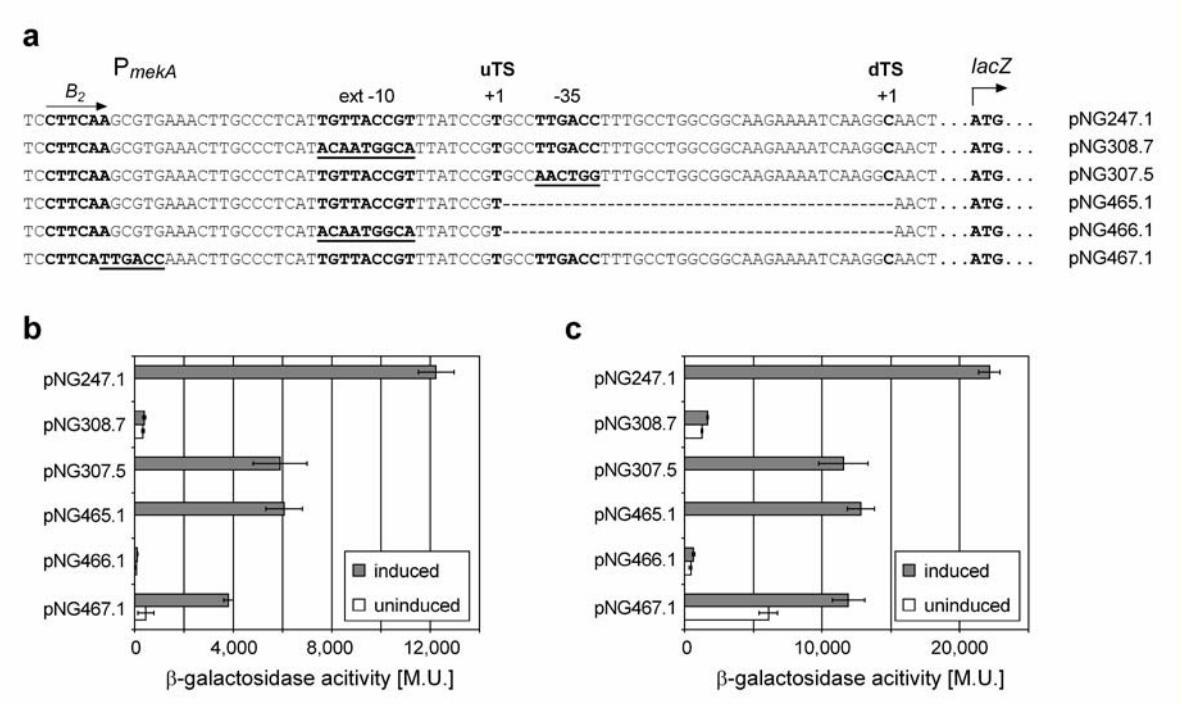

Fig. 6 Mutations in the P_{mekA} region. [a] Box B_2 of the tandem repeat, the two observed transcriptional start sites, uTS and dTS, the deduced promoter elements, and the start codon of *lacZ* are written in bold letters. The vectors were brought into [b] *P. putida* KT2440 and [c] *E. coli* JM109. β-galactosidase activities were measured in the presence and absence of MEK. The mean values of the determined Miller Units (M.U.) of three independent experiments are shown. Standard deviation is indicated by error bars

Further analysis of the P_{mekA} promoter region by complementary base exchange mutagenesis was performed in order to analyze the role of the deduced promoter elements (Fig. 6a). Besides these mutations (pNG308.7 and pNG307.5), the region between the uTS and dTS was completely deleted (pNG465.1 and pNG466.1). Additionally, a perfect -35 hexamer was inserted with a distance of 17 bps to the proposed -10 sequence upstream of the uTS (pNG467.1). All these vectors were brought into *E. coli* JM109 as well as *P. putida* KT2440 and compared to pNG247.1. The β-galactosidase activities of the transformants were determined in the presence and absence of the inducer MEK (Fig. 6b, c).

In most cases, the effects of the mutations were nearly the same in *P. putida* and in *E. coli*. Complementary base exchange of the extended -10 (pNG308.7) resulted in a strong decrease of β-galactosidase activity. However, the basal level increased, resulting in almost constitutive activity. Due to the complementary exchange a new potential -10 promoter element, TACAAT, resembling the consensus sequence of the σ^{70} has been generated located from position -17 to -12 relative to the uTS. Upsteam and in a distance of 17 bp, a TTCAAG hexamer could be found, which also resembled the -35 consensus sequence of σ^{70}. This formation of a new promoter might be responsible for the constitutive activity of the mutant. Complementary exchange of the -35 hexamer upstream of the dTS as well as complete deletion of the region between uTS and dTS (pNG307.5 and pNG465.1) decreased the β-galactosidase activities by nearly 50%. Again, the complementary exchange of the extended -10 in the mutant lacking the region between both transcription start sites (pNG466.1) led to weak but constitutive activities. Introduction of a perfect -35 hexamer 17 bps upstream of the proposed -10 sequence (pNG467.1) decreased the overall β-galactosidase activity but increased the basal activity. However, the extent of the latter effect was different in the compared microorganisms. Whereas with *P. putida* only a slight increase of the basal activity to ca. 12% of the full induced level could be observed, about 51% were reached with *E. coli*. The decrease of overall activity might be due to the A→T mutation in box B_2 of the downstream direct repeat.

All these mutants showed that the region between uTS and dTS is necessary to reach high β-galactosidase activities, but cannot act as an independent promoter region.

Block scanning mutagenesis of the putative MekR binding site in the *mekA* promoter region

The region with the tandem repeat was shown to be essential for transcriptional activity of P_{mekA}. In analogy to the binding pattern of other members of the AraC/XylS-family of regulators, the conserved boxes, CACC ($A_{1/2}$) and CTTCAA ($B_{1/2}$), in the tandem repeat can be assumed to represent the putative recognition elements of MekR. Due to inclusion body formation with overexpressed MekR in *E. coli* as well as in *P. putida*, it was not possible to identify the binding sites of MekR by footprinting experiments. Therefore, a block scanning mutagenesis was performed by substitution of defined blocks with the according complementary nucleotides. The boxes A_1, B_1, A_2 and B_2, as well as the variable spacer regions between them (S_1 and S_2) and between the two repeats (S_{1-2}) were mutated separately (Fig. 7a).

A reciprocal exchange of the spacer regions S_1 and S_2 was also analyzed. After fusion of these mutated promoter regions to *lacZ*, the corresponding expression vectors were brought into *P. putida* KT2440 and *E. coli* JM109, respectively, and compared by measurement of the β-galactosidase activities (Fig. 7b, c). The mutation of each of the four boxes had strong negative effects on β-galactosidase activity to a greater or lesser extent. Interestingly, the CTTCAA→GAAGTT mutation of box B_2 (pNG436.1) raised the basal activity in *E. coli* to about 55% of the induced level. A generation of a potential -35 promoter element (TTGCGT) in a distance of 18 bps to the deduced -10 promoter element might explain the weak constitutive behavior. However, this effect could not be observed with *P. putida*. Possibly, the RNA polymerase of *E. coli* could better recognize these promoter elements as the corresponding enzyme of *P. putida*. The complementary exchange of S_1 (pNG431.1) had a stronger negative effect than the exchange to the sequence of S_2 (pNG439.1). This behavior could also be observed vice versa (pNG435.1 and pNG394.1). Finally, the complementary exchange of S_{1-2} had also a strong negative effect on β-galactosidase activity. Taken together, the tandem repeat including S_{1-2} seems to play a significant role in transcription activation. The only exchange having not such a drastic effect was the ATTTC→TAAAG mutation in S_2 (pNG435.1), reaching about 75% and 45% of the wild-type activity in *P. putida* and *E. coli*, respectively.

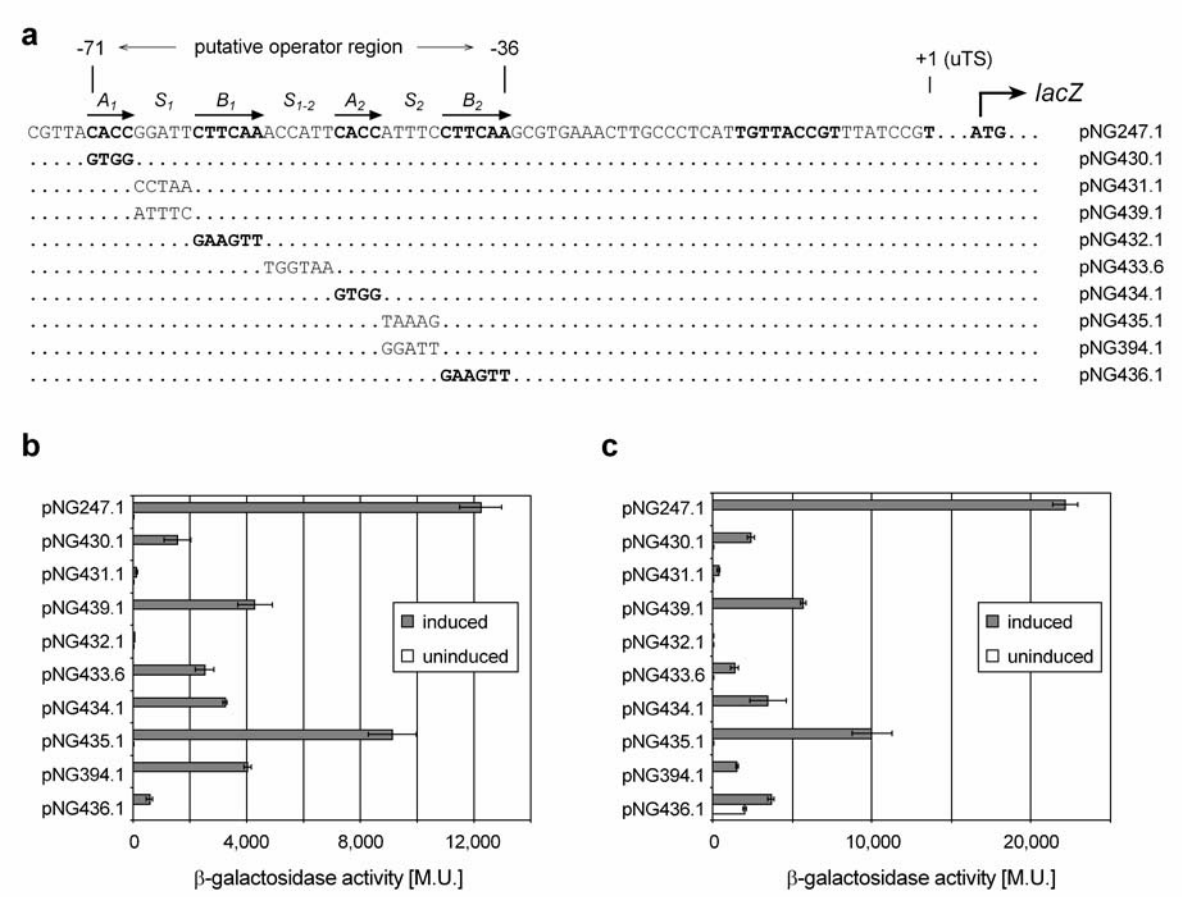

Fig. 7 Block scanning mutagenesis of the putative MekR binding site. [a] The identified tandem repeat (boxes A_1, A_2, B_1 and B_2), the upstream transcriptional start site (uTS), the deduced extended -10 promoter element, and the start codon of *lacZ* are written in bold letters. Spacer regions between the repeat boxes S_1, $S_{1\text{-}2}$, and S_2 are indicated. After the vectors were brought into [b] *P. putida* KT2440 and [c] *E. coli* JM109, β-galactosidase activities were measured in the presence and absence of MEK. The mean values of the determined Miller Units (M.U.) of three independent experiments are shown. Standard deviation is indicated by error bars

