Anaerobic degradation of phenol and aniline by the sulfate-reducing bacterium *Desulfatiglans anilini*

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SUMMARY

The anaerobic degradation pathways of aniline and phenol by sulfate-reducing bacteria are not yet fully understood. In this thesis, the sulfate-reducing bacterium *Desulfatiglans anilini*, which can grow with aniline or phenol as carbon and electron source, was investigated. In particular, this study is focused on the metabolic reactions and enzymes involved in anaerobic degradation of aniline or phenol by *D. anilini* and improvement of the growth of *D. anilini* with aniline.

The study on phenol degradation in *D. anilini* describes the genes coding for the enzymes responsible for the anaerobic conversion of phenol to benzoyl-CoA which were identified by analyzing the genome of *D. anilini*, comparing the transcription of putative genes in phenol-grown or benzoate-grown cells by RT-PCR and comparing the translation of putative genes in phenol-grown or benzoate-grown cells by total proteome analysis. Further evidence suggests that phenylphosphate is an intermediate of phenol degradation in *D. anilini* as shown in *in-vitro* enzyme assays of phenylphosphate synthase. The results obtained revealed that phenol is most likely phosphorylated to phenylphosphate, followed by carboxylation to 4-hydroxybenzoate, which is analogous to the well-known phenol degradation pathway in the nitrate-reducing bacterium *Thauera aromatica*.

To investigate the pathway of anaerobic aniline degradation by *D. anilini*, improving its growth is essential for further studies. A potential carboxylation activity was measured with carbon dioxide (CO_2) as the co-substrate for activating aniline in the presence of ATP with cell-free extracts of *D. anilini*. Hydrogen sulfide, which is a product of sulfate reduction in sulfate-reducing bacterial cultures, was found to inhibit the enzymatic activity of the potential carboxylase during aniline activation and the phenylphosphate synthase during phenol activation. Hydrogen sulfide was also found to inhibit not only enzyme activities, but also growth of *D. anilini* when grown with aniline or phenol. A sulfide-consuming phototrophic bacterium *Thiocapsa roseopersicina* was co-cultured with *D. anilini* in a co-cultivation device to continuously remove hydrogen sulfide from the *D. anilini* culture. The doubling time of *D. anilini* was 15 days in the co-cultivation device, as compared to 26 days in the absence of a sulfide-oxidizing partner.

The proteins specifically induced with aniline or 4-aminobenzoate were identified by comparing the total proteomes of *D. anilini* after growth with aniline, 4-aminobenzoate, benzoate or phenol. The genes coding for aniline-induced proteins are located in one single gene cluster (locus tag from H567DRAFT_03866 to H567DRAFT_03876) in the genome of *D. anilini*, including the genes coding for pyruvate water dikinase (H567DRAFT_03868), phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) and phenylphosphate carboxylase beta subunit (H567DRAFT_03872). These three enzymes and phenylphosphate carboxylase beta subunit (H567DRAFT_02059) were successfully cloned and overexpressed in *E. coli*. The enzyme assays with recombinant proteins demonstrated that phenylphosphoamidate is carboxylated to 4-aminobenzoate by enzymes encoded by the genes H567DRAFT_03872 and H567DRAFT_02059. Aniline is activated to 4-aminobenzoate by a combined reaction of the enzymes H567DRAFT_03868, H567D RAFT_03871, H567DRAFT_03872 and H567DRAFT_02059. We conclude that aniline is initially phosphorylated to phenylphosphoamidate by pyruvate water dikinase (H567DRAFT_03868) and phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567D RAFT_03871), and phenylphosphoamidate is subsequently carboxylated to 4-aminobenzoate by phenylphosphate carboxylase beta subunit (H567DRAFT_03872 and H567DRAFT_02059).

ZUSAMMENFASSUNG

Die Stoffwechselwege des anaeroben Abbaus von Anilin und Phenol wurden bislang noch nicht vollständig aufgeklärt. In der vorliegenden Arbeit wurde das Sulfat-reduzierende Bakterium *Desulfatiglans anilini* untersucht, das mit Anilin oder Phenol als einziger Kohlenstoff- und Energiequelle wachsen kann. Hierbei liegt der Schwerpunkt der Arbeit auf der Aufklärung der Identität der beteiligten Enzyme des anaeroben Abbaus von Anilin oder Phenol, sowie der Verbesserung des Wachstums von *D. anilini* in Laborkulturen.

Bei der Untersuchung des Abbaus von Phenol durch *D. anilini* wurden die Gene, die für Enzyme der anaeroben Umwandlung von Phenol zu Benzoyl-CoA verantwortlich sind im Genom von *D. anilini* identifiziert und deren Trankription und Translation jeweils mittels RT-PCR und Gesamtproteomanalyse untersucht. Des Weiteren wurden in Enzymtests für Phenylphosphat Synthase Hinweise darauf gefunden, dass Phenylphosphat ein Zwischenprodukt des Abbaus von Phenol darstellt. Insgesamt deuten die Ergebnisse darauf hin, dass Phenol zu Phenylphosphat aktiviert wird. Analog zum bereits bekannten Weg des Phenol Abbaus im Nitrat-reduzierenden Bakterium *Thauera aromatica* wird Phenylphosphat anschließend zu 4-Hydroxybenzoat carboxyliert.

Um den anaeroben Abbau von Anilin in *D. anilini* näher zu untersuchen, war es erforderlich das Wachstum des Stammes unter Laborbedingungen zu verbessern. Eine potentielle Carboxylase Aktivität mit CO_2 als Kosubstrat für die Aktivierung von Anilin in Gegenwart von ATP konnte in zellfreien Extrakten von *D. anilini* gemessen werden. Schwefelwasserstoff als Endprodukt der Sulfatreduktion in Sulfat-reduzierenden Bakterien hemmte in Enzymtests die Enzymaktivität sowohl der Carboxylase bei der Aktivierung von Anilin als auch die Aktivität der Phenylphosphat Synthase bei der Aktivierung von Phenol. Darüber hinaus hemmte Schwefelwasserstoff auch das Wachstum von *D. anilini* während des Wachstums mit Anilin oder Phenol. Das Sulfid-oxidierende phototrophe Bakterium *Thiocapsa roseopersicina* wurde mit *D. anilini* in einer speziellen Vorrichtung kokultiviert, um Schwefelwasserstoff kontinuierlich aus der *D. anilini* Kultur zu entfernen. Die Verdopplungszeit von *D. anilini* konnte dadurch von 26 Tagen in der Reinkultur auf 15 Tage in der Kokultur mit dem Sulfid-oxidierenden Partnerbakterium reduziert werden.

Die spezifisch induzierten Proteine mit Anilin oder 4-Aminobenzoat wurden durch Vergleich der Gesamtproteome von *D. anilini* nach Wachstum mit Anilin, 4-Aminobenzoat, Benzoat oder Phenol identifiziert. Die Gene, die für Anilin-induzierte Proteine kodieren befinden sich in einem einzigen Gencluster (locus tag von H567DRAFT_03866 bis H567DRAFT_03876) in der Genomsequenz von *D. anilini*. Dieses Gencluster beinhaltet die Gene für pyruvate water dikinase (H567DRAFT_03868), phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) und phenylphosphate carboxylase beta subunit (H567DRAFT_03872). Diese drei Enzyme und zusätzlich die phenylphosphate carboxylase beta subunit (H567DRAFT_02059) wurden erfolgreich kloniert und in *Escherichia coli* überexprimiert. Enzymtests mit den rekombinanten Proteinen zeigten, dass Phenylphosphoamidat durch die von den Genen H567DRAFT_03872 und H567DRAFT_02059 kodierten Enzyme zu 4-Aminobenzoat carboxyliert wird. Anilin hingegen wird im Enzymtest nur in einer kombinierten Reaktion mit allen vier rekombinanten Enzymen (H567DRAFT_03868, H567D RAFT_03871, H567DRAFT_03872 und H567DRAFT_02059) zu 4-Aminobenzoat aktiviert. Aus diesen Ergebnissen wird geschlossen, dass Anilin zuerst durch Pyruvat,Wasser-Dikinase (H567DRAFT_03868) und Phosphoenolpyruvat-Synthase/Pyruvat,Phosphate Dikinase (H567D RAFT_03871) zu Phenylphosphoamidat phosphoryliert wird, und Phenylphosphoamidat anschließend durch Phenylphosphat-Carboxylase beta Untereinheit (H567DRAFT_03872 und H567DRAFT_02059) zu 4-Aminobenzoat carboxyliert wird.

CHAPTER 1

General introduction

1.1. Microbial degradation of aromatic compounds

1.1.1. General characteristics of aromatic compounds

The most common aromatic compounds are derivatives of benzene, which contain a ring of resonance bonds. Aromatic compounds are chemically stable, and for their degradation, the resonance energy that stabilizes the ring structure needs to be overcome to break the ring. There are three major types of aromatic compounds: substituted aromatics, heterocyclic and polycyclic aromatic hydrocarbons. Aromatic compounds are mainly found in the living organisms in the aromatic side chains of the amino acids histidine, phenylalanine, tryptophan and tyrosine. Besides living organisms, aromatic compounds also play an important role in industry, most of them are derived from the refining of oil or by distillation of coal tar. Aromatic compounds can be used to produce various chemicals and polymers, some of which are environmental pollutants such as aniline, phenol, toluene, xylene and many others. To remove aromatic pollutants from contaminated sites, bioremediation is a powerful technology, which can use a wide range of microorganisms to convert hazardous aromatic compounds into harmless compounds.

1.1.2. Aerobic degradation of aromatic compounds

Some aerobic bacteria can oxidize aromatic compounds with O_2 by oxygenases [1, 2]. Monoxygenases incorporate one hydroxyl group into aromatic substrates and dioxygenases incorporate two oxygen atoms of one dioxygen molecule into aromatic substrates. The strategies that microorganisms use for the breakdown of aromatic compounds vary depending on their chemical structure. However, most aromatic compounds are transformed into a few key central intermediates by peripheral pathways. These central intermediates are then activated for oxidative ring cleavage. Some common central intermediates are catechol, protocatechuate, 2,5-dihydroxybenzoate and 2,5-dihydroxyphenyl acetate [3, 4]. Table 1 lists some aromatic compounds that are degraded via above mentioned central intermediates in aerobic microorganisms.

Table 1. The list of aromatic compounds that can be degraded via common central intermediates by aerobic microorganisms.

Aromatic substrates	Central intermediate	References
Phenol, p-Cresol, p-Toluene	Catechol	[5]
Benzene		[6]
Caffeate 4-Hydroxybenzoate	Protocatechuate	
3-Hydroxybenzoate	2,5-dihydroxybenzoate	[7]
Naphthalene		[8, 9]
Salicylate		[10, 11]
l-Phenylalanine, l-Tyrosine, 3-Hydroxyphenylacetate	2,5-dihydroxyphenyl acetate	[12]

The key central intermediates are converted into intermediary metabolites such as pyruvate, acetyl-CoA and succinate. For example, the ring of catechol or protocatechuate can be cleaved in the *ortho* position (between the hydroxyl groups) or in the *meta* position (adjacent to one of the hydroxyl groups) by dioxygenase. The *ortho*-cleavage pathway of catechol and protocatechuate is also called beta-ketoadipate pathway, as beta-ketoadipate is a key intermediate in this pathway [13, 14]. As shown in Fig. 1, catechol and protocatechuate are ring-cleaved by incorporating both atoms of O_2 into *ortho* position, yielding the products *cis, cis*-muconate and 3-hydroxy-*cis, cis*-muconate [3], followed by conversion into beta-ketoadipate and then into tricarboxylic acid cycle intermediates.

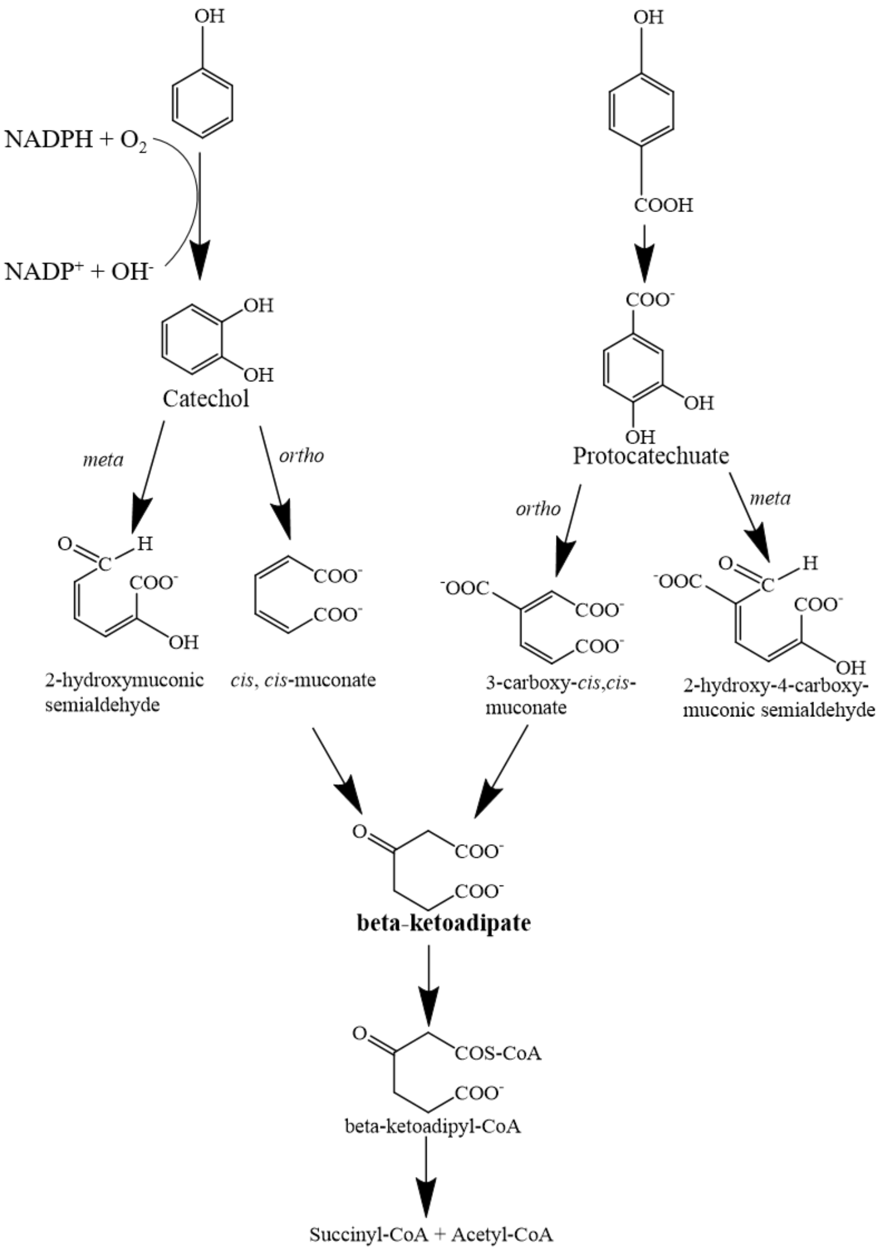

Figure 1. *Beta*-ketoadipate pathway of catechol and protocatechuate, adapted from Harwood C.S. et al [14] with certain modifications.

1.1.3. Anaerobic degradation of aromatic compounds

Some bacteria can also degrade aromatic compounds in the absence of O_2 in soils, sediments or ground waters [15]. Utilization of aromatic compounds as growth substrates was studied in several microorganisms, for instance, the phototrophic bacterium *Rhodopseudomonas palustris* [16], the nitrate-reducing bacterium *Thauera aromatica* [17-20], several sulfate-reducing bacteria [21-24] or iron-reducing bacteria [25]. The absence of O_2 forces anaerobic bacteria to use a completely different strategy to cleave the aromatic ring compared to aerobic bacteria. Similar to aerobic bacteria, the degradation pathways of aromatic compounds can be divided into two parts, peripheral pathways and central pathways. A variety of aromatic compounds can be converted into a few central intermediates, for instance, benzoyl-CoA, resorcinol, or phloroglucinol [26, 27]. These central intermediates are then ring-cleaved in the central pathways. The hydroxyl substituents at the ring of resorcinol and phloroglucinol weaken the mesomeric system of these aromatic compounds, which turns them easily reducible by ferredoxin or NAD(P)H [27-30]. Resorcinol was found to be degraded by the classical reductive dearomatization in sulfate-reducing [31] or fermenting bacteria [32] and the oxidative HHQ pathway in nitrate-reducing bacteria [33]. Phloroglucinol is reduced by an NADPH-dependent phloroglucinol reductase in fermenting bacteria [28].

Benzoyl-CoA is the most common central intermediate in the anaerobic metabolism of aromatic compounds, including p-cresol, phenol, benzyl alcohol, toluene, ethylbenzene and many others [34]. The strategies that are used by anaerobic bacteria to transform aromatic compounds via the peripheral pathways to benzoyl-CoA may differ based on the ring substituents or redox potentials of the electron acceptors supplied [35]. Novel reactions comprise, among others, carboxylation of phenolic compounds [36, 37], fumarate addition [38, 39], or oxidation of methyl substituents [40, 41]. Nitrate-reducing bacteria use often strategies different from those used by sulfate-reducing, iron-reducing or fermenting bacteria in general, since the average standard redox potential of nitrate/N_2 (+ 750 mV) is close to that of O_2/H_2O (+ 810 mV) and thus much higher than the one of SO_4^{2-}/HS^- (- 218 mV) or $Fe(OH)_3/Fe^{2+}$ (+ 100 mV, pH 7.0) [35].

In the central benzoyl-CoA pathway, benzoyl-CoA is first reduced to cyclohex-1,5-diene-1-carboxyl-CoA by benzoyl-CoA reductases (BCRs) [27, 34, 42, 43]. In facultative anaerobes, like *Thauera aromatica*, the BCRs are class I BCRs, which have an αβγδ-composition and three $[4Fe\text{-}4S]^{+1/+2}$ clusters [44] to hydrolyze two molecules of ATP to ADP and Pi per molecule substrate reduced [45]. In the strictly anaerobic bacteria *Rhodopseudomonas palustris* and *Geobacter metallireducens*, an ATP-independent class II BCR containing W and FeS clusters was identified [46]. Both class I and class II BCR can convert benzoyl-CoA to cyclohex-1, 5-diene-1-carboxyl-CoA, which is metabolized through the pathway shown in Figure 2A. Except for this extensively studied benzoyl-CoA pathway, another benzoyl-CoA pathway was described in *Rhodopseudomonas palustris* (Figure 2B). Here, cyclohex-1, 5-diene-1-carboxyl-CoA is reduced by 2 electrons to cyclohex-1-ene-1-carboxyl-CoA. In the subsequent degradation steps, similarly to the benzoyl-CoA pathway in *Thauera aromatica*, hydratase and dehydrogenase are involved to yield 2-ketocyclohexane-1-carboxyl-CoA, which is then hydrolytically cleaved into pimeloyl-CoA [47, 48].

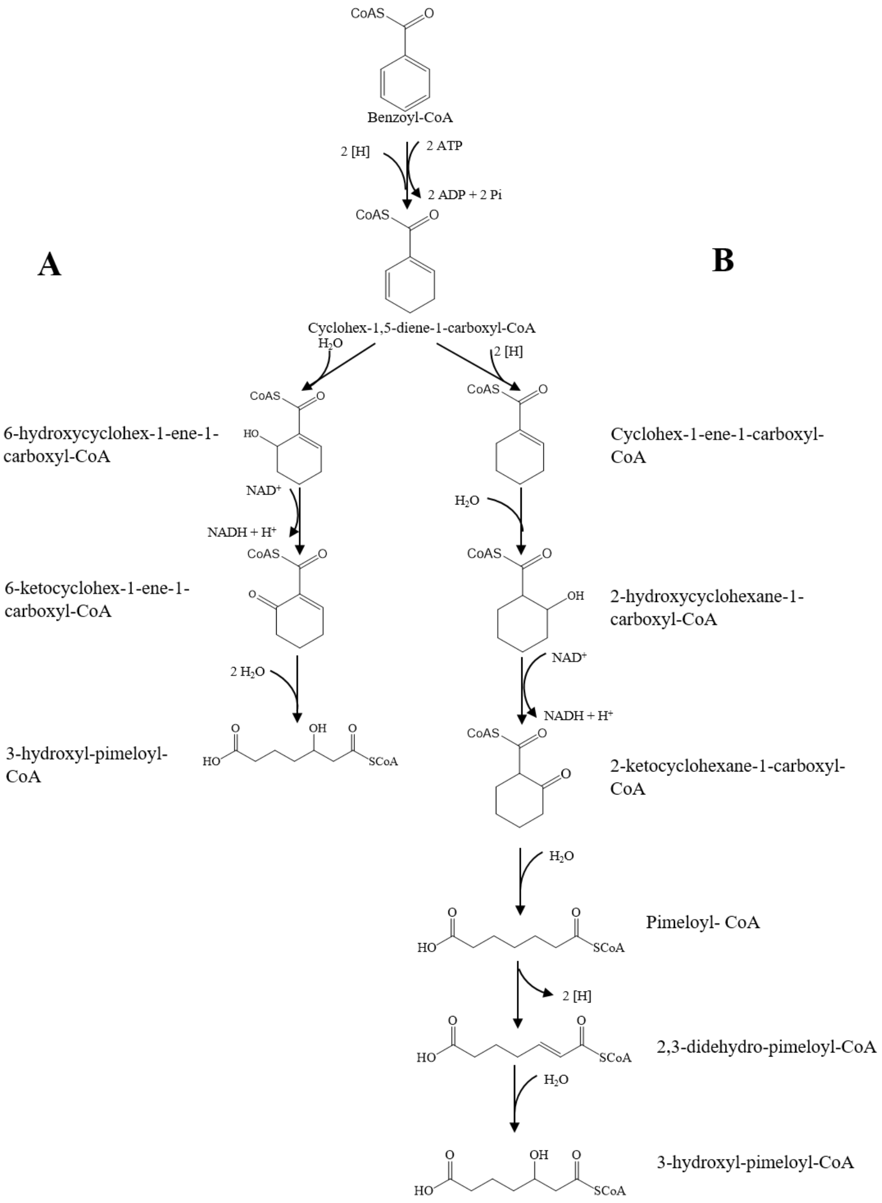

Figure 2. Central benzoyl-CoA degradation pathway in *Thauera aromatica*, *Rhodopseudomonas palustris* and *Geobacter metallireducens* (A); in *Rhodopseudomonas palustris* (B), adapted from Pelletier D. A. et al [47] with certain modifications.