Analysing the range of possible inducers

Structurally related ketones to MEK (2-butanone) with smaller and longer hydrocarbon chains were tested in order to determine the range of possible inducers of the MekR/P_{mekA} expression system. Besides MEK, the ketones acetone (Ac), 2-pentanone (2P), 3-pentanone (3P), 2-hexanone (2H), and 3-hexanone (3H) were used in different concentrations (Fig. 8a). Besides MEK, 2P and 3P were able to induce *lacZ* expression in *P. putida* significantly when low amounts were used (Fig. 8b). After doubling of inducer amount only MEK resulted in higher expression levels. High

concentrations of 2P and 3P, and especially of 2H and 3H impaired *lacZ* expression to a high extent, which was most probably be due to the strong inhibition of growth after addition of the ketones. Acetone led to weak *lacZ* expression independently of the used amount. Similar results were obtained in *E. coli* (Fig. 8c). Again, Ac, 2H and 3H had only marginal inducing effects on *lacZ* expression independently of the used amounts. Growth of *E. coli* was strongly impaired with the latter two. High β-galactosidase activities were achieved with MEK, 2P and 3P. Again, doubling of the inducer amounts led to a strong decrease in β-galactosidase activity with exception of MEK, which seems to be better tolerated as the other organic compounds.

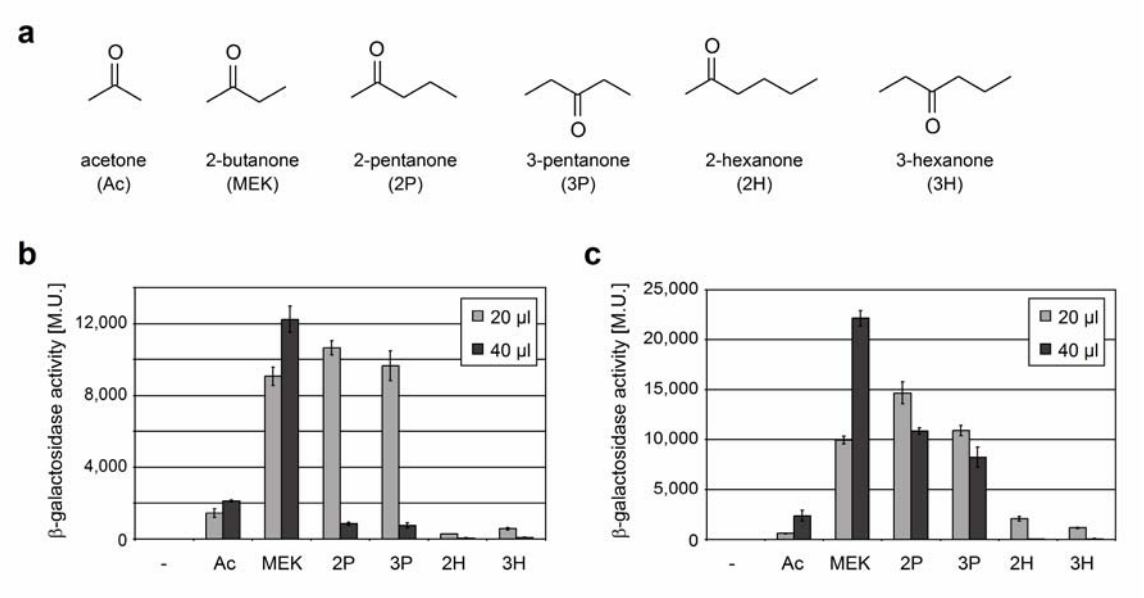

Fig. 8 Analysis of the range of inducers for the MekR/P_{mekA} expression system. Chemical structures of the used ketones with growing hydrocarbon chain are shown in panel [a]. β-galactosidase activities of [b] *P. putida* KT2440 / pNG247.1 and [c] *E. coli* JM109 / pNG247.1 uninduced (-) and induced by acetone (Ac), 2-butanone (MEK), 2-pentanone (2P), 3-pentanone (3P), 2-hexanone (2H), and 3-hexanone (3H) are presented by the mean values of the determined Miller Units (M.U.) after 4 h of induction of at least three independent experiments. Error bars indicate the standard deviation. Addition of 20/40 µl inducer to 5 ml culture medium corresponds to initial concentrations of 55/110 mM Ac, 45/90 mM MEK, 37/74 mM 2P and 3P, 32/64 mM 2H and 3H, respectively

The MekR/P$_{mekA}$ expression system is repressed by glucose but is not subject to CCR by single action of Crc and PtsN

In analogy to the XylS/Pm and XylR/Pu expression systems of the *P. putida* pWW0 plasmid where Crc- and PTS^{Ntr}-dependent CCRs could be observed, the novel MekR/P$_{mekA}$ expression system was checked to be subject to CCR by these global regulators. The role of the PTS^{Ntr} system, which is supposed to be involved in the regulation of C/N balance and CCR by glucose, was investigated on the novel MekR/P$_{mekA}$ expression system by treatment with glucose. A Crc-dependent CCR would be provoked in LB medium due to the amino acids present in this medium. Therefore, the *P. putida* deletion strains KT2440/*crc* and KT2440/*ptsN* as well as the wild-type strain KT2440 were transformed with pNG247.1 and analyzed for their β-galactosidase activities in the absence and presence of the inducer MEK and glucose (Fig. 9). The β-galactosidase activities in all the strains were repressed by glucose by an extent of about 30-40%. However, neither of the deletions of the genes *crc* and *ptsN* did affect the β-galactosidase levels with and without glucose proposing that the MekR/P$_{mekA}$ expression system is not subject to CCR exerted by Crc or PtsN alone.

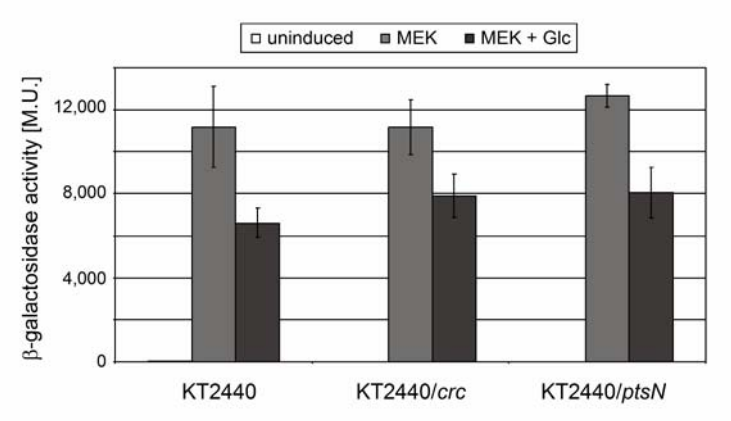

Fig. 9 Carbon catabolite repression (CCR) on the MekR/P$_{mekA}$ expression system. *P. putida* strains KT2440, KT2440/*crc* and KT2440/*ptsN* transformed with pNG247.1 were grown in LB medium and analyzed for β-galactosidase activities under uninduced, induced (with MEK) and CCR conditions (MEK and glucose). Presented are the mean values of at least three independent experiments. The standard deviation is given by error bars

Comparison to other expression systems

Several expression systems based on transcriptional regulators of the AraC/XylS family have been approved and optimized for the use in different bacteria and have also become commercially available (Brautaset et al. 2009). Well studied examples are the AraC controlled promoter P_{BAD} from the arabinose operon of *E. coli* (Guzman et al. 1995; Sukchawalit et al. 1999; Huang et al. 2000; Lim et al. 2000; Loessner et al. 2007; Qiu et al. 2008) and the XylS controlled Pm promoter of the *meta* operon of the *P. putida* TOL plasmid pWW0 (Mermod et al. 1986; Blatny et al. 1997a; Blatny et al. 1997b; Winther-Larsen et al. 2000a; Winther-Larsen et al. 2000b; Sletta et al. 2004; Bakke et al. 2009). For comparison of the novel MekR/P_{mekA} expression system to these well-established systems, the promoter regions of P_{BAD} and Pm were fused to *lacZ* and brought into a pBBR1MCS-based broad-host-range vector. After transformation of *P. putida* KT2440 and *E. coli* JM109 with pNG247.1 (P_{mekA}-*lacZ*), pNG413.1 (P_{BAD}-*lacZ*), and pNG418.3 (Pm-*lacZ*), β-galactosidase activities were measured in the absence and presence of the cognate inducers (Fig. 10).

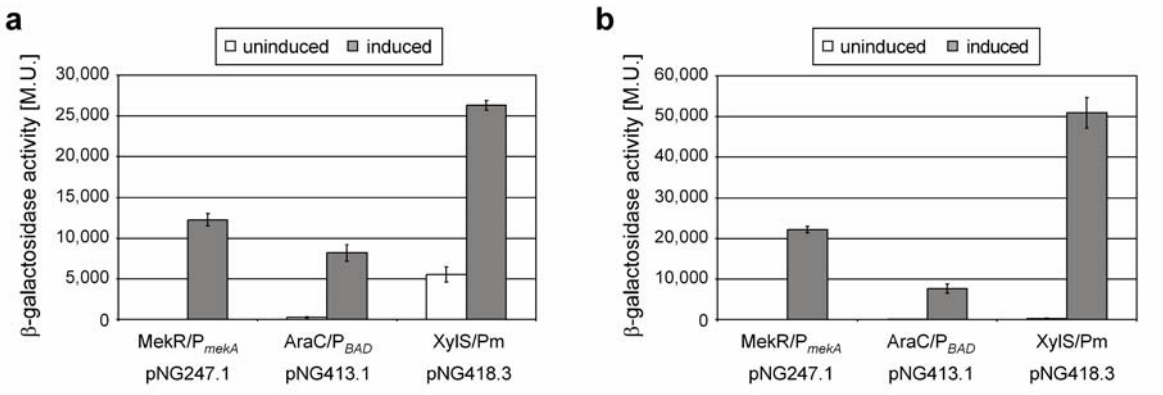

Fig. 10 Comparison of the novel MekR/P_{mekA} expression system (pNG247.1) to the well-established AraC/P_{BAD} (pNG413.1) and XylS/Pm (pNG418.3) expression systems. The vectors were brought into [a] *P. putida* KT2440 and [b] *E. coli* JM109. The β-galactosidase activities were measured in the presence and absence of the corresponding inducers: 40 µl MEK (approx. 90 mM), 0.2% (w/v) L-arabinose and 2 mM m-toluic acid. Mean values of the determined Miller Units (M.U.) of at least three independent experiments are shown. Error bars indicate the standard deviation

In total, the XylS/Pm expression system led to the highest β-galactosidase activities in both microorganisms. In *P. putida* and in *E. coli* the MekR/P_{mekA} expression system reached about 45% of the level obtained with the XylS/Pm system. With the AraC/P_{BAD} expression system even lower activities were obtained, namely 31% in *P. putida* and 15% in *E. coli*. However, the basal activity of the XylS/Pm system in *P. putida* was rather high reaching about 21% of the induced level. Thus, induction with m-toluic acid raised the expression only 5-fold. On the other hand, the basal activity in *E. coli* was much lower resulting in a nearly 170-fold higher level after induction. A similar value of a 250-fold induction could be observed with luciferase activity in *E. coli* (Winther-Larsen et al. 2000b). The AraC/P_{BAD} expression system was induced 30-fold in *E. coli* and about 60-fold in *P. putida*. As expected, growth was not negatively affected by arabinose in neither of the microorganisms. Due to the extremely low basal activities in both microorganisms, the MekR/P_{mekA} expression system showed the highest increase after induction. Addition of MEK induced the system about 1,200-fold in *P. putida* and about 1,000-fold in *E. coli*.