1.2. Microbial degradation of aniline and phenol

1.2.1. Importance of aniline and phenol

Aniline is an aromatic amine compound, which can be used in the production of a variety of important chemicals such as dyes, additives of rubbers, photographic chemicals, pharmaceuticals, pesticides, and explosives. Phenol is also an aromatic organic compound with a hydroxyl group bound to the phenyl group. Phenol is an important industrial commodity as a precursor of many materials and useful compounds. Two-thirds of phenol are used to synthesize plastics. It is also very important for the production of polycarbonates, epoxies, Bakelite, nylon, detergents, herbicides such as phenoxy herbicides, and numerous pharmaceutical drugs.

As industrially important compounds, the extensive applications of aniline and phenol pose a serious risk to water reservoirs and soils. Because of the toxicity of aniline and phenol to human and marine life, many treatment methods have been studied to remove aniline and phenol from wastewater or soils. Compared to traditional physico-chemical methods, the application of biodegradation processes with specific microorganisms to utilize aniline and phenol in waste water appears to be an attractive treatment strategy for removing environmental contaminations as it can completely mineralize organic pollutants and can be applied in *in situ* processes [49].

1.2.2. Aerobic degradation of aniline and phenol

Both aniline and phenol were reported to be initially degraded to the central intermediate catechol, followed by further degradation via *meta-cleavage* or *ortho-cleavage* pathway depending on the type of bacterium. Some strains that utilize aniline or phenol as well as the respective degradation pathways are summarized in table 2. Three enzymes are involved in the conversion of aniline to catechol, a glutamine synthetase-like enzyme, an aniline dioxygenase and a glutamine amidotransferase [50]. The glutamine synthetase-like enzyme catalyzes the ligation of L-glutamate and aniline to form γ-glutamylanilide, in an ATP dependent reaction [51]. Then γ-glutamylanilide is converted into catechol by aniline dioxygenase. The function of the third enzyme glutamine amidotransferase is to convert γ-glutamylanilide to aniline to prevent its accumulation, as high concentrations of γ-glutamylanilide are cytotoxic [51]. The enzyme that can hydroxylate phenol to catechol is phenol hydroxylase, which is an NADPH-dependent flavoprotein incorporating one oxygen atom of molecular oxygen into the aromatic ring of phenol to form catechol [52, 53].

Table 2. Strains that utilize aniline or phenol as growth substrates aerobically and the initial degradation steps involved.

Aromatic compound	Strain	Initial degradation step	Reference
Aniline	*Pseudomonas putida* UCC22	Aniline is degraded to catechol and further degraded via the *meta-cleavage* pathway	[54]
	Acinetobacter sp. YAA		[55]
	Pseudomonas sp. AW-2		[56]
	Delftia tsuruhatensis AD9		[57]
	Frateuria sp. ANA-18	Aniline is degraded to catechol and further degraded via the *ortho-cleavage* pathway	[58]
	Delftia sp. XYJ6		[59]
Phenol	*Pseudomonas fluorescens* PU1	Phenol is degraded to catechol and further degraded via the *meta-cleavage* pathway	[60]
	Sulfolobus solfataricus 98/2		[61]
	Acinetobacter calcoaceticus NCIB 8250	Phenol is degraded to catechol and further degraded via the *ortho-cleavage* pathway	[62]
	Pseudomonas stutzeri strain SPCZ		[63]

1.2.3. Anaerobic degradation of phenol

Anaerobic phenol degradation has been studied in detail with the denitrifying bacterium *Thauera aromatica* [19]. Phenol is initially activated to phenylphosphate [64], which is then carboxylated to 4-hydroxybenzoate and further degraded [30, 37, 65]. The enzymes involved in these two steps were also investigated. The first enzyme is phenylphosphate synthase (encoded by *pps* genes), which can convert phenol and ATP to phenylphosphate, AMP, and phosphate [66]. The second enzyme is phenylphosphate carboxylase (encoded by ppc genes), which can carboxylate phenylphosphate to 4-hydroxybenzoate. 4-hydroxybenzoate is then catalyzed by 4-hydroxybenzoate CoA ligase to 4-hydroxybenzoyl CoA [67], which is converted to the central intermediate benzoyl-CoA by 4-hydroxybenozyl-CoA reductase [68].

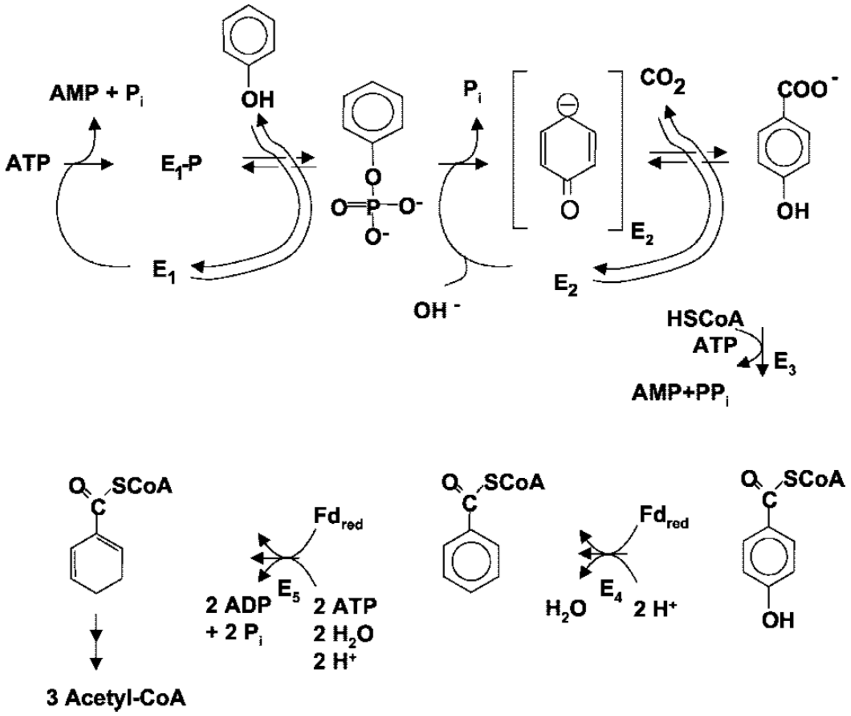

Figure 3. Anaerobic degradation of phenol in *T. aromatic*, adapted from Schuhle K. [36]. E1: phenylphosphate synthase; E2: phenylphosphate carboxylase; E3: 4-hydroxybenzoate CoA ligase; E4: 4-hydroxybenzoyl-CoA reductase; E5: benzoyl-CoA reductase; $\mathrm{Fd_{red}}$: reduced ferredoxin.

The pathways of anaerobic phenol degradation by iron-reducing, fermenting and sulfate-reducing bacteria were also studied [69] [70, 71] [72]. The same phenol degradation pathway was proposed for the iron-reducing bacterium *Geobacter metallireducens* GS-15 [69]. In *Geobacter metallireducens* GS-15, the phenol-induced gene cluster (*pps-ppc*) was identified. In the sulfate-reducing bacterium *Desulfatiglans anilini*, the phenol degradation pathway appears to be the same as in *Thauera aromatica* [72]. However, there are also some differences for the phenol induced gene cluster in *Geobacter metallireducens* GS-15 compared to the corresponding gene cluster in *T. aromatica*: it is not induced specifically in phenol-grown cells and it only contains a *ppc β* homologue. Phenol degradation was not studied at the biochemical and proteome level before in sulfate-reducing bacteria. In the fermenting bacterium *Sedimentibacter hydroxybenzoicus*, phenol is most likely carboxylated by an ATP-dependent 4-hydroxybenzoate decarboxylase [70, 71].

1.2.4. Anaerobic degradation of aniline

An aerobic aniline-degrading bacterium, strain HY99, was isolated and found to degrade aniline under both aerobic and anaerobic nitrate-reducing conditions [73]. Under strictly anoxic conditions, *Desulfatiglans anilini* is the only bacterium known to degrade aniline under anoxic sulfate-reducing conditions [31, 74]. In the latter organism, aniline was proposed to be carboxylated to 4-aminobenzoate, followed by activation of 4-aminobenzoate to 4-aminobenzoyl-CoA which is reductively deaminated to benzoyl-CoA. Activities of 4-aminobenzoyl-CoA synthase and benzoyl-CoA synthase could be measured in cell-free extracts of *D. anilini* [74]. However, it is still unknown how aniline is converted to 4-aminobenzoate and the enzymes involved in this step.

1.3. Isolation and characterization of *Desulfatiglans anilini*

Desulfatiglans anilini was originally isolated from marine enrichment cultures inoculated with sediment from the North Sea coast [31]. It is a rod-shaped, gram-negative, non-sporing, non-motile sulfate-reducing strain which can use aniline as sole electron donor and carbon source. Except for aniline, it also utilizes several other aromatic compounds like phenol, p-cresol and several hydroxyl- and amino-benzoates as growth substrates. It requires vitamin B12 and thiamine as growth factors. The growth of *D. anilini* with most of the aromatic compounds are quite slow with doubling time exceeding 72 h. Phenol proved to be the best growth substrate.

The genome of *D. anilini* was sequenced by the U.S. Department of Energy Joint Genome Institute and is publicly available in the Integrated Microbial Genomes system (IMG) database. The total size of the genome is 4,667,344 bp. The sequences were annotated by IMG. In summary, 4105 genes were predicted, of which 4040 were protein coding genes, 3133 protein coding genes with function prediction. 1033 protein coding genes were predicted to encode enzymes. 65 RNA genes were predicted.