Discussion

The novel MekR/P_{mekA} expression system of *P. veronii* MEK700 was shown to be suitable for application in *E. coli* and *P. putida* due to its extremely low basal activities and due to a several magnitudes fold raise of expression after induction with MEK. It showed similar properties as the well-established XylS/Pm and AraC/P_{BAD} expression systems. In contrast to arabinose, addition of m-toluic acid or MEK to the growth medium provoked a decrease in the specific growth rate μ, while the β-galactosidase activity remained unaffected. A similar behavior has been reported by García-González et al. (2005). Relative high amounts of MEK had to be added and the culture tubes had to be sealed airtight in order to see inducing effects. Although MEK posseses a good solubility in water, it is highly volatile. Therefore, we cannot say something about the actual molarities in the solution. The same is valid for the other tested ketones. Too high inducer levels impaired growth in *E. coli* as well as in *P. putida*. However, the low tolerance towards MEK was similar to the tolerance towards m-toluic acid, the inducer of the well-established XylS/Pm system.

Like the Pm promoter of the *P. putida* TOL plasmid pWW0 (Bakke et al. 2009), P_{mekA} does not exhibit a -35 element, and the -10 promoter element, although exhibiting the extended -10 region, seems to be rather weak. The requirement for MekR to promote transcription initiation was previously shown by Onaca et al. (2007) and confirmed in this study. Autoregulatory properties which have been shown for some members of the AraC/XylS family, e.g. AraC and SoxS of *E. coli* and YbtA of *Yersinia pestis* (Martin et al. 1986; Hamilton and Lee 1988; Nunoshiba et al. 1993; Li and Demple 1994; Fetherston et al. 1996), were not obvious for MekR, as typical binding patterns could not be found in the *mekR* promoter region. Such sequences are most often made up of tandem repeats each comprising two recognition elements separated by a short variable linker. AraC, XylS/BenR, and SoxS bind for example to the consensus sequences $AGCN_7TCCATA$, $TGCAN_6GGNTA$, and $GCACN_7CAAA$ (Li and Demple 1996; Gallegos et al. 1997; González-Pérez et al. 1999; Silva-Rocha and de Lorenzo 2012). Indeed, a putative binding site of MekR with a consensus sequence of $CACCN_5CTTCAA$ could be identified in the P_{mekA} region. The centers of the two putative recognition elements, CACC and CTTCAA, are separated by 10 bps corresponding to a complete turn of the DNA double helix. In analogy to other members of the AraC/XylS family, MekR might bind as a dimer to the identified tandem repeat, thereby inserting the two helix-turn-helix recognition helices of each monomer into adjacent major grooves of the DNA. Such a behavior could be shown for MarA of *E. coli*, thereby bending the DNA between the two recognition elements (Rhee et al. 1998). Since DNA bending rigidity is sequence dependent (Geggier and Vologodskii 2010), the nucleotide sequence of the spacer region might play an important role for activation. Indeed, not only the mutation of the putative recognition elements impaired transcriptional activity provoked by MekR, but also the mutations of the spacer regions. A lowering of the AT-content by the mutations led to an even higher decrease of β-galactosidase activity. For XylS a sequential binding of the monomers could be observed which induced DNA bending of the spacer region between the tandem repeat prior to activation (Domínguez-Cuevas et al. 2010). In case of MekR, this spacer region ($S_{1\text{-}2}$) was also important for activation since the complementary mutation of the corresponding sequence in the P_{mekA} region strongly impaired β-galactosidase activity.

Class II promoter structures and requirements for the RNA polymerase α subunit C-terminal domain as well as the C-terminal end of the σ subunit for full activation (Browning and Busby 2004) have been shown for a variety of AraC/XylS family members (Jair et al. 1995; Jair et al. 1996a; Jair et al. 1996b; Landini et al. 1997; Landini et al. 1998; Lonetto et al. 1998; Landini and Busby 1999; Holcroft and Egan 2000; Bhende and Egan 2000; Domínguez-Cuevas et al. 2008; Taliaferro et al. 2012). The location of the identified tandem repeat in the P_{mekA} region seems to overlap with the -35 hexamer indicating a class II promoter in analogy to other AraC/XylS family members.

Primer extension experiments proposed two transcriptional start sites in the P_{mekA} region. However, sequence analysis, mutations and deletions in the P_{mekA} region challenged the existance of the second transcriptional start site (dTS). Although mRNA structures suggested by *in silico* experiments can differ from the real *in vivo* structures, our results propose that the *mekAB*-mRNA has a high potential to form a stable stem-loop structure close to its 5′ end. This feature is common to a number of stable prokaryotic mRNAs (Varani 1995; Grunberg-Manago 1999). A 5′-terminal stem-loop can prolong the half-life of the mRNA by protecting its 5′-terminus to become a substrate for pyrophosphohydrolase, which is the rate-determining step in mRNA degradation (Deana et al. 2008). Previously it was shown, that the HIV-1 reverse transcriptase stalls at certain stem-loop formations of the RNA template resulting in the build-up of intermediate-length polymerization products (Olsen et al. 1994). Taking this stable mRNA-fold and the accuracy of plus or minus one nucleotide of the primer extension experiments into consideration, it is possible that the AMV reverse transcriptase used in the primer extension experiments could also have been stalled at this stem-loop. This would lead to a shorter cDNA and thus to the second band in the sequencing gel which could be misinterpreted as a second transcription start site. We showed that this region is necessary to reach high β-galactosidase activities, but cannot act as an independent promoter region. Thus, the decreased β-galactosidase activity observed after deletion of the region between the uTS and dTS can be explained by a probably less stable mRNA. A loss of the

5'-terminal stem-loop structure could lower the half-life of the *lacZ* mRNA leading to a lower β-galactosidase activity.

A CCR exerted by glucose could be observed for the MekR/P_{mekA} expression system with cells growing in complete medium. However, a major player like Crc or PtsN ($EIIA^{Ntr}$) could not be identified. The level of β-galactosidase activity did not increase in a Δ*crc* genetic background, showing that the organic acids present in LB medium did not lead to a Crc-dependent CCR. Sequence analysis for short unpaired AA(C/U)AA(C/U)AA motifs, that represent the recognition sites for Crc in the mRNA (Moreno et al. 2009; Sonnleitner et al. 2009), revealed such sequences 89 to 99 bps upstream of the *mekR* start codon. However, these sequences seem to be located near to the putative -10 promoter element of P_{mekR} rather than in the leader region of the *mekR*-mRNA. In a proteomic approach, PtsN was observed to be a global regulator but not a key player in CCR exerted by glucose (Cases et al. 2001). Also in case of the MekR/P_{mekA} expression system the deletion of *ptsN* could not relieve the inhibition by glucose. As CCR in *Pseudomonas* is not yet as well understood as in *E. coli* or *B. subtilis*, we can only suppose other mechanisms or even several distinct global control systems acting together on the MekR/P_{mekA} expression system to explain the negative influence of glucose.

Future experiments will focus on the optimization of the MekR/P_{mekA} system in order to reach higher expression levels as they have been observed with the XylS/Pm system. Since the -10 promoter element seems to be rather weak, site-directed mutagenesis of this region might lead to higher expression levels. However, the usage of random mutagenesis of promoters to improve protein expression have been shown to have a higher potential than targeted modifications of individual functional elements (Bakke et al. 2009) and should also be taken into consideration.

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Supplemental Material

Table S1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
Strains		
E. coli JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, Δ(lac-proAB), F' [traD36 proAB⁺ lacI^q lacZΔM15]*	Yanisch-Perron et al. 1985
P. putida KT2440	wild type	ATCC 47054
P. putida KT2440/*crc*	derivative of KT2440 with *crc::GmR*	Aranda-Olmedo et al. 2005
P. putida KT2440/*ptsN*	derivative of KT2440 with *ptsN::KmR*	Aranda-Olmedo et al. 2005
Plasmids		
pJOE5332.10	P_{mekA}-*mekAB*-P_{mekR}-*mekR* (pBBR1MCS2 derivative)	Onaca et al. 2007
pJOE6533.4	pBBR1MCS2 derivative	Altenbuchner, unpublished
pJOE6038.1	*araC* P_{BAD} expression vector	Warth and Altenbuchner 2013
pSUN272.1	*lacZ* expression vector	Sun and Altenbuchner 2010
pJB658	*xylS* Pm expression vector	Blatny et al. 1997b
pNG176.6	intermediate cloning vector	this study
pNG217.1	P_{mekA}-*lacZ* Am^R	this study
pNG247.1	P_{mekR}-*mekR* P_{mekA}-*lacZ* Am^R	this study
pNG413.1	*araC* P_{BAD}-*lacZ* Am^R	this study
pNG418.3	*xylS* Pm-*lacZ* Am^R	this study
Mutated derivatives of pNG247.1 [a] with oligonucleotide primers used for construction [b]		
pNG307.5	+5/+10 TTGACC → AACTGG	s7159 / s7160 + s7161 / s7162
pNG308.7	-16/-8 TGTTACCGT → ACAATGGCA	s7159 / s7165 + s7166 / s7162
pNG370.10	Δ to position -49	s6469 / s6472
pNG371.2	Δ to position -74	s7167 / s6472
pNG373.1	Δ to position -64	s7469 / s6472
pNG394.1	-46/-42 ATTTC → GGATT	s7159 / s7835 + s7834 / s7162
pNG430.1	-71/-68 CACC → GTGG	s7159 / s8232 + s8233 / s7162
pNG431.1	-67/-63 GGATT→ CCTAA	s7159 / s8234 + s8235 / s7162
pNG432.1	-62/-67 CTTCAA → GAAGTT	s7159 / s8236 + s8237 / s7162
pNG433.6	-56/-51 ACCATT → TGGTAA	s7159 / s8238 + s8239 / s7162
pNG434.1	-50/-47 CACC → GTGG	s7159 / s8240 + s8241 / s7162
pNG435.1	-46/-42 ATTTC → TAAAG	s7159 / s8242 + s8243 / s7162
pNG436.1	-41/-36 CTTCAA → GAAGTT	s7159 / s8244 + s8245 / s7162
pNG437.1	Δ to position -69	s8231 / s6472
pNG439.1	-67/-63 GGATT → ATTTC	s7159 / s8278 + s8277 / s7162
pNG449.4	Δ to position -71	s8297 / s6472
pNG465.1	Δ +2 to +37	s7159 / s8441 + s8442 / s7162
pNG466.1	-16/-8 TGTTACCGT → ACAATGGCA Δ +2 to +37	s7159 / s7165 + s7166 / s7162 s7159 / s8443 + s8442 / s7162
pNG467.1	-36/-31 AGCGTG → TTGACC	s7159 / s8444 + s8445 / s7162

[a] range of substitutions is indicated by a slash; deletions by a 'Δ'; positions are relative to the upstream transcription start site (uTS)

[b] oligonucleotide sequence shown in Table S2; underlined primers were used for subsequent fusion PCR

Table S2 Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′ direction)[a, b]
s5703	AAAAAA**GAATTC**AAATAGCTCGCCAGCCAAT
s5704	AAAAAA**GGATCC**CTATCCGCTGGTTTTAATTC
s5760	AAAAAA**AAGCTT**TCCGGTAGTCAATAAACCG
s5678	AAAAAA**CATATG**TTTTATGCCTCATATTGTTATG
s5959	Cy5-GCTGCAAGGCGATTAAGTTGG
s6198	AAAAAA**GAATTC**CCACAAGCGGCCGTCT
s6199	AAAAAA**GAATTC**CTAGTATCCTGATGATTGAATG
s6200	AAAAAA**GAATTC**GCCAGGTCATCTCTTGAAC
s6201	AAAAAA**GAATTC**CCGCAAGAAGAGCAAGGAT
s6430	AAAAAA**TTAATTAA**GTCGTGACCGCCTACGG
s6431	AAAAAA**TTAATTAA**ACTTATGAGCTCAGCCAATC
s6432	AAAAAA**ACCGGT**CCGCTACGGCCTGTATGT
s6433	AAAAAA**AGATCTTGTACA**TTATTATTTTTGACACCAGACC
s6435	TGCCGTGGGTTTC**AATATT**G
s6468	GTAAAACGACGGCCAGTGA
s6469	AAAAAA**AGATCT**ACCATTCACCATTTCCTTCAA
s6472	ACGGCGTTAAAGTTGTTCTG
s6592	TAAGTCGTGACCGCCTACGG
s6593	AGGCAGACAAGGTATAGGGC
s6880	AAAAAAA**CCGGTGGATCCCAATTGAGATCT**TACGCGTACGAG
s6881	AAAAAA**ACCGGTCATATG**ACCATGATTACGGA
s7159	GATCGAGTCTTGTGTGCGC
s7160	TTTTCTTGCCGCCAGGCAAAccagttGGCACGGATAAACGGTAACA
s7161	TGTTACCGTTTATCCGTGCCaactggTTTGCCTGGCGGCAAGAAAA
s7162	AGCCTCCAGTACAGCGCG
s7165	AAAGGTCAAGGCACGGATAAtgccattgtATGAGGGCAAGTTTCACGCT
s7166	AGCGTGAAACTTGCCCTCATacaatggcaTTATCCGTGCCTTGACCTTT
s7167	AAAAAA**AGATCT**TTACACCGGATTCTTCAAACC
s7469	AAAAAA**AGATCT**TTCTTCAAACCATTCACCATTT
s7472	GGCTGCGAGACTGGGTTAT
s7475	GATGTGCTGCAAGGCGATTA
s7834	CACCggattCTTCAAGCGTGAAACTTGCCCTCATTG
s7835	CACGCTTGAAGaatccGGTGAATGGTTTGAAGAATCC
s8231	AAAAAA**AGATCT**CCGGATTCTTCAAACCATTCA
s8232	TATCCGTTAgtggGGATTCTTCAAACCATTCACC
s8233	AAGAATCCccacTAACGGATATCTTCAAACGCC
s8234	GTTACACCctaaCTTCAAACCATTCACCATTTCC
s8235	GTTTGAAGttaggGGTGTAACGGATATCTTCAAAC
s8236	CCGGATTgaagttACCATTCACCATTTCCTTCAAG
s8237	TGAATGGTaacttcAATCCGGTGTAACGGATATCT
s8238	ATTCTTCAAtggtaaCACCATTTCCTTCAAGCGTG
s8239	AAATGGTGttaccaTTGAAGAATCCGGTGTAACGG
s8240	CAAACCATTgtggATTTCCTTCAAGCGTGAAACTT
s8241	GAAGGAAATccacAATGGTTTGAAGAATCCGGTG
s8242	CATTCACCtaaagCTTCAAGCGTGAAACTTGCC
s8243	GCTTGAAGctttaGGTGAATGGTTTGAAGAATCC
s8244	ACCATTTCgaagttGCGTGAAACTTGCCCTCATT
s8245	TTCACGCaacttcGAAATGGTGAATGGTTTGAAGA
s8278	CGTTACACCatttcCTTCAAACCATTCACCATTTCC
s8279	TTTGAAGAATggGGTGTAACGGATATCTTCAAAC
s8297	AAAAAA**AGATCT**CACCGGATTCTTCAAACC
s8441	ATGTTTATAGTTACGGATAAACGGTAACAATGAG
s8442	CGTTTATCCGTAACTATAAACATAACAATATGAGG
s8443	ATGTTTATAGTTACGGATAATGCCATTGTATGAG
s8444	GCAAGTTTggtcaaTGAAGGAAATGGTGAATGGTTT
s8445	TCCTTCAttgaccAAACTTGCCCTCATTGTTACC

[a] Restriction sites are indicated in boldface

[b] Mutated nucleotides are in lower case

4.4 Ethylene glycol metabolism by *Pseudomonas putida*

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Abstract

In this study, we investigated the metabolism of ethylene glycol in the *Pseudomonas putida* strains KT2440 and JM37 by employing growth and bioconversion experiments, directed mutagenesis, and proteome analysis. We found that strain JM37 grew rapidly with ethylene glycol as a sole source of carbon and energy, while strain KT2440 did not grow within 2 days of incubation under the same conditions. However, bioconversion experiments revealed metabolism of ethylene glycol by both strains, with the temporal accumulation of glycolic acid and glyoxylic acid for strain KT2440. This accumulation was further increased by targeted mutagenesis. The key enzymes and specific differences between the two strains were identified by comparative proteomics. In *P. putida* JM37, tartronate semialdehyde synthase (Gcl), malate synthase (GlcB), and isocitrate lyase (AceA) were found to be induced in the

presence of ethylene glycol or glyoxylic acid. Under the same conditions, strain KT2440 showed induction of AceA only. Despite this difference, the two strains were found to use similar periplasmic dehydrogenases for the initial oxidation step of ethylene glycol, namely, the two redundant pyrroloquinoline quinone (PQQ)-dependent enzymes PedE and PedH. From these results we constructed a new pathway for the metabolism of ethylene glycol in *P. putida*. Furthermore, we conclude that *Pseudomonas putida* might serve as a useful platform from which to establish a whole-cell biocatalyst for the production of glyoxylic acid from ethylene glycol.

Introduction

Ethylene glycol is an important starting material for many applications in the chemical industry and is readily available in large quantities at low cost. Besides its application in antifreeze solutions, it is used for the chemical synthesis of glyoxal, glycolic acid and glyoxylic acid. Glyoxylic acid serves as an important building block for the synthesis of many molecules of industrial interest. It is used for the production of agrochemicals, cosmetic ingredients, polymers, pharmaceuticals as well as for flavors and fragrance products. Different routes for the chemical synthesis of glyoxylic acid have been developed (29, 34). Besides the formation of byproducts, all these chemical approaches share the major drawback of low yields. Industrial biotechnology using biocatalytic-based approaches provides an interesting alternative to overcome these difficulties. Examples for the enzymatic production of glyoxylic acid are the conversion of glycolic acid by glycolic acid oxidase from spinach, selectively yielding up to 98 % glyoxylic acid in a whole cell system. Further, a two-step chemo-enzymatic route using base catalysis and glycolic acid oxidase, for the synthesis of glyoxylic acid from glyoxal, and the synthesis of glycolic acid by using glycerol oxidase from *Aspergillus japonicus* have been described. (13, 20, 35).

An attractive alternative to these procedures is a combined one-step whole cell biotransformation using ethylene glycol as a readily available and economically priced substrate. Various organisms which can utilize ethylene glycol as a sole source

of carbon and energy under oxic as well as anoxic conditions have been described (12, 13). Metabolic sequential oxidation of ethylene glycol into glyoxylic acid via the intermediate glycolic acid has been shown to be present in a variety of different bacterial species (7, 10, 16, 22). These reactions can either be catalyzed by dehydrogenases, oxidases, or a combination of these enzymes (5, 20, 21). Among the alcohol dehydrogenases (ADHs), the pyrroloquinoline quinone (PQQ)-dependent enzymes are of specific interest, as some of these enzymes are known to be involved in the oxidation of various short chain alcohols (1, 11). One of the best studied examples of these enzymes is *ExaA* (formerly QedH) from *Pseudomonas aeruginosa,* which is a homolog of *PedE* and *PedH* from *P. putida* U (2). This periplasmic enzyme has been found to be essential for the growth utilizing ethanol as a carbon source, as it catalyzes the initial oxidation of the substrate into acetaldehyde (19).

In addition to ethanol, ExaA has been recently reported to accept a broad spectrum of substrates including primary and secondary alcohols as well as diols such as 1,3-propanediol (8). From these results, together with the fact that a broad substrate spectrum represents a common feature of alcohol dehydrogenases, one could speculate that ethylene glycol could serve as an alternative substrate for ExaA. As the absence of an appropriate cofactor regeneration system limits the use of the purified enzyme, the use of a whole cell system is essential for applications based on this pathway. In this regard, *P. aeruginosa* is problematic due to its pathogenic potential. In contrast, strains of *Pseudomonas putida* are generally considered as model organisms for biotechnological approaches due to their metabolic versatility and the low pathogenic potential. Sequence analysis showed that all *P. putida* strains sequenced so far harbor at least one homolog of the *exaA* gene. Therefore, these strains might serve as a good platform to establish a fermentative route from ethylene glycol to glyoxylic acid.

To test this hypothesis, we investigated, characterized and compared the metabolism of ethylene glycol in the two *P. putida* strains KT2440 and JM37. This was approached via growth and bioconversion experiments, as well as directed mutagenesis and proteome analysis.