1.4 Aims of this thesis

As mentioned in the previous introduction part, the mechanisms of aniline or phenol activation by sulfate-reducing bacteria are still unknown. Since sulfate-reducing bacteria have a tighter energy budget than nitrate-reducing bacteria [35], it would be interesting to know whether sulfate-reducing bacteria use the same strategy to activate aniline or phenol which consumes even more energy. *Desulfatiglans anilini* was chosen as the model strain to study, however, growth of *D. anilini* with aniline was rather slow. For studying the biochemistry of aniline degradation in this strain it is therefore vital to have an efficient cultivation system at hand for producing enough cell mass for biochemical experiments. Furthermore, improving growth of *D. anilini* with aniline could help to find economical solutions for bioremediation of environmental sites contaminated with aniline. The aims of this research project are therefore to improve growth of *D. anilini* with aniline as growth substrate, to investigate pathways of anaerobic aniline or phenol degradation by the sulfate-reducing bacterium *D. anilini* and to identify the intermediates, genes and key enzymes involved in aniline or phenol degradation pathways.

CHAPTER 2

Enzymes involved in the anaerobic degradation of phenol by the sulfate-reducing bacterium *Desulfatiglans anilini*

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2.1. Abstract

Background: The sulfate-reducing bacterium *Desulfatiglans anilini* can grow with phenol as sole source of carbon and energy under strictly anaerobic, sulfate-reducing conditions. In the nitrate-reducing bacterium *Thauera aromatic*a, the enzymes involved in phenol degradation have been well elucidated, whereas the anaerobic phenol degradation pathway by *D. anilini* was not studied in detail yet.

Results: The pathway of anaerobic phenol degradation by the sulfate-reducing bacterium *Desulfatiglans anilini* was studied by identification of genes coding for phenylphosphate synthase (encoded by *pps* genes) and phenylphosphate carboxylase (encoded by *ppc* genes) in the genome of *D. anilini*, by analysis of the transcription and translation of *pps-ppc* genes, and by measurement of phenylphosphate synthase activity in cell-free extracts of phenol-grown cells. The majority of genes involved in phenol degradation were found to be organized in one gene cluster. The gene cluster contained genes *ppsα* (phenylphosphate synthase alpha subunit), *ppsβ* (phenylphosphate synthase beta subunit), *ppcβ* (phenylphosphate carboxylase beta subunit), as well as 4-hydroxybenzoyl-CoA ligase and 4-hydroxylbenzoyl-CoA reductase-encoding genes. The genes *ppsγ* (phenylphosphate synthase gamma subunit), *ppcα* (phenylphosphate carboxylase alpha subunit) and *ppcδ* (phenylphosphate carboxylase delta subunit) were located elsewhere in the genome of *D. anilini*, and no obvious homologue of *ppcγ* (phenylphosphate carboxylase gamma subunit) was found in the genome. Induction of genes *pps* and *ppc* during growth on phenol was confirmed by reverse transcription polymerase chain reaction. Total proteome analysis revealed that the abundance of enzymes encoded by the gene cluster under study was much higher in phenol-grown cells than that in benzoate-grown cells. In *in-vitro* enzyme assays with cell-free extracts of phenol-grown cells, phenylphosphate was formed from phenol in the presence of ATP, Mg^{2+}, Mn^{2+}, K^+ as co-factors.

Conclusions: The genes coding for enzymes involved in the anaerobic phenol degradation pathway were identified in the sulfate-reducing bacterium *D. anilini*. The results indicate that the first steps of anaerobic phenol degradation in *D. anilini* are phosphorylation of phenol to phenylphosphate by phenylphosphate synthase and carboxylation of phenylphosphate by phenylphosphate carboxylase.

2.2. Key words

Phenol degradation, Sulfate-reducing bacterium, *Desulfatiglans anilini***,** Phenylphosphate synthase, Phenylphosphate carboxylase

2.3. Background

Phenol is an important industrial commodity used as a precursor for the production of plastics, polycarbonates, epoxies, detergents and pharmaceutical drugs. Its wide use and toxicity has caused serious contaminations of waters and soils. Biological phenol degradation is an economic and effective method to deal with these contaminants without causing secondary pollution problems [75].

Anaerobic phenol degradation has been studied in detail with the denitrifying bacterium *Thauera aromatica.* Initially, phenol is phosphorylated to phenylphosphate [64], which is subsequently carboxylated to 4-hydroxybenzoate [37, 65]. The enzyme involved in the first reaction is phenylphosphate synthase (encoded by *pps* genes), which converts phenol and ATP to phenylphosphate, AMP, and phosphate [66]. Phenylphosphate synthase contains three subunits (α, β, γ), and their encoding genes are located adjacent to each other in one operon (Fig. 1). The α-subunit (70 kDa) containing a conserved histidine residue alone can catalyze the exchange of free [^{14}C] phenol and the phenol moiety of phenylphosphate, but not the phosphorylation of phenol. The β-subunit (40 kDa) is required in the phosphorylation of phenol, which can transfer a diphosphoryl group to the conserved histidine residue in the α-subunit [76]. The reaction is stimulated by the addition of γ-subunit (24 kDa), but the exact function of the γ-subunit is unknown [66].

The enzyme involved in the subsequent carboxylation of phenylphosphate with CO_2 to 4-hydroxybenzoate is phenylphosphate carboxylase (encoded by *ppc* genes), which consists of four subunits (α, β, γ, δ) [36, 37, 65]. The exchange of $^{14}CO_2$ and the carboxyl group of 4-hydroxybenzoate was catalyzed by three of the subunits (α, β, γ; 54, 53, and 10 KDa). Phenylphosphate carboxylation was restored when the δ subunit (18 KDa) was added [36]. The δ subunit is assigned to the hydratase/phosphatase protein family and can catalyze alone a very slow hydrolysis of phenylphosphate. The genes coding for these four subunits are located adjacent to each other in one operon (Fig.1). K^+ and divalent metal cations (Mg^{2+} or Mn^{2+}) are required for phenylphosphate carboxylase activity, and oxygen is an inhibitor for phenylphosphate carboxylase activity. 4-hydroxybenzoate is then catalyzed by 4-hydroxybenzoate CoA ligase to 4-

hydroxybenzoyl CoA [67], which is converted to the central intermediate benzoyl-CoA by 4-hydroxybenozyl-CoA reductase [68].

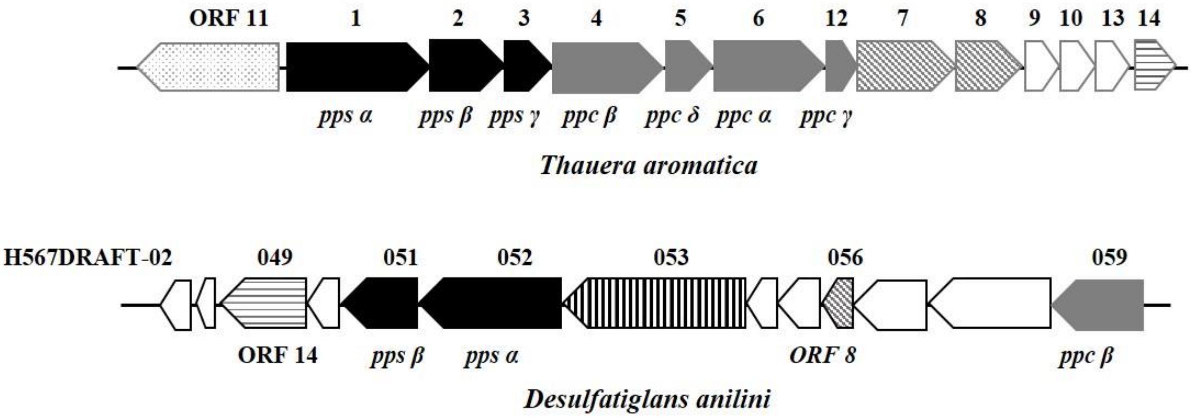

Figure 1. Organization of genes encoding phenylphosphate synthase (ppsαβγ) and phenylphosphate carboxylase (ppcαβγδ) in *T. aromatica* and the proposed anaerobic phenol degradation gene cluster in *D. anilini*. The directions of transcription are indicated by arrows. Similar ORFs are presented by the same shades and patterns.

The same phenol degradation pathway was proposed earlier for the iron-reducing bacterium *Geobacter metallireducens* GS-15 [69]. The initial steps of phenol degradation in *G. metallireducens* are accomplished by phenylphosphate synthase and phenylphosphate carboxylase as known from *Thauera aromatica*. The phenol induced gene cluster (*pps-ppc*) was identified in the genome of *G. metallireducens*, which revealed some differences compared to the corresponding gene cluster in *T. aromatica*: it is not induced specifically in phenol-grown cells and it only contains a *ppc β* homologue. In the fermenting bacterium *Sedimentibacter hydroxybenzoicus*, phenol is most likely carboxylated by an ATP-dependent 4-hydroxybenzoate decarboxylase [70, 71]. In a newly isolated strain of the sulfate-reducing bacterium *Desulfatiglans anilini*, the phenol degradation pathway appears to be the same as in *Thauera aromatica* [72]. Yet, phenol degradation was not studied at the biochemical and proteome level before in sulfate reducing bacteria. In the present study, we identified the catabolic enzymes and their genes involved in anaerobic degradation of phenol in the sulfate reducing bacterium *Desulfatiglans anilini*.

2.4. Results

2.4.1. Anaerobic growth with phenol or benzoate

The growth of *Desulfatiglans anilini* on phenol or benzoate was investigated. 2 mM phenol or 2 mM benzoate was supplied to *D. anilini* cultures as the only source of electrons with Na_2SO_4 as the electron acceptor (Fig. 2). The doubling time of *D. anilini* on benzoate is 4.4 days, which is slightly shorter than that on phenol (6.6 days). Culture samples were taken at different time points, and metabolites were analyzed by HPLC. No intermediate organic degradation products were detected in the growth medium. 2 mM phenol or benzoate was consumed in around 20 days along with approximately 8 mM Na_2SO_4 being reduced. Cells of late logarithmic phase cultures that had been pre-grown for 10 generations on the respective substrates were harvested and used for the following experiments.