Materials and Methods

Strains, plasmids, and growth conditions

A list of the strains and plasmids used in this study is provided in Table 1. For maintenance of *P. putida* strains, cells were grown on *Pseudomonas* Isolation Agar (BD, Franklin Lakes, NJ USA) at 30°C and subsequently stored at 4°C until further use. For liquid growth, LB (35) or a modified M12 medium was used. One liter of modified M12 medium contained 1 g $(NH_4)_2SO_4$, 0.1 g NaCl, 0.2 g $MgSO_4 \bullet 7\ H_2O$, 0.02 g $CaCl_2 \bullet 2\ H_2O$, 1 ml of SL4 trace element solution (31), 2 g KH_2PO_4, and was finally adjusted to a pH of 6.7. Additionally, 10 ml of a sterile vitamin solution containing 100 mg/l biotin, 200 mg/l folic acid, 20 mg/l aminobenzoic acid, 20 mg/l riboflavin, 40 mg/l calcium panthothenate, 140 mg/l nicotinic acid, 40 mg/l pyridoxine hydrochloride, 200 mg/l *myo*-inositol, and 40 mg/l thiamine hydrochloride was added. Unless indicated otherwise, precultures were grown overnight in test tubes with 5 ml medium using a rotary shaker (CH-4103, INFORS AG, Switzerland) at 30°C and 180 rpm. For growth experiments, 20 ml of M12 minimal media supplemented with 10 mM ethylene glycol, glycolic acid or glyoxylic acid as carbon and energy source, were inoculated with precultures to an initial optical density (OD_{600}) of 0.01 and growth was monitored by measuring the OD_{600}.

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
JM109	*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi, Δ(lac-proAB), F' [traD36 proAB⁺ lacI^q lacZΔM15]*	(39)
P. putida		
KT2440	wild type	ATCC 47054
ΔUPP4	Δ*upp*	(18)
GN104	Δ*upp* ΔPP_2679	This study
GN116	Δ*upp* ΔPP_2674	This study
GN127	Δ*upp* ΔPP_2674 ΔPP_2679	This study
GN133	Δ*upp* Δ*prp*	This study
GN140	Δ*upp* Δ*prp* Δ*gcl*	This study
GN187	Δ*upp* Δ*prp* Δ*gcl* Δ*glcB*	This study
GN259	Δ*upp* Δ*prpB* Δ*gcl* Δ*glcB* Δ*aceA*	This study
JM37	*Pseudomonas putida* strain, originally isolated from from a m-xylene enriched soil sample.	(27)

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Plasmids		
pJOE6261.2	pIC20HE backbone with a kanamycin resistance gene and a copy of *upp* from *P. putida* KT2440	(18)
pNG91.2	pJOE6261.2 with the up- and downstream regions of PP_2674 cloned into BamHI site	This study
pNG98.4	pJOE6261.2 with the up- and downstream regions of PP_2679 cloned into BamHI site	This study
pNG122.1	pJOE6261.2 with the up- and downstream regions of *prpB* cloned into BamHI site	This study
pNG123.6	pJOE6261.2 with the up- and downstream regions of *gcl* cloned into NdeI site	This study
pNG141.8	pJOE6261.2 with the up- and downstream regions of *glcB* cloned into BamHI site	This study
pNG214.4	pJOE6261.2 with the up- and downstream regions of *aceA* cloned into BamHI site	This study

Generation of mutants

For deletion of various chromosomal genes in *P. putida* KT2440 the previously described *upp* / 5-fluorouracil (5-FU) counterselection system was used (18). Briefly, the up- and downstream regions including the start and stop codons of the target gene were cloned into pJOE6261.2. Transformants were first selected on LB agar plates supplemented with 50 µg/ml of kanamycin (Kan). One of the Kan^r $5\text{-}FU^s$ clones, obtained, was incubated in LB at 30°C for 24 h and subsequently plated on M9 minimal plates (35) containing 20 µg/ml 5-FU and 0.2 % glucose. $5\text{-}FU^r$ and Kan^s clones were checked by colony PCR and a clone tested positive for deletion of the corresponding gene was selected for further experiments. Oligonucleotides for the different strain constructions are shown in Table S1.

Bioconversion experiments

For bioconversion experiments, 1 ml from precultures were transferred to 1 liter Erlenmeyer flasks containing 250 ml LB medium and cells were grown to stationary phase. The cultures were harvested by centrifugation at 4°C and 13700 × *g* for 20 min (SLA-3000, Sorvall) and washed with 40 ml of M12 medium without carbon source. Finally, cells were re-suspended in M12 medium containing 50 mM ethylene glycol, 25 mM glycolic acid or 25 mM glyoxylic acid and the OD_{600} was adjusted to 7.5. In order to provide a stable pH of 6.7 during the entire experiment, 0.1 M sodium phosphate buffer was used for experiments with ethylene glycol and 0.2 M for experiments with glycolic acid or glyoxylic acid. 50 ml of this cell suspension was transferred to 250 ml Erlenmeyer flasks and incubated in a rotary shaker at 30°C and 150 rpm for 120 h. Samples (1.5 ml) were taken at regular time intervals and cells were removed by centrifugation at 20000 × *g* for 1 min (Centrifuge 5417 C, Eppendorf) for subsequent HPLC analysis of the supernatant.

HPLC-Analysis

For HPLC-analysis, samples were mixed with the internal standard xylitol at a final concentration of 10 mM and filtered through a 0.2 µm filter. HPLC analysis was carried out on an Agilent System (1200 series) using the cation exchange resin column Aminex HPX-87H (300×7.8 mm, Bio-Rad, USA) at 60°C, with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 ml/min. The substrates and products were quantified using the corresponding standards and a refractive index detector (Agilent 1200 series, G1262A). The detector temperature was set to 35 °C. For the identification of unknown products, the corresponding fractions were pooled and further analyzed by GC-MS.

GC-MS-Analysis

GC/MS-analysis was performed using a Shimadzu system (GC2010, GCMS-QP2010) equipped with a FS-Supreme-5 capillary column (30 m×0.25 mm, 0.25 µm film thickness, CS-Chromatographie, Germany). Prior to GC-MS-analysis, 500 µl of the samples were mixed with 10 µl HCl (37 %), supplemented with 50 µg NaCl and extracted with ethyl acetate (2 × 500 µl). The organic phases were combined, evaporated using a stream of nitrogen and derivatized using 40 µl TMS agent (70°C for 30 min). Chromatography was performed using helium as the carrier gas at a linear velocity of 30 cm/sec. The temperature program started with an initial temperature of 60°C for 3 min, followed by a ramp to 200°C at a rate of 8°C/min. The column temperature was maintained for 1 min, raised to 300°C with a rate of 50°C/min and held for 5 min. The injector and detector temperature were 250°C and 200°C, respectively. The volume injected was 1 µl (split ration 1:50). Components were characterized in full scan mode and identified by their mass fragmentation pattern by comparison with the corresponding reference substances.

Culture conditions and sample preparation for proteomics experiments

For proteomics experiments, precultures of *P. putida* strains KT2440, GN259 and JM37 were grown in 20 ml LB medium overnight. From these precultures, 100 µl were transferred into 100 ml of M12 medium in 500 ml Erlenmeyer flasks and incubated at 30°C and 180 rpm using 10 mM glucose as sole source of carbon and energy. After 12 h of incubation, cultures were either treated with 80 mM ethylene glycol or 10.8 mM glyoxylic acid or left untreated as control experiments. After an additional incubation for 5 h, cells were harvested by centrifugation at 3200 × *g* and washed in three steps with 20 ml, 10 ml, and 1.5 ml of 5 mM magnesium acetate in 10 mM Tris-HCl at pH 8. For 2D-DIGE analysis, cell pellets were resuspended in 1 ml DIGE Lysis Buffer (30 mM Tris-HCl pH 8.5, 7 M urea, 2 M thiourea, 4 % CHAPS) and lysed by sonication on ice (5 × 30 s) (*Branson Sonifier 450, USA)*. Lysed samples were centrifuged at 14,000 × *g* for 10 min at 4°C followed by ultracentrifugation (Beckman Coulter, Germany) at 4°C and 100,000 × *g* for 1 h. Protein concentration of the supernatant was determined using the Bradford assay (6).

2D-DIGE analysis

All 2D-DIGE experiments were performed with three biological replicates. In each replicate, a treated and an untreated sample were compared. Minimal labelling was performed with Cy-dyes according to the manufacturer's instructions (GE Healthcare, Germany). In one experiment (Fig. S3) G-dyes (NH DyeAGNOSTICS GmbH, Germany) were used instead of Cy-dyes (GE-Healthcare, Germany). After minimal labelling, samples were loaded on 24 cm Immobiline DryStrips pH 4-7 or pH 3-11 by rehydration loading in a DryStrip re-swelling tray (GE Healthcare, Germany) according to the manufacturer's instructions. Isoelectric focussing was performed on an IPGphor3 (GE Healthcare, Germany) using the following protocol: Step 1: 150 V for 2 h; Step 2: 300 V for 2 h; Step 3: 300-1000 V for 8 h; Step 4: 1000-8000 V for 3 h; Step 5: 8000 V for 7 h. Afterwards strips were equilibrated according to the manufacturer's protocol. The second dimension was run on a 12 % SDS gel in an EttanDaltsix electrophoresis unit (GE Healthcare, Germany). After electrophoresis 2D-DIGE gels were scanned on a Typhoon Trio+ Imaging System (GE Healthcare, Germany) with excitation and emission wavelengths corresponding to the respective dyes.

Protein expression analysis

Gel images were analyzed using Progenisis SameSpots 4.0 software (Nonlinear Dynamcis, UK). For image alignment and spot detection, the default settings of the software were used. Cy3 and Cy5 images from each gel were normalised to the corresponding internal standard (Cy2). Three biological replicates for each condition were grouped and fold changes were calculated between the groups. Fold changes were statistically analysed by a one-way ANOVA, taking into account sample size, mean difference, and variance. Spots showing a differential expression of at least 1.8-fold (or 0.6-fold, respectively) with a p-value of <0.05 were considered significant and selected for spot picking. Spot picking was performed using an Ettan TM Spot Picker (GE Healthcare, Germany). Spot picking was validated by subsequent silver staining of gels (9). Spots not precisely picked by the picking robot were excised manually. All excised protein spots were in-gel-digested with trypsin according to Shevchenko *et al.* (35) and analysed by mass spectrometry (for further details see supplementary methods).

Results

Growth- and bioconversion experiments with *Pseudomonas putida* strains JM37 and KT2440

In order to test the ability of the *P. putida* strains JM37 and KT2440 to use ethylene glycol or its putative degradation intermediates glycolic acid and glyoxylic acid as sole source of carbon and energy, growth experiments were performed. These experiments revealed that strain JM37 is able to grow with ethylene glycol, glycolic

acid and glyoxylic acid, finally yielding optical densities of 0.6, 0.54, and 0.41, respectively. Notably, the initial lag-phase during growth with ethylene glycol was longer in comparison to the other substrates (Fig. 1). In contrast to JM37, strain KT2440 did not show growth with ethylene glycol, glycolic acid, or glyoxylic acid, even after incubation for 24 h (Fig. 1) or 48 h (data not shown).