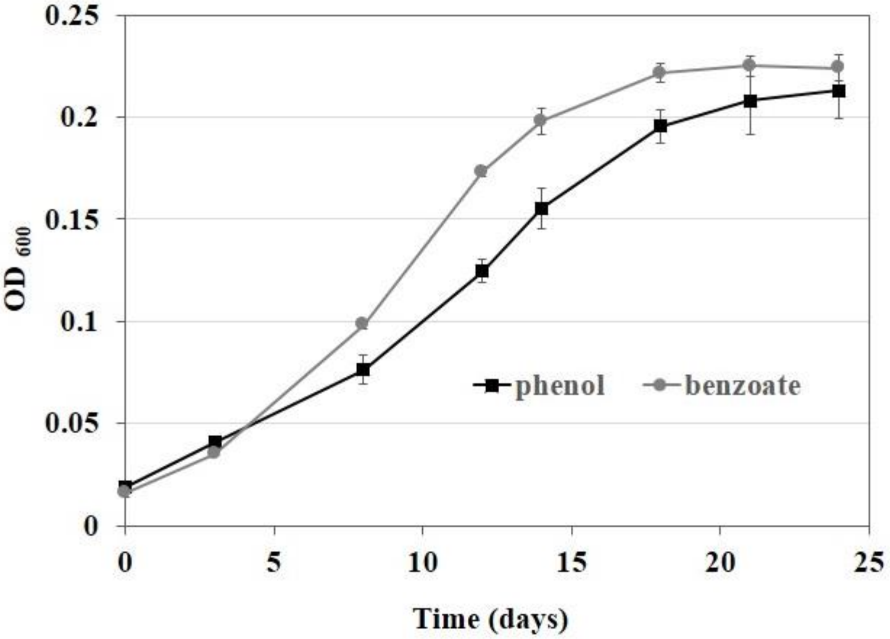

Figure 2. Anaerobic growth of *D. anilini* with 2 mM phenol or 2 mM benzoate plus sulfate (10 mM) as electron acceptor.

2.4.2. Similarity search of genes involved in phenol metabolism

The enzymes reported to be involved in phenol degradation by *T. aromatica* (ppsα, ppsβ, ppsγ, ppcα, ppcβ, ppcγ, ppcδ, ORF7-11 and ORF13-14) [77, 78] or *G. metallireducens* (BamQ, BamA, BamR and BamB) were used for a similarity search in the genome of *D. anilini* by IMG/M blast or NCBI blast. As shown in Table 2, the proteins with locus tags H567DRAFT_02052, H567DRAFT_02051, H567DRAFT_02059, H567DRAFT_02049 and H567DRAFT_02056 displayed the highest identities of 38%-56% to *ppsα, ppsβ, ppcβ, ORF14 and ORF8* of *T. aromatica* individually. The genes coding for the above-mentioned proteins are located in one gene cluster, indicating that this gene cluster could be involved in phenol degradation. However, the proteins that showed highest similarities to the genes *ppsγ*, *ppcδ* and *ppcα* in *T. aromatica* were the acetoin utilization protein AcuB (locus tag H567DRAFT_03126), KDO 8-P phosphatase (locus tag H567DRAFT_00862) and phenylphosphate carboxylase beta subunit (locus tag H567DRAFT_03563) individually, whose encoding genes are not located in the same gene cluster. Genome analysis did not reveal obvious homologues of *ppcγ*. For the ORFs 7, 9, 10, 11, 13, the genes showing high similarities were not localized in this gene cluster.

For the downstream metabolism of phenol, the degradation pathway of benzoyl-CoA consists of benzoyl-CoA reductase (BamB-I) [79, 80], a cyclohexadienoyl-CoA hydratase (BamR) [81], a hydroxyenoyl-CoA dehydrogenase (BamQ) [79] , and an oxoenoyl-CoA hydrolase (BamA) [82]. The genes coding for proteins which display the highest similarities to *BamQ*, *BamA* and *BamR* of *G. metallireducens* are located adjacent to each other in the genome of *D. anilini*.

Table 2. Identities of putative genes involved in phenol or benzoate-degradation

Enzymes in *T. aromatica* or *G. metallireducens* [a]	Annotation from IMG in *D. anilini*	Gene locus (H567DRAFT_) [b]	Mol. weight (kDa)	Sequence coverage (%) [c]	Score [d]	Identities (%) [e]
ppsα	pyruvate, water dikinase	02052	70.027	49.1	323.31	42
ppsβ	pyruvate, water dikinase	02051	39.928	21.2	93.396	38
ppsγ	acetoin utilization protein AcuB	03126	25.893	35.4	202.27	25
ppcβ	phenylphosphate carboxylase beta subunit	02059	52.24	68.2	323.31	49
ppcδ	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase (KDO 8-P phosphatase)	00862	-	-	-	46
ppcα	phenylphosphate carboxylase beta subunit	03563	51.922	12.9	76.233	33
ORF 14	phenylacetate--CoA ligase	02049	50.501	9	68.567	39
ORF 8	4-hydroxy-3-polyprenylbenzoate decarboxylase	02056	-	-	-	56
BamQ*	6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase	01120	39.75	42.1	282.42	49
BamA*	6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase	01121	42.886	72.7	323.31	51
BamR*	cyclohexa-1,5-diene-1-carbonyl-CoA hydratase	01122	28.001	55.7	323.31	37
BamB*	tungsten-dependent benzoyl-CoA reductase subunit bamB	00366	75.838	46.1	323.31	80

a.Abbreviations of the enzymes involved in phenol or benzoate (*) anaerobic degradation pathways in *T. aromatica* or *G. metallireducens* (*). b. IMG gene locus tag from the genome of *D. anilini*. c. Sequence coverage represents the extent of peptides obtained during MS-MS identification of respective protein in the total proteome analyses. d. The protein score from an MS/MS search is derived from the ions scores. Score and sequence coverage of the peptide finger print match as indicated by the MASCOT-search engine. e. The highest identity of protein sequence in *D. anilini* to that in *T. aromatica* or *G. metallireducens*. - : Not found in total proteome analyses result.

2.4.3. Reverse transcription PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) experiments were performed with mRNA extracted from cells grown on phenol or benzoate (Fig. 3) to test whether the putative genes of *ppsα*, *ppsβ*, *pps*γ, *ppcα*, *ppcβ*, and *ppcδ* in the genome of *D. anilini* were induced by phenol. With cDNA from phenol-grown cells, all genes under study were amplified. With cDNA from benzoate-grown cells, no amplifications of *ppsβ*, *ppcα* and *ppcδ* were obtained. This result indicates that transcription of all genes (*ppsα*, *ppsβ*, *pps*γ*, ppcα*, *ppcβ*, and *ppcδ*) was induced in phenol-grown cells, but not all of them were induced in benzoate-grown cells. Hence, this result confirmed the involvement of these genes in phenol degradation by *D. anilini*. In a control PCR experiment, genomic DNA instead of cDNA from mRNA was used as the template, and DNA fragments of the expected sizes were obtained in all cases (Fig. S1). The gene coding for dissimilatory adenylylsulfate reductase alpha subunit precursor was transcribed in both phenol-grown and benzoate-grown cells and used as the house-keeping gene reference.

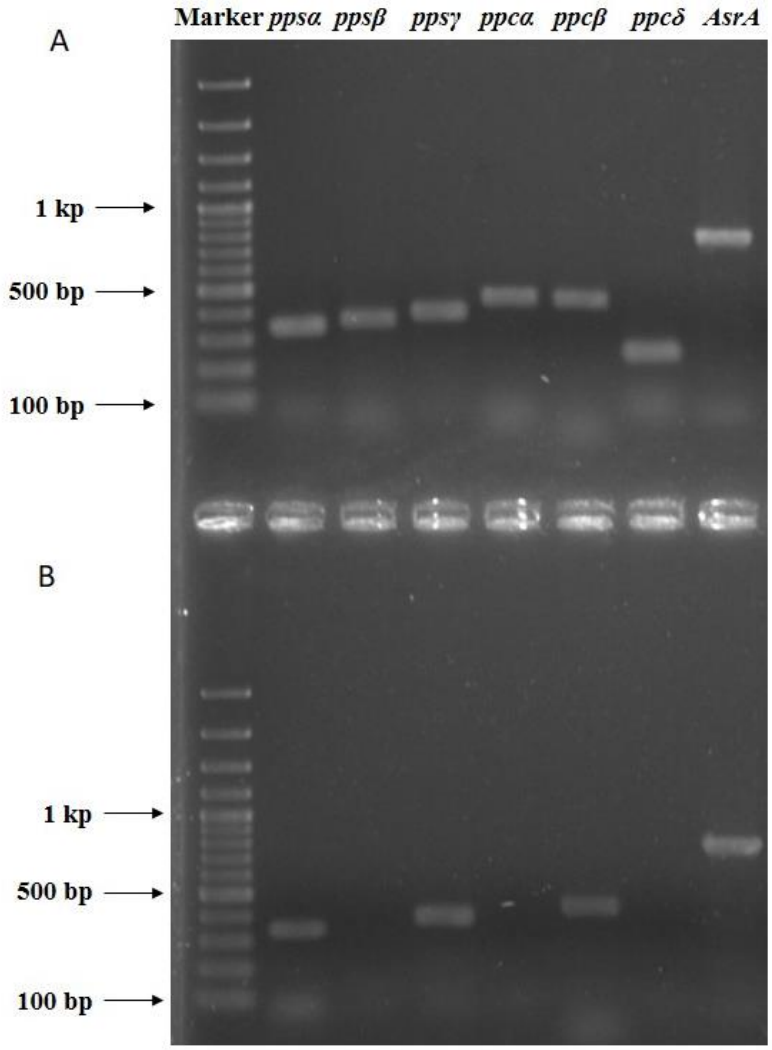

Figure 3. Reverse transcriptase PCR of genes *ppsα, ppsβ, ppsγ, ppcα, ppcβ and ppcδ* in *D. anilini*. (A) Reverse transcriptase PCR products of *ppsα, ppsβ, ppsγ, ppcα, ppcβ, ppcδ* and *AsrA* (dissimilatory adenylylsulfate reductase alpha subunit precursor; housekeeping gene) using the cDNA from phenol-grown cells as template. (B) Reverse transcriptase PCR products of *ppsα, ppsβ, ppsγ, ppcα, ppcβ, ppcδ* and *AsrA* (dissimilatory adenylylsulfate reductase alpha subunit precursor; housekeeping gene) using the cDNA from benzoate-grown cells as template.

2.4.4. Total proteomics analysis

To further verify the expression of putative genes involved in phenol degradation by *D. anilini*, the proteome of phenol-grown cells was compared to that of benzoate-grown cells by total proteomics analysis. Equal amounts of protein from phenol-grown cells or benzoate-grown cells were analyzed, and all protein abundances were quantified by label-free protein quantification (LFQ) (Fig. 4). The LFQ of enzymes encoded by the putative phenol degradation gene cluster (locus tag from H567DRAFT_02049 to H567DRAFT_02059) in phenol-grown cells were much higher than that in benzoate-grown cells (Fig. 4A). The gene cluster under study is a 14.4 kb gene cluster in the genome of *D. anilini*. The putative gene *ppcα* (locus tag H567DRAFT_03563) which is not located in this gene cluster was specifically induced in phenol-grown cells. Nevertheless, the putative gene *ppsγ* (locus tag H567DRAFT_03126) did not exhibit higher abundance in phenol-grown cells than in benzoate-grown cells. These results further verified the participation of the gene cluster in phenol degradation. Hence, phenol is presumed to be degraded through a similar pathway as in *T. aromatica.*

The total proteome data of phenol-grown cells and benzoate-grown cells also demonstrate the existence of the putative enzymes to be involved in anaerobic benzoyl-CoA degradation. The label-free quantification (LFQ) intensity of these enzymes (H567DRAFT_01120 6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase, H567DRAFT_01121 6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase, H567DRAFT_01122 cyclohexa-1, 5-diene-1-carbonyl-CoA hydratase) did not show significant differences between phenol-grown cells or benzoate –grown cells (Fig. 4B).

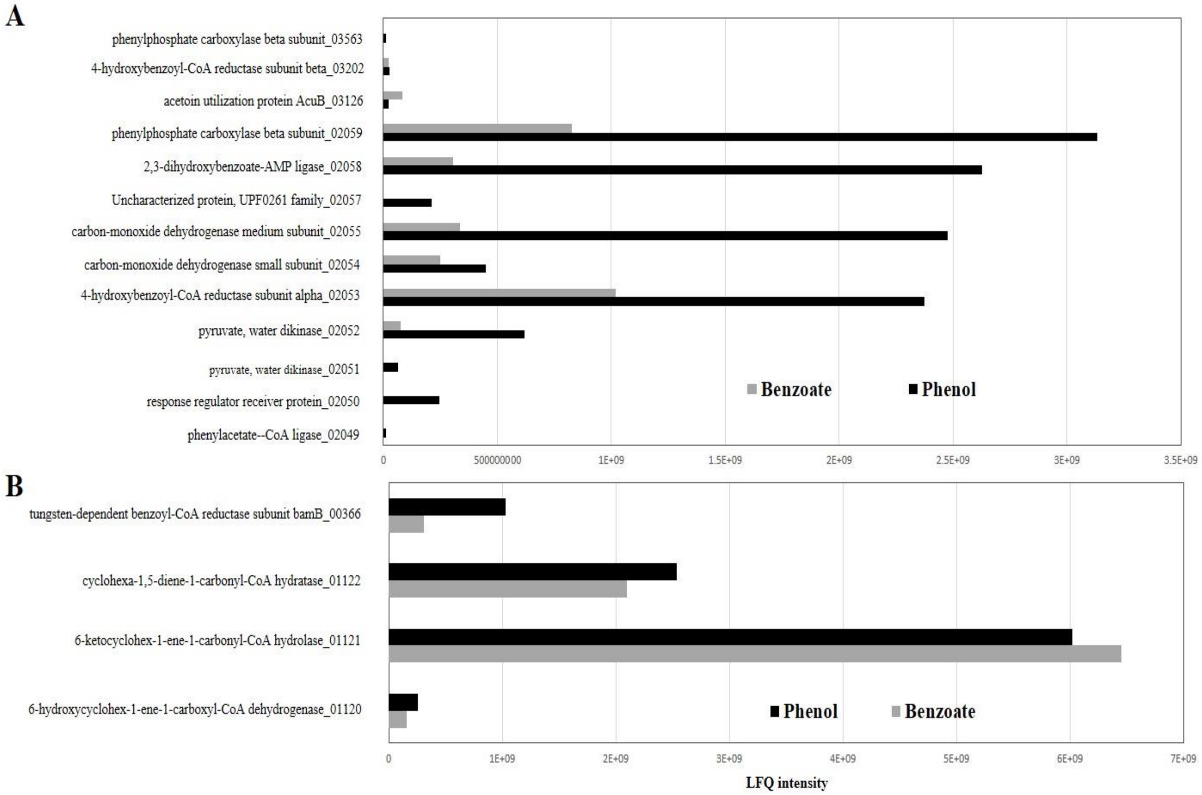

Figure 4. Total proteome analysis of cell-free extracts from the cells of *D. anilini* grown on phenol (black bars) and benzoate (grey bars). (A) label-free quantification intensity (LFQ-intensity) of the enzymes encoded by *pps-ppc* gene cluster (locus tag 02049-02059) and putative *ppsγ* (locus tag 03126) and ppcα (locus tag 03563) in *D. anilini* grown with phenol or benzoate. (B) LFQ-intensity of enzymes (6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase, 6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase, cyclohexa-1, 5-diene-1-carbonyl-CoA hydratase, tungsten-dependent benzoyl-CoA reductase subunit bamB) involved in benzoyl CoA metabolism in *D. anilini* grown with phenol or benzoate. The LFQ intensity value of the enzymes from phenol-grown cells are labeled at the end of the columns. The lowest LFQ intensity value observed in total proteomics analysis was 109120, and the highest LFQ intensity observed is 16493000000.

2.4.5. Phenylphosphate synthase activity

In-vitro enzyme activity assays were carried out with cell-free extracts of phenol- or benzoate-grown cells. The enzyme activity of phenylphosphate synthase was observed by detecting the formation of phenylphosphate over time, using phenol and ATP as the substrates with cell-free extracts of phenol-grown cells. Fig. 5 shows that these cell-free extracts were able to convert phenol to phenylphosphate with ATP as a co-substrate and Mg^{2+}, Mn^{2+}, and K^{+} as cofactors. Extracts of phenol-grown *T. aromatica* cells catalyzed the MgATP-dependent formation of [^{14}C] phenylphosphate from [U-^{14}C] phenol at a specific rate of 1.5 nmol min^{-1} mg^{-1} of protein [66]. In our study, the rate of phenylphosphate formation catalyzed by phenylphosphate synthase was tested as 0.52 nmol min^{-1} mg^{-1} of protein which is lower than that of *T. aromatica* cells. The activity was inhibited by oxygen, and no activity was measured with cell free extracts of benzoate grown cells.

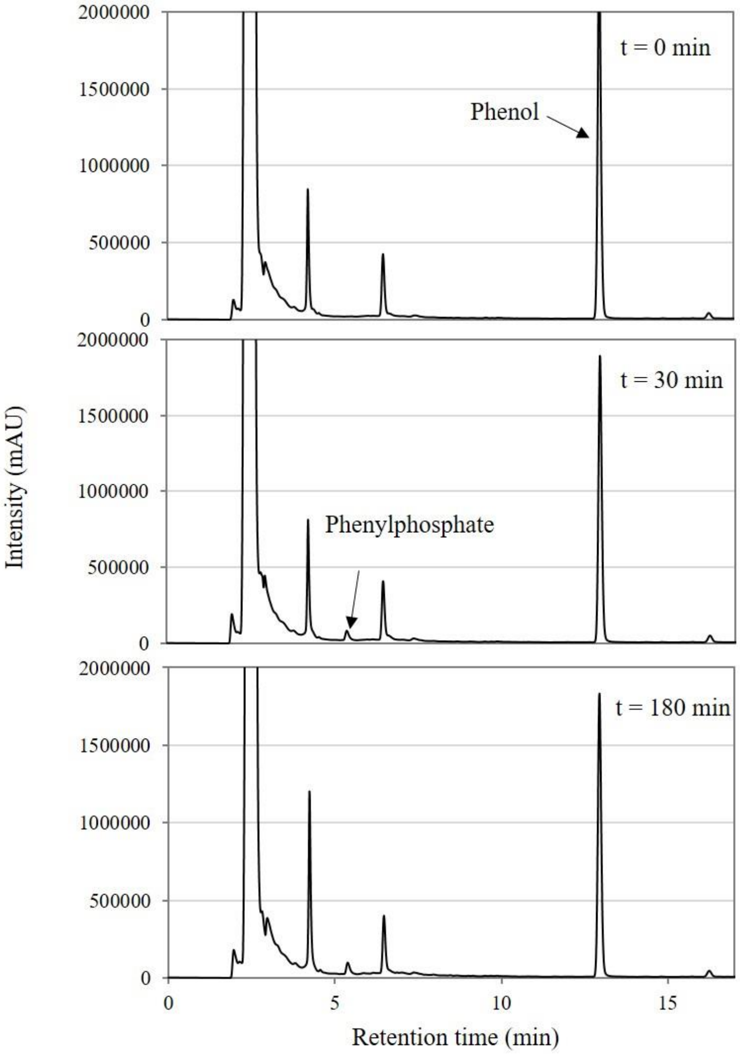

Figure 5. Time course of enzymatic phenylphosphate formation from phenol and ATP by the putative phenylphosphate synthase discontinuously assayed by HPLC with crude cell-free extract as sample.

2.4.6. Phenylphosphate carboxylase activity

The activity of phenylphosphate carboxylase was assayed either by spectrophotometric assays or HPLC [3]. In spectrophotometric assays no change of absorption could be measured as a consequence of phenylphosphate consumption at 235 nm or 4-hydroxybenzoate production at 280 nm. No substrate consumption or product formation was observed by HPLC, neither with CO_2 nor with CO as co-substrate.

2.5. Discussion

In the present study, the initial steps of phenol activation in a sulfate-reducing bacterium were studied by characterizing the transcription and expression of *pps*- and *ppc*- like ORFs and *in-vitro* phenylphosphate synthase assays. The results revealed that the phenol degradation pathway in this sulfate-reducing bacterium *D. anilini* (Fig. 6) is analogous to the known phenol degradation pathway in the nitrate-reducing bacterium *T. aromatica*.

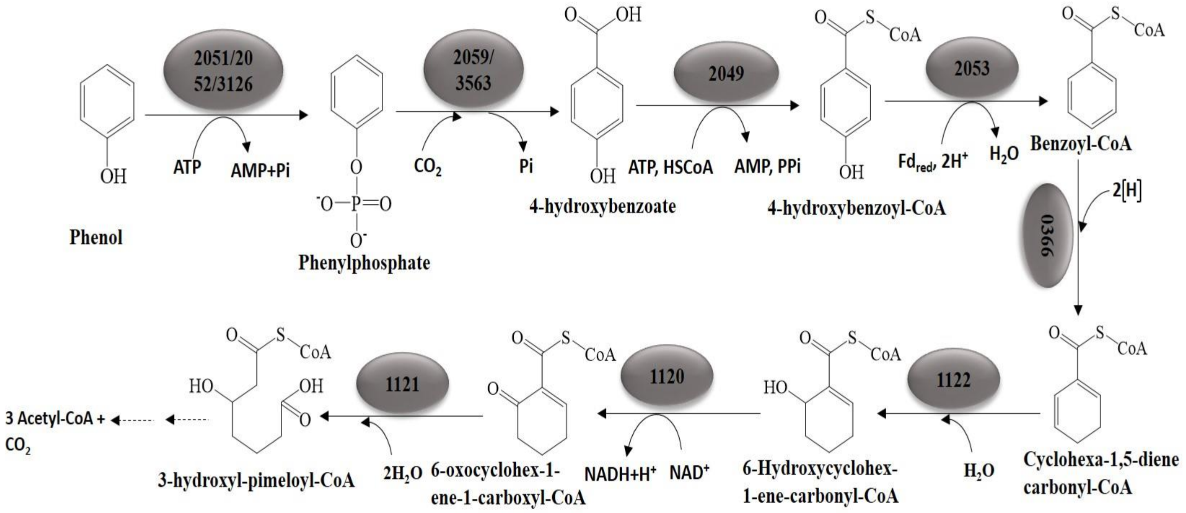

Figure 6. Proposed pathway of anaerobic phenol degradation by *D. anilini*. Numbers in dark ellipses indicate the locus tags of the enzymes.