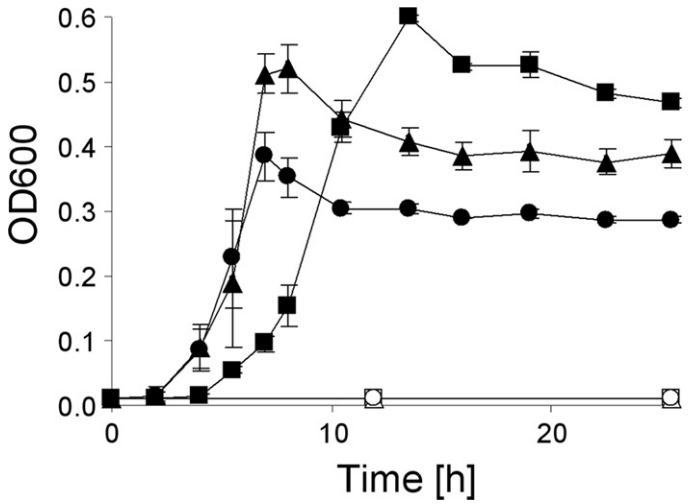

Figure 1: Growth of *P. putida* strains JM37 (filled symbols) and KT2440 (empty symbols) in M12 minimal medium with 50 mM of sodium phosphate buffer pH 6.7 and 10 mM ethylene glycol (squares), 10 mM glycolic acid (triangles) or 10 mM glyoxylic acid (circles). Cultures were incubated at 30°C with shaking at 180 rpm and growth was measured as OD_{600}. Experiments were performed in triplicates. Error bars indicate the standard deviation.

Experiments with resting cells of strain JM37 showed complete conversion of 50 mM ethylene glycol, 25 mM glycolic acid and 25 mM glyoxylic acid within 24 h of incubation and a maximal conversion rate of 3.2 mM/h for ethylene glycol (Fig. 2A, Fig. S1AB). Increasing optical densities during these experiments indicated growth permitting conditions (data not shown). For the identification of intermediates emerging from ethylene glycol metabolism, we analysed the supernatants from bioconversion experiments by HPLC. In these experiments no accumulation of putative intermediates could be observed.

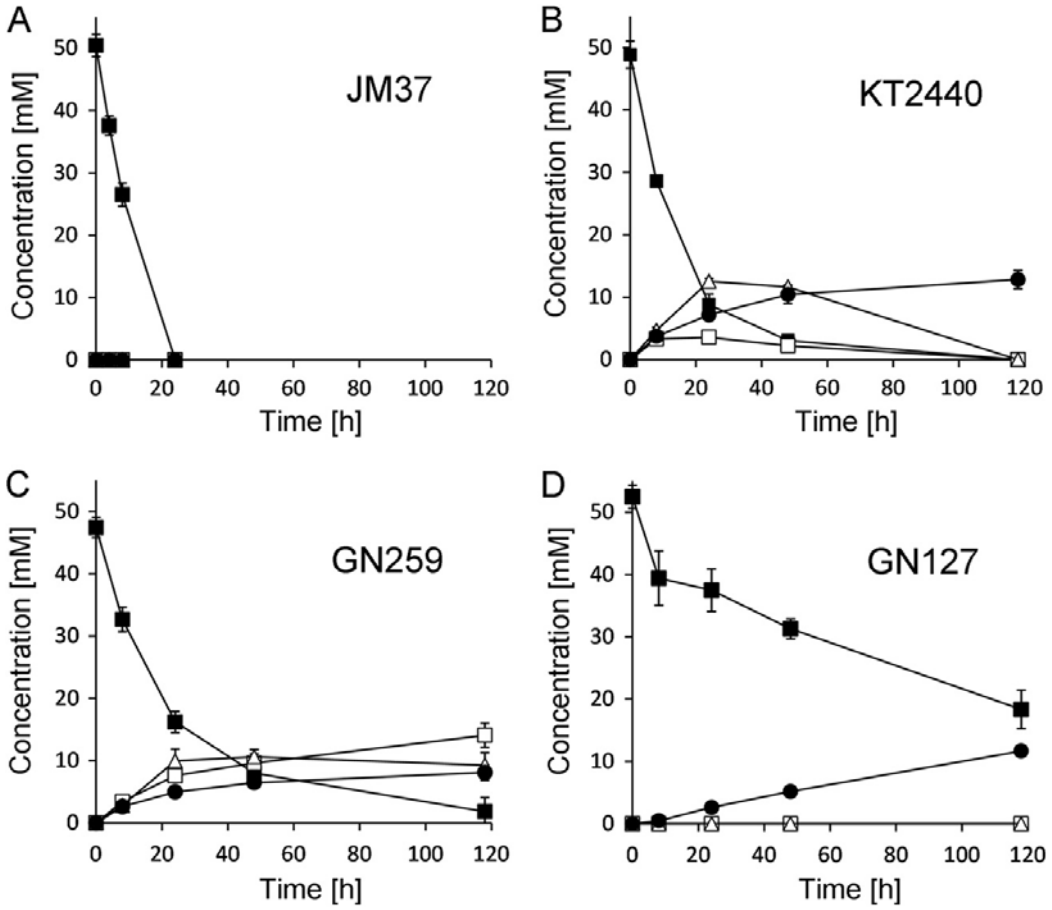

Figure 2: Bioconversion of 50 mM ethylene glycol in resting cell suspension experiments (OD_{600} = 7.5). LB grown cells were washed and adjusted to an OD_{600} of 7.5 in M12 medium (100 mM sodium phosphate buffer, pH 6.7). The consumption of ethylene glycol (■) and the formation of the metabolites glycolic acid (□), glyoxylic acid (Δ) and oxalic acid (●) was analyzed by HPLC/RI. **A** *P. putida* strain JM37. **B** *P. putida* strain KT2440. **C** Deletion mutant GN259 (Δ*upp* Δ*prpB* Δ*gcl* Δ*glcB* Δ*ace*) derived from strain KT2440. **D** Deletion mutant GN127 (Δ*upp* Δ*pedE* Δ*pedH*) derived from strain KT2440. Error bars represent the standard deviation of three independent experiments.

Even though strain KT2440 did not grow with any of the substrates, resting cell suspension experiments revealed complete conversion of ethylene glycol, glycolic acid and glyoxylic acid (Fig. 2B, Fig. S1CD). However, compared to experiments with strain JM37, strain KT2440 showed reduced conversion rates for all substrates and no increase in optical density during the time course of the experiment. As a consequence of this, the time for complete substrate conversion was significantly increased (>48 h) with 8.8 mM ethylene glycol still residing in supernatants of KT2440 after 24 h (Fig. 2B). At this time point, no substrate was left in supernatants of strain JM37 (Fig. 2A). Another difference compared to experiments with strain JM37

was the detection of glycolic acid, glyoxylic acid, and oxalate in supernatants of strain KT2440 during incubation with ethylene glycol. The maximal temporal accumulation of 3.6 mM glycolic acid and 12.6 mM glyoxylic acid was observed at 24 h after starting the experiments by the addition of 50 mM ethylene glycol (Fig. 2B). In contrast, the concentration of oxalate was found to continuously increase over time, finally reaching 12.8 mM after 118 h of incubation.

When 25 mM glycolic acid was used, temporal accumulation of 2.5 mM glyoxylic acid was found after 24 of incubation. Furthermore, 8.5 mM oxalic acid was detected as the final product after 118 h of incubation (Fig. S1C). When 25 mM glyoxylic acid was used, oxalic acid was found as the only product with a final concentration of 11.3 mM after 118 h of incubation (Fig. S1D). Interestingly, we found in all bioconversion experiments a carbon imbalance when substrate degradation and product formation was compared. Moreover, we found that the imbalance increased over the time course of the experiment, finally reaching 72 %, 63 %, and 51 % of the initial carbon concentration with ethylene glycol, glycolic acid, and glyoxylic acid, respectively (Figure 2B, Fig. S1CD).

Bioconversion experiments with mutants of *P. putida* strain KT2440 defective in canonical degradation pathways for glyoxylic acid

The carbon imbalance together with the fact that no growth could be detected with any of the substrates indicated the activity of additional degradation pathway other than those leading to the formation of oxalic acid. For constant accumulation of the central metabolite, glyoxylic acid, the disruption of all potential degradation pathways is necessary. To achieve this, we generated the mutant strain GN259 which lacks the genes for glyoxylate carboligase (*gcl*), malate synthase (*glcB*), isocitrate lyase (*aceA*) and 2-methylisocitrate lyase (*prpB*). The additional deletion of *prpB* was chosen due to recent enzymatic evidence, that isocitrate lyase from *Mycobacterium tuberculosis* (ICL1) is able to function as a 2-methylisocitrate lyase and thus, these two enzymes might functionally complement each other (17). Strain GN259 was then used in bioconversion experiments with ethylene glycol, glycolic acid, and glyoxylic acid as substrates. These experiments revealed a significant reduction in the ethylene

glycol conversion rate compared to experiments with strain KT2440 (Fig. 2BC). Consequently, 16.2 mM of ethylene glycol could be identified in supernatants of strain GN259 after 24 h of incubation, representing twice the amount compared to supernatants of strain KT2440. While strain KT2440 showed complete conversion of ethylene glycol, 1.8 mM ethylene glycol was still present in supernatants from experiments with strain GN259 after 118 h. Most importantly, strain GN259 showed constant accumulation of glycolic acid over the time course of the experiment. Whereas the concentration of glycolic acid increased constantly over time, reaching a final concentration of 14.1 mM after 118 h, the concentration of glyoxylic acid increased only over the first 24 h of the experiment and then remained at a constant level of about 10 mM. Oxalic acid constantly accumulated during the entire experiment to a final concentration of 8.1 mM after 118 h. Finally, strain GN259 also showed an improved carbon balance during bioconversion experiments with ethylene glycol (83 %) when compared to strain KT2440.

Proteome analysis for identification of pathways involved in ethylene glycol metabolism

In order to gain insight into the pathways involved in the metabolism of ethylene glycol and glyoxylic acid, quantitative proteomics experiments were carried out. We compared the proteomes of the untreated *P. putida* strains KT2440, JM37, and GN259 with their ethylene glycol and glyoxylic acid treated counterparts. Figures S2 to S9 show 2D-DIGE images of the different strains and conditions. In the presence of ethylene glycol 50 up- or down-regulated proteins were identified for strain KT2440 and 21 proteins for JM37, respectively. All quantitative proteomics results are given in the tables of supplementary figures S2 to S9. For a subset of eight spots, quantitative data are shown in table 2.

In ethylene glycol treated cells of strain JM37 a significant induction of enzymes involved in glyoxylic acid conversion, namely isocitrate lyase AceA (1.9-fold), glyoxylate carboligase Gcl (9.6-fold) and malate synthase GlcB (3.7-fold) was observed. When cells were treated with glyoxylic acid, significant induction of Gcl (16-fold) and GlcB (17-fold) was detected. Conversely, in strain KT2440 isocitrate

lyase AceA (2.8-fold), but not Gcl or GlcB, was found to be significantly up-regulated for both substrates. Besides enzymes involved in the degradation of gyloxylic acid, the PQQ-dependent ADHs PP_2674 and PP_2679 were up-regulated 34-fold and 21-fold respectively in strain KT2440 in the presence of ethylene glycol (Table 2).