The first step for phenol activation in *D. anilini* is a phosphorylation of phenol to phenylphosphate catalyzed by phenylphosphate synthase. Homologues of the three genes coding for the three subunits of phenylphosphate synthase (*ppsαβγ*) in *T. aromatica* are present in the genome of *D. anilini*. The genes *ppsα* (locus tag H567DRAFT_02052) and *ppsβ* (locus tag H567DRAFT_02051) are two adjacent genes showing homologies to pyruvate, water dikinase, and are transcribed and translated during growth on phenol. The putative gene *ppsγ* (locus tag H567DRAFT_03126) was transcribed both in phenol- and in benzoate-grown cells. The expression of the putative gene *ppsγ*, which is not located in the same gene cluster with *ppsα* and *ppsβ*, is lower in phenol-grown cells than in benzoate-grown cells. The enzyme activity of phenylphosphate synthase was observed *in vitro* in cell-free extracts of phenol-grown cells by measuring the conversion of phenol to phenylphosphate. Since the conversion of phenol to phenylphosphate can be accomplished without the presence of *ppsγ*, further verifications are needed to prove the participation of gene *ppsγ* in phenol degradation in *D. anilini*.

After activation of phenol to phenylphosphate, the latter is carboxylated to 4-hydroxybenzoate by phenylphosphate carboxylase. Homologues of three genes coding for the three subunits (*ppcαβδ*) of phenylphosphate carboxylase in *T. aromatica* are present in the genome of *D. anilini*. No homologue for *ppcγ* was found in the genome of *D. anilini*. The gene cluster under study carries only a homologue for the β-subunit of phenylphosphate carboxylase. The genes, *ppcα, ppcβ, ppcδ*, were all transcribed during growth on phenol. The expression of these genes except for *ppcδ* was observed in phenol-grown cells. However, attempts to demonstrate phenylphosphate carboxylase activity in cell-free extracts of phenol-grown cells failed. Earlier studies have shown, that the activity of phenylphosphate carboxylase in *Thauera aromatica* (earlier known as *Pseudomonas* strain K 172) is extremely oxygen sensitive and its activity in oxygen-saturated buffer had a half-life of 30 s [3,7]. It is therefore likely, that phenylphosphate carboxylase is also present in *D. anilini*, but inactivated during cell-lysis despite all precautions made for excluding oxygen from the samples. The fact that all necessary enzymes for the degradation 4-hydroxybenzoate were expressed during growth with phenol leads to conclude that phenylphosphate, whose presence as an intermediate was proven, needs to undergo a carboxylation or carbonylation with phenylphosphate carboxylase as the most likely responsible enzyme.

4-hydroxybenzoate is subsequently transformed to the central intermediate benzoyl-CoA via 4-hydroxybenzoyl-CoA, the enzymes involved are 4-hydroxybenzoyl-CoA ligase and 4-hydroxybenzoyl-CoA reductase. The gene (locus tag H567DRAFT_02049) coding for 4-hydroxybenzoyl-CoA ligase in the genome of *D. anilini* shows homologies to genes coding for phenylphosphate-CoA ligase. The genes coding for 4-hydroxybenzoyl-CoA ligase and the α-subunit of 4-hydroxybenzoyl-CoA reductase are located in the gene cluster under study. This gene cluster carries a carbon monoxide dehydrogenase medium subunit (locus tag H567DRAFT_02055) and a carbon monoxide dehydrogenase small subunit (locus tag H567DRAFT_02054), which can catalyze the reduction of CO_2 to CO. The resulting CO is combined with a methyl group to form acetyl-CoA by acetyl-CoA synthase through the Wood-Ljungdahl pathway in some anaerobic microbes [83, 84]. In *D. anilini* this enzyme system is needed for cleaving acetyl-CoA to CO and a methyl group to allow complete oxidation of the substrate to CO_2 with sulfate as electron acceptor [85]. Since carbon monoxide dehydrogenase (CODH) was strongly expressed in phenol-grown cells in comparison to benzoate-grown cells (Fig. 4), CODH may also play a role in the upper phenol degradation pathway. For example, it could convert CO_2 to CO and employ CO as a co-substrate to activate phenylphosphate, as reported in anaerobic acetone degradation by sulfate-reducing bacteria [86]. Another presumption is that CODH may have a reductive dehydroxylation function, by dehydroxylating 4-hydroxybenzoate or 4-hydroxybenzoyl-CoA to benzoate or benzoyl-CoA respectively. The function of 2, 3-dihydroxybenzoate-AMP ligase (locus tag H567DRAFT_02058), which is induced during growth with phenol, is unknown. In benzoate-grown cells, some of the genes in this gene cluster were still transcribed and translated. A possible reason may be that 10 generations of adaptation to benzoate are not enough to switch the metabolism from phenol to benzoate utilization completely.

The results obtained in this work support the hypothesis that sulfate-reducing bacteria use for phenol degradation a similar strategy as nitrate-reducing bacteria do. But it remains unclear how a sulfate reducer with its small energy budget can afford the high energy expenditure for phenol carboxylation and hydroxybenzoic acid activation which together require up to four ATP equivalents. The lack of *ppcγ* in the genome of *D. anilini* is in accordance with the results in the iron-reducing bacterium *Geobacter metallireducens* GS-15 [69], indicating a different catalytic mechanism of phenylphosphate carboxylase in sulfate-reducing and in iron-reducing bacteria. One possible activation mechanism that could be considered is a hypothetical phenylphosphate carbonylation to 4-hydroxybenzaldehyde with CO that is produced by the CO dehydrogenase located in the abovementioned gene cluster.

2.6. Conclusion

In this study, the genes coding for the enzymes involved in the anaerobic conversion of phenol to benzoyl-CoA were identified in the sulfate-reducing bacterium *D. anilini*. Analysis of the genome, transcriptome and proteome revealed that phenol is most likely activated to phenylphosphate, followed by carboxylation to 4-hydroxybenzoate, which is analogous to the well-known phenol degradation pathway in nitrate-reducing bacteria. Further evidence for phenylphosphate as an intermediate of phenol degradation in *D. anilini* is provided by *in-vitro* enzyme assays. Activity of phenylphosphate carboxylase could not be demonstrated by *in-vitro* enzyme assays, however all necessary genes for this enzyme complex were specifically expressed during growth with phenol. This work contributes to completing the picture of the phenol degradation pathway in anaerobic bacteria depending on different electron acceptors.

2.7. Materials and methods

2.7.1. Bacterial strains and culture media

Desulfatiglans anilini DSM 4660 was isolated from marine sediments [31] and described as a sulfate-reducing bacterium oxidizing aniline. *D. anilini* was grown in bicarbonate-buffered (30 mM) and sulfide-reduced (2 mM) brackish water medium [87]. After autoclaving at 121°C and 1 atm overpressure and cooling to room temperature under a stream of N_2/CO_2, 1 mL selenite tungstate solution/L and 1 mL 7 vitamins solution/L [88] were added. A heat-sterilized stock solution of a trace element mixture (SL 13) [89] was added to the basal medium (1:1000 v/v). Resazurin (0.4 mg mL^{-1}) was used as redox indicator. The pH was adjusted to 7.2. Benzoate or phenol was added anoxically from filter-sterilized anoxic stock solutions to 2 mM final concentration as growth substrate plus sulfate (10 mM) as terminal electron acceptor.

2.7.2. Adaptation of *D. anilini* to utilization of phenol or benzoate

D. anilini was inoculated into fresh medium with each 1 mM phenol or benzoate at an initial OD_{600} of around 0.04. The OD_{600} was monitored once per week until a maximum OD_{600} of around 0.18 was reached after 27 days. Cells were subsequently transferred to fresh medium four more times reaching over 135 days from initial culture.

2.7.3. Reverse transcription PCR analysis

For extracting RNA of *D. anilini*, 20 mL of phenol- or benzoate-grown cells in log-phase were collected by centrifuging at 11,700 x g for 20 minutes. Cells were washed with ultrapure water and centrifuged again. The pellets were used to extract RNA by using the TRIzol®Reagent with the PureLink®RNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer’s protocol. RNA Clean & Concentrator Kit (ZYMO RESERACH, Irvine, California, USA) was used to remove contaminating DNA from RNA samples. The first strand cDNA was synthesized from total RNA using the SuperScript®III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Waltham, Massachusetts, USA) with random hexamer primers. Genomic DNA of *D. anilini* was isolated from 1.5 ml of a dense culture using the Gentra Puregene Cell Kit (Qiagen). The concentrations of DNA, RNA and cDNA were measured with a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad, Hercules, California, USA). The standard PCR mixture had a volume of 25 µL and contained 2.5 µL of 10 × PCR buffer, 5 nmol dNTPs, 50 pmol of each primer (Microsynth, Balgach, Switzerland), 4 nmol $MgCl_2$, 0.2 µL Tag-polymerase (5 U/µL, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 2 µL cDNA or 10 to 50 ng genomic DNA as template. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 31 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. Primer pairs used to amplify approximately 200-500 bp fragments of genes are listed in table 1. The PCR purification products' qualities were analyzed by electrophoresis in a 1.0 % agarose gel at 110 V for 30 minutes.

Table 1. Primers used in reverse transcription PCR analysis**.**

PCR product	Gene locus tag	Primer sequence
ppsα	H567DRAFT_ 02052	ppsα-1: AAGATCCTCACCAAGCACGG
		ppsα-2: GGGGAACCCGGTGATTTCAT
ppsβ	H567DRAFT_ 02051	ppsβ-1: TCATGTTCTCGCTCAACCCC
		ppsβ-2: AGATCGATTCAGGGAACGGC
ppsγ	H567DRAFT_ 03126	ppsγ-1: CGGACAGGGATCTCAAACGG
		ppsγ-2: CGTTCGTAGCTGGTCAGGAT
ppcα	H567DRAFT_ 03563	ppcα-1: ACCC TGTGGCAGCAGTTATC
		ppcα-2: TAGAATCCCAGCTCCGACCA
ppcβ	H567DRAFT_ 02059	ppcβ-1: TGACCATGGCCGTTTCCTAC
		ppcβ-2: TCTTGACCATTTCGGGGTCG
ppcδ	H567DRAFT_ 00862	ppcδ-1: AAGTCGTCATCATCACGGGC
		ppcδ-2: AAATCGGCCATTTCACGGAC
Asrα	H567DRAFT_02821	Asrα-1: ATGACCATTTCTCAGGCGCA
		Asrα-2: GTCGCGCTTCATCATTTCCC

2.7.4. Preparation of cell-free extracts

Cultures grown on phenol or benzoate were harvested at the end of the exponential growth phase (OD_{600} = 0.15– 0.20) in an anoxic chamber (Coy, Ann Arbor, USA) by centrifugation (20,300 x g for 30 min at 4°C, Dupont Sorvall, Midland, Canada). For total proteomics analysis, cells were washed twice by repeated centrifugation in anoxic 200 mL 50 mM potassium phosphate buffer containing 3 mM dithiothreitol, pH 7.5, and resuspended in 3–4 mL of the same buffer. Cells were broken anoxically by three passages through a cooled MiniCell French pressure cell (SLM Aminco, Cat. No. FA003, Urbana, Illinois, USA) operated at 137 MPa pressure. Cell debris was removed by centrifugation at 30,300 x g for 30 min at 4°C to obtain the crude extract. The soluble protein fraction was obtained by ultra-centrifugation (150,000 x g for 60 min, Optima^{TM} TL Ultracentrifuge, Beckman Coulter, Brea, California, USA) of the crude extract to remove insoluble membrane particles. The protein concentration was estimated with the Bradford assay using bovine serum albumin as protein standard [90].

2.7.5. Total proteomics analysis and database search

The supernatants containing soluble proteins were used for total proteomics analysis, whose concentrations were 2.7 mg mL^{-1} (phenol-grown cells) and 2.9 mg mL^{-1} (benzoate-grown cells), from which 500 µL supernatant was submitted to peptide-fingerprinting-mass spectrometry at the Proteomics Facility of the University of Konstanz. Total proteome analysis was performed using a LTQ Orbitrap Discovery with an Eksigent 2D-nano HPLC (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The mass spectrometry data was analyzed by the Mascot search engine [v2.2.2 from Matrix Science] [91], to identify and characterize proteins from the protein database of the IMG annotated genome of *D. anilini*. Quantitative analysis of the identified proteins was done by label-free quantification using the LFQ algorithms included in the Proteome Discoverer software package V1.3 (Thermo Scientific). Relative protein abundances were expressed as label-free quantification intensity (LFQ-intensity).

2.7.6. Phenylphosphate synthase activity

Cell extracts from cells grown on phenol or benzoate were prepared in 50 mM imidazole-HCl buffer (pH 7.0), 0.5 mM dithiothreitol, 0.5 mg DNase I, followed with a French press and ultra-centrifugation (30 min; 30,300 x g) to remove cell debris. The standard enzyme assay mixture (2 mL) for phenylphosphate synthase contained approximately 1 mg protein, 2 mM ATP, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM KCl and 1 mM phenol. The enzyme tests were performed at 30 °C under strictly anaerobic conditions. To analyze the enzyme product, 300 µL samples were withdrawn at different time points and the reaction was stopped by addition of an equal volume of dichloromethane and centrifuged (11,700 x g for 10 min). The supernatant was transferred into 200 µL HPLC vials and analyzed by HPLC.

2.7.7. Phenylphosphate carboxylase activity

Cell extracts were prepared in 50 mM imidazole-HCl buffer (pH 7.0), 0.5 mM dithiothreitol, 10% glycerol, 0.5 mg DNase, using a French press, followed by ultra-centrifugation (30 min; 30,300 x

g) to remove cell debris. The enzyme assays were performed at 30 °C under strictly anoxic conditions. The standard assay mixture (2 mL) contained 50 mM imidazole-HCl buffer (pH 7.0) with 0.5 mM dithiothreitol, 0.7 mg protein, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM KCl, 1 mM phenylphosphate and 30 mM $NaHCO_3$ (or 10% CO). The reaction products were monitored by UV/Vis absorption spectra or HPLC analysis. For spectrophotometric assays, 50 µL samples were withdrawn from the assay mixture at different time points, added into 950 µL of 1 M KOH solution and the absorption was determined at two wavelengths for quantification of phenylphosphate (phenolate ion at pH 14; 235 nm; $\varepsilon = 9400\ M^{-1}\ cm^{-1}$) and the product 4-hydroxybenzoate (280 nm; $\varepsilon = 16300\ M^{-1}\ cm^{-1}$) [3]. For HPLC analysis, 300 µL samples were withdrawn from the assay mixture at different time points and the reaction was stopped by addition of an equal volume of acetonitrile and centrifuged (11,700 x g for 10 min). The supernatant (200 µL) was transferred to HPLC vials and analyzed by HPLC.

2.7.8. Analytical methods

The concentrations of phenol, phenylphosphate and 4-hydroxybenzoate were determined with a reversed-phase HPLC (Shimadzu, Kyoto, Japan) system equipped with a UV-visible diode array detector and a 4 um Max-RP 80Å LC column (250*4.6mm, Synergi) (Phenomenex, Torrance, California, USA) at 25°C. Eluents were prepared by mixing ultrapure water with 0.1% H_3PO_4 (buffer B), and acetonitrile with 0.1% H_3PO_4 (buffer A) and filtration through 0.2 µm. A gradient of buffer B increasing from 80% to 60% was used at a flow rate of 1 mL min^{-1}. 50 uL samples were injected into the column. The compounds were identified by comparing the retention times and UV-spectra of peaks to the retention time and UV-spectra of the respective standards. Figure 5 was prepared by exporting the chromatogram data of the 200 nm PDA-channel from the Shimadzu LC solutions software to ASCII – format (time (s) and intensity (mAU)) and the time data was converted from s to min. Then, the data was converted to a Microsoft – Excel diagram to obtain a better resolution of the chromatogram layout.

2.7.9. Chemicals

All standard chemicals were of analytical quality and were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) or Sigma (St. Louis, USA). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

2.8. Abbreviations

D. anilini: Desulfatiglans anilini DSM 4660; *G. metallireducens: Geobacter metallireducens* GS-15; *T. aromatica: Thauera aromatica*; pps: phenylphosphate synthase; ppc: phenylphosphate carboxylase; ppsα: phenylphosphate synthase alpha subunit; ppsβ: phenylphosphate synthase beta subunit; ppsγ: phenylphosphate synthase gamma subunit; ppcα: phenylphosphate carboxylase alpha subunit; ppcβ: phenylphosphate carboxylase beta subunit; ppcγ: phenylphosphate carboxylase gamma subunit; ppcδ: phenylphosphate carboxylase delta subunit; AsrA: dissimilatory adenylylsulfate reductase alpha subunit precursor; BamB-I: benzoyl-CoA reductase; BamR: cyclohexadienoyl-CoA hydratase; BamQ: hydroxyenoyl-CoA dehydrogenase; BamA; oxoenoyl-CoA hydrolase; HPLC: High-performance liquid chromatography; ASCII: American Standard Code for Information Interchange; IMG/M: Integrated Microbial Genomes & Microbiomes; NCBI: National Center for Biotechnology Information; ORF: Open reading frame*;* RT-PCR: Reverse transcription polymerase chain reaction; LFQ: Label-free quantification intensity; CODH: carbon monoxide dehydrogenase; UV/Vis: Ultraviolet–visible spectroscopy; MS: mass spectrometry

2.9. Declarations

Acknowledgements

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Availability of data and materials

All data presented are included in the main article. The genome sequence of *Desulfatiglans anilini* DSM 4660 and its gene annotations are available on the Integrated Microbial Genomes database under the IMG genome ID 2526164742.

Authors' contribution

XX and NM designed experiments, analyzed the data and wrote the manuscript. Experiments were conducted by XX. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

2.10. Supplementary materials

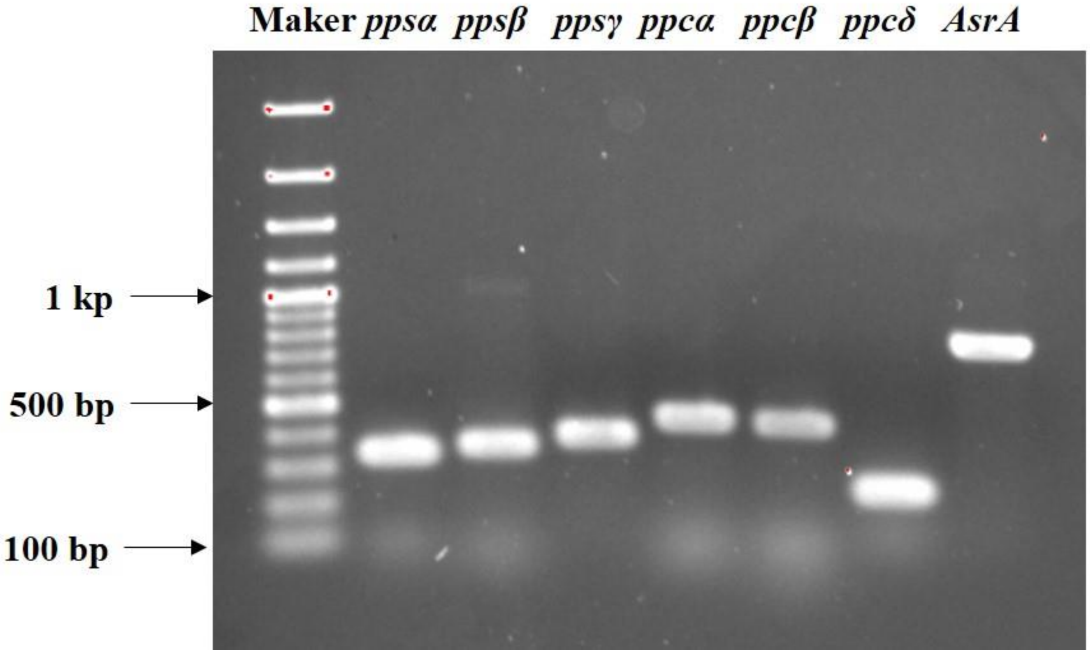

Fig. S1. PCR products using genomic DNA as template.

CHAPTER 3

Sulfide inhibits activation of aniline in *Desulfatiglans anilini*

Xiaoman Xie, Nicolai Müller

To be submitted

3.1. Abstract

Desulfatiglans anilini is a sulfate-reducing bacterium capable of oxidizing aniline, however growth and aniline turnover rates are slow making it difficult to analyze the metabolism of the strain. This study was designed to investigate the effect of sulfide on aniline degradation in *D. anilini* cultures and to improve growth and aniline turnover rate of *D. anilini*. Carbon dioxide (CO_2) was found to be an essential co-substrate for activation of aniline. A carboxylation activity could be measured in cell-free extracts with aniline, $NaHCO_3$, and ATP as substrates. The effects of hydrogen sulfide on this carboxylase activity for aniline activation and on phenylphosphate