Table 2: Selected proteins regulated in response to ethylene glycol or glyoxylic acid treatment.

		Fold change in expression of the following strain after the indicated treatment[a]					
#	**Protein**	**KT2440**		**GN259[b]**		**JM37**	
		EG	**GXA**	**EG**	**GXA**	**EG**	**GXA**
1	Isocitrate lyase (AceA)	2.8	2.8	k.o.	k.o.	1.9	-
2	Glyoxylate carboligase (Gcl)	-	-	k.o.	k.o.	9.6	16
3	Malate synthase G (GlcB)	-	-	k.o.	k.o.	3.7	17
4	Quinoprotein alcoholdehydrogenase (PedH)	21	n.d.	n.d.	n.d.	n.d.	n.d.
5	Quinoprotein alcoholdehydrogenase (PedE)	34	-	8.2	-	31	0.5
6	Aldehyde dehydrogenase family protein (PedI)	5.2	-	-	-	7.9	-
7	Aldehyde dehydrogenase family protein (PP_0545)	-	-	5.5	-	2	-
8	Transcriptional regulator (PedR1)	4.8	-	-	-	4.8	-
9	PAS/PAC sensor hybrid histidine kinase (PedS1)	7.7	-	-	-	-	-

[a] as determined by 2D-DIGE. EG, ethylene glycol; GXA, glyoxylic acid. Each experiment comprised three biological replicates. Proteins were considered upregulated when they showed an increase of >1.8-fold or down-regulated with a decrease to <0.6-fold. Regulation was assumed to be significant with a p-value ≤ 0.05. Data for EG treatment of KT2440 were taken from Figs. S2 and S3 in the supplemental material except for PedH, for which data were obtained from Fig. S4 in the supplemental material. Data for all other strains and conditions were taken from Fig. S5 to S9 in the supplemental material. If a protein was detected in more than one spot of a gel, the spot with the highest fold change is shown in this table. Fold changes of all differentially regulated protein spots and proteins identified therein are given in Fig. S2 to S9 in the supplemental material and the corresponding tables.

b a KT2440 mutant (Δ*upp* Δ*prpB* Δ*gcl* Δ*glcB* Δ*ace*)

Protein spot number

k.o. Indicates genes deleted in the mutant GN259

n.d. Not determined

- Spots not regulated in this experiment

Under the same condition, the homologous protein to PP_2674 was also found to be up-regulated 31-fold in strain JM37. Sequence analysis revealed that PP_2674 and PP_2679 share 84 % and 52 % amino acid sequence identity with the ethanol dehydrogenase ExaA from *Pseudomonas aeruginosa* as well as 98 % and 99 % sequence identity to the previously described enzymes PedE and PedH from *Pseudomonas putida* U (Table 3) (2).

Table 3: Amino acid sequence comparison of the two quinoproteins PedE and PedH, the aldehyde dehydrogenase PedI as well as the response regulator PedR1 of *P. putida* KT2440 with the homologues of *P. putida* U and *P. aeruginosa* PAO1. Sequence similarities compared to *P. putida* KT2440 are given as percent amino acid identity and the names of the corresponding enzymes are given in brackets.

***P. putida* KT2440**	***P. putida* U**	***P. aeruginosa* PAO1**
PedR1 (PP_2665)	91 % (PedR1)	86 % (ErbR)
PedE (PP_2674)	98 % (PedE)	84 % (ExaA)
PedH (PP_2679)	99 % (PedH)	52 % (ExaA)
PedI (PP_2680)	97 % (PedI)	88 % (ExaC)

Comparison of the genetic context in the surrounding of these genes in *P. putida* KT2440 further revealed a high structural similarity to the phenylethanol degradation cluster (Ped) from *P. putida* U (Fig. 3).

Due to this homology, we will further use the Ped nomenclature of *P. putida* U for the corresponding genes PP_2264 to PP_2680 in *P. putida* KT2440 (Fig. 3).

Interestingly, PedH has a rather basic isoelectric point (pI) of 8.75 compared to PedE with an pI of 6.52. Thus, PedH eludes detection on the 2D-DIGE gels used routinely in our experiments that cover the pH-range of pH 4 to 7. It was detected only in a single ethylene glycol experiment carried out with strain KT2440 in which the protein extracts were separated with a pH-gradient of pH 3–11 (Fig. S4). In addition to the PQQ-dependant ADHs PedE and PedH, also two aldehyde dehydrogenases (ALDH) PedI and PP_0545 were found to be up-regulated in response to ethylene glycol. While only one of these ALDHs was found to be up-regulated in strains KT2440 (PedI) and GN259 (PP_0545), both enzyme were induced in JM37 (Table 2). In contrast, PedE as well as the two ALDHs PedI and PP_0545 were not found to be induced after treatment with glyoxylic acid in any tested strain (Table 2, Fig. S5, S7, and S9).

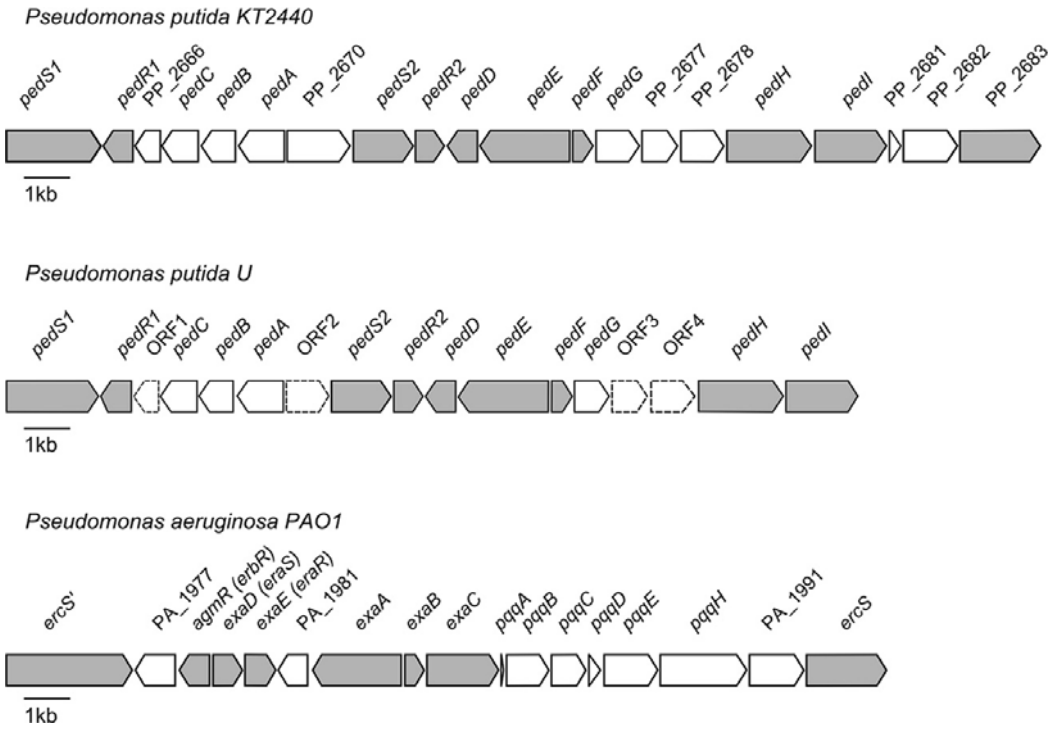

Figure 3: Comparison of the gene cluster controlled by the ErbR regulator in *P. aeruginosa* PAO1 with the homologous clusters of *P. putida* U and KT2440. Genes labeled in grey show significant similarities between the three strains. *P. putida* homologues of the *P. putida U* proteins PedR1 (ErbR), PedS1, PedE and PedI were found to be regulated under ethylene glycol treatment in *P. putida* KT2440. ORFs indicated by dotted lines do not reflect the actual size of this region.

In *P. aeruginosa* the expression of the homologous ethanol degradation pathway is controlled by a complex regulatory system including the transcriptional regulator ErbR (30). In our experiments, we found that the expression of PedE, PedH and PedI was co-regulated with the ErbR homologue PedR1 (4.8-fold) in JM37 and KT2440 after ethylene glycol treatment (Table 2). The PAS/PAC sensor kinase PedS1 could only be detected in KT2440. No regulation of PedR1 could be detected in the mutant GN259. Surprisingly, this mutant still showed up-regulation of PedE, even though to a much lower degree compared to the wild-type strain (Table 2).

Bioconversion experiments with mutants of *P. putida* strain KT2440 harboring deletions for the quinoprotein alcohol dehydrogenases PedE and PedH

As described above, our proteome analysis showed an induction of the two quinoprotein ADHs PedE and PedH in cells treated with ethylene glycol. In order to test their importance in the metabolism of ethylene glycol, we constructed mutant strains, in which *pedE* (strain GN116) and *pedH* (strain GN104) or both genes (strain GN127) were deleted. These deletion mutants were then subjected to bioconversion experiments in resting cell suspensions. In these experiments we found that single deletions had no effect and showed similar conversion rates as strain KT2440 (data not shown). In contrast, the double deletion mutant GN127 was significantly impaired in the conversion of ethylene glycol, as 35 % of the initial substrate concentration could be found in supernatants even after incubations for 118 h (Fig. 2D). While oxalate accumulated in supernatants of the double deletion mutant over time to a final concentration of 11.7 mM, no traces of glycolic acid and glyoxylic acid could be detected. When glycolic acid or glyoxylic acid was used as a substrate, no differences between strain GN127 and strain KT2440 could be observed (Fig. S1CDEF).

Discussion

The ability to grow with ethylene glycol as a sole source of carbon and energy has been demonstrated for various kinds of microorganisms, some of them initially oxidizing it to glyoxylic acid via glycolic acid (10, 12). In this work we investigated the potential of the *Pseudomonas putida* strains KT2440 and JM37 for microbial oxidation of ethylene glycol into the more valuable product glyoxylic acid. In contrast to strain KT2440, strain JM37 showed rapid growth with ethylene glycol, glycolic acid and glyoxylic acid as sole source of carbon and energy. The observation of a longer initial lag-phase of strain JM37 during growth on ethylene glycol suggests the induction of additional enzymes like dehydrogenases and oxidases, which are reported to be important for the first steps of ethylene glycol metabolism via glyoxylic acid (5, 7). Despite the differences in growth, resting cell suspension experiments revealed complete conversion of ethylene glycol, glycolic acid and glyoxylic acid for *P. putida* KT2440 and JM37, albeit with substantial differences in

the time needed for complete conversion. While strain JM37 accumulated no potential metabolic intermediates during conversion of ethylene glycol - glycolic acid and glyoxylic acid were found in supernatants of strain KT2440. This clearly indicates their role as intermediates in the metabolism of ethylene glycol in strain KT2440. Previous studies demonstrated glycolaldehyde to be a product of ethylene glycol oxidation (5, 21). Surprisingly, this first expected intermediate in a three step oxidation of ethylene glycol to glyoxylic acid was not found to accumulate in our experiments. A possible explanation for this observation could be that the formation of glycolic acid from ethylene glycol is actually catalyzed in two consecutive steps by the same enzyme. Alternatively, free glycolaldehyde might not be detected due to the formation of Schiff base adducts with amino groups of nearby proteins (28). In both cases the expected glycolaldehyde concentrations would be very low and thus could be below the detection limit of our system.

Even though further experiments are needed to prove this hypothesis, we propose that strain KT2440 actually metabolizes ethylene glycol via glycolaldehyde to glycolic- and glyoxylic acid (Fig. **4).** Subsequently, some of the glyoxylic acid is oxidized to oxalic acid, whereas about three quarters of the substrate could not be found in any of the identified products.

From our differential proteomics approach, increased expression levels of tartronate semialdehyde synthase (Gcl), malate synthase (GlcB) and isocitrate lyase (AceA) were found in strain JM37 after treatment with ethylene glycol or glyoxylic acid. In these experiments, isocitrate lyase showed the smallest fold change after treatment with ethylene glycol and was not significantly up-regulated after treatment with glyoxylic acid. This suggests that the "glycerate pathway" and the "malate pathway" play a key role for glyoxylic acid conversion in JM37. It is reported, that the pathway initiated by Gcl enables growth on glyoxylic acid, the pathways using GlcB and AceA can only lead to the production of energy (Fig. 4) (3, 23, 25). In a recent study, Li and coworkers tried to prevent the metabolism of glyoxylic acid in strain JM37 by disrupting all the aforementioned degradation pathways. However, all single mutations in *aceA*, *glcB* and *glc* as well as all corresponding double mutations had no effect on glyoxylic acid degradation (27). The authors suggested that an additional

degradation pathway might exist, since a number of such alternative pathways have been described for different microorganisms (4, 24, 37). Despite the possibility of such an alternative in JM37, our proteome analysis showed no induction of enzymes which could be related to one of these pathways. As we cannot rule out that possible alternative metabolic pathways could be constitutively expressed, this issue needs some further investigations which are currently under way in our laboratory.

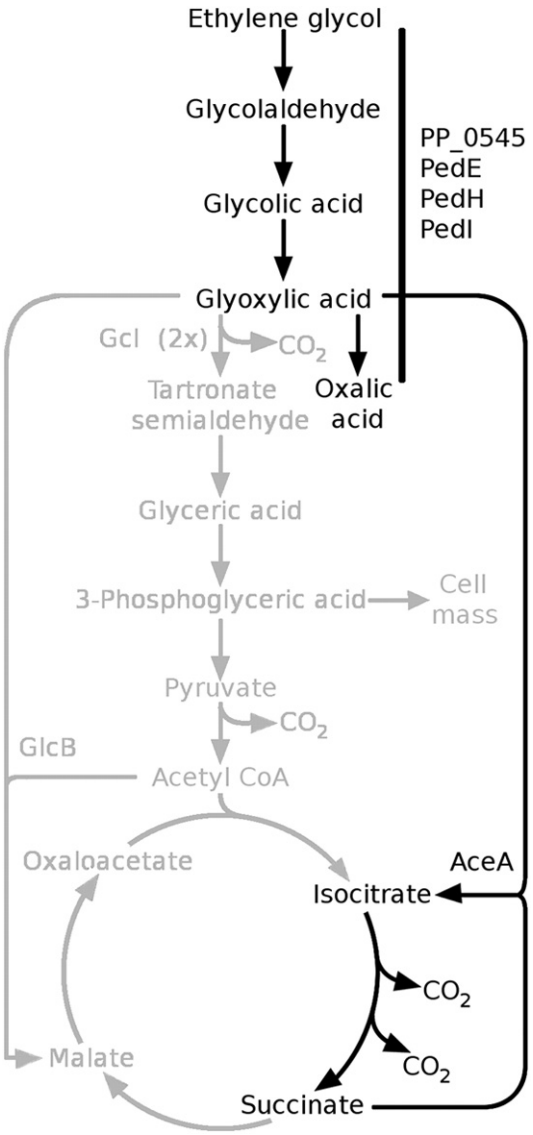

Figure 4: Postulated pathway for the metabolism of ethylene glycol in *Pseudomonas putida* strains KT2440 and JM37. The enzymes and/or metabolites identified in response to ethylene glycol in KT2440 are depicted in black. Additional pathways identified in strain JM37 are shown in grey. Detailed descriptions of the pathways are given in the text.

Interestingly, in strain KT2440 AceA, but not Gcl (PP_4297) or GlcB (PP_0356), was up-regulated after treatment with ethylene glycol or glyoxylic acid. On the one hand, the absence of Gcl induction might explain the lack of growth with ethylene glycol, glycolic acid or glyoxylic acid found for strain KT2440. On the other hand, the induction of AceA suggests that this enzyme is active under the conditions used. This enzyme catalyzes the first step of the glyoxylate shunt for the generation of energy but does not support cell mass generation. Together with the absence of detectable growth, the activity of AceA, could therefore be the explanation for the observed carbon imbalance observed in all bioconversion experiments. This is further supported by the observation that conversions with the deletion mutant GN256 resulted in an improved carbon balance compared to strain KT2440. In addition, this strain accumulated the intermediates glycolic acid and glyoxylic acid over the entire time course of the experiment. This underlines the importance of at least one of these pathways for ethylene glycol metabolism via the central intermediate glyoxylic acid in strain KT2440. We currently cannot rule out that more than one pathway might be or become active during incubation. However, our data clearly supports the hypothesis that the degradation of ethylene glycol leads to the formation of oxalic acid and the production of energy from glyoxylic acid via the glyoxylate shunt.

In addition to proteins involved in the degradation of glyoxylic acid, our proteome analysis of ethylene glycol induced cells of KT2440 and JM37 revealed the up-regulation of the two PQQ-dependent ADHs PedE and PedH as well as the NADH-dependent ALDHs PedI and PP_0545. As already indicated by the name, the quinoprotein alcohol dehydrogenases PedE and PedH harbor the cofactor pyroloquinoline quinine (PQQ) as a prosthetic group. These periplasmic enzymes were shown to play a crucial role in the degradation of primary alcohols (32, 36) and were described to be induced by their corresponding substrates. The best studied example of a PQQ-dependent ADH is ExaA, an ethanol dehydrogenase from *P. aeruginosa* (33). In this organism ExaA was found to catalyze the initial oxidation of ethanol to acetaldehyde, thus enabling growth on ethanol (36). In *P. aeruginosa*, the transcriptional regulator ErbR (formerly AgmR) controls the expression of ExaB (cytochrome c), ExaC (aldehyde dehydrogenase) and the operon for PQQ biosyn-

thesis (*pqqABCDEH*) directly, whereas ExaA expression is regulated indirectly via the sensor kinase/response regulator pair EraS/R (formerly ExaD/E) (15). Upstream of ErbR, the two histidine kinases ErcS and ErcS′ as well as the response regulator ErdR were identified as additional regulatory elements (30).

Our results indicate that the oxidation of ethylene glycol in KT2440 could be controlled by such an ErbR-type regulon. This hypothesis is supported by several lines of evidence: *i*) induction of ErbR and ErcS′ homologues (PedR1, PedS1); *ii*) induction of several enzyme homologues known to be regulated by ErbR in *P. aeruginosa* (e.g. PedE, PedI); iii) similarities in the corresponding genetic context of *P. putida* KT2440, *P. aeruginosa* and *P. putida* U (Fig. 3). In addition, we found a strongly reduced PedE expression and no induction of PedI in the *aceA*-deleted *P. putida* strain GN259 after treatment with ethylene glycol and no induction when compared to strain KT2440. This observation is in agreement with a recent study, demonstrating that the deletion of *aceA* in *P. aeruginosa* ATCC 17933 results in a down-regulation of all genes necessary for ethanol oxidation, including ExaA (26). The deletion of the ErbR homologue PedR1 and subsequent characterization of the PedE and PedH induction pattern in the presence of ethylene glycol could finally prove this hypothesis and the corresponding experiments are currently in progress. Despite similarities of the ErbR-like regulon between *P. aeruginosa* and the putative homologue in different *P. putida* strains, some major differences exist. One such difference is the presence of two quinoproteins in *P. putida* strains (e.g. PedE and PedH) compared to only one in *P. aeruginosa*. Arias and coworkers showed that PedE and PedH from strain *P. putida* U are involved in the conversion of phenylethanol and alcohols with a chain length of C6-C9 (2). However, only a double mutant strain defective in both PQQ-dependent enzymes was unable to grow with the aforementioned substrates. Interestingly, growth with alcohols with chain length shorter than C6 was not altered in this double mutant, indicating specificity and redundancy of this system. In analogy to this, our bioconversion experiments revealed that either PedE or PedH from *P. putida* strain KT2440 can catalyze the conversion of ethylene glycol in this organism. This can be concluded from the observation that only the double mutant strain GN127 was significantly affected in

the conversion of ethylene glycol, whereas the single mutants exhibited a similar rate as the parental strain. The fact that this mutant was not altered in the conversion rate of glycolic acid and glyoxylic acid highlights the importance of PedE and PedH for the first steps of ethylene glycol metabolism. The residual ethylene glycol conversion rate observed in strain GN127 is most likely due to the activity of unspecific dehydrogenases, partly compensating for the activity of the deleted PQQ-dependent ADHs. The absence of glycolic acid and glyoxylic acid accumulation in this mutant can be explained by the rapid oxidation of emerging low amounts of intermediates by subsequent enzymes of the oxidation chain, most likely the two ALDH PedI and PP_0545.

In summary, we were able to reconstruct novel pathways for ethylene glycol metabolism in the *Pseudomonas putida* strains KT2440 and JM37 (Figure 4). In KT2440 this pathway is initiated by the PQQ-dependant ADHs PedE and PedH and the ALDHs PedI and PP_0545 via the intermediates glycolaldehyde and glycolic acid to glyoxylic acid. Subsequently, glyoxylic acid is either further oxidized to the "dead end" product oxalic acid or metabolized via AceA leading to the generation of energy. In strain JM37, the activity of two additional pathways, namely Gcl and GlcB, leads to rapid metabolism of ethylene glycol without accumulation of the corresponding intermediates or oxalic acid. The elimination of key enzymes for glyoxylic acid metabolism in strain KT2440 resulted in improved carbon balance, most likely due to the deletion of *aceA* and accumulation of glyoxylic acid over the entire experimental time course. From these results, we propose that *P. putida* KT2440 is a promising candidate for further metabolic engineering approaches in order to establish a biocatalytic route for the production of glyoxylic acid from ethylene glycol.

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Supplemental Material

Online-Resource for Supplemental files 1 and 2:

http://aem.asm.org/content/suppl/2012/11/14/AEM.02062-12.DCSupplemental.html

Supplemental file 1:

Oligonucleotide primers (Table S1), overview of strain constructions (Table S2), bioconversion of 25 mM glycolic acid or glyoxylic acid in resting cell experiments (Fig. S1), images of performed 2D-DIGE experiments and corresponding tables (Fig. S2 to S9).

Supplemental file 2:

Protein identification by mass spectrometry.

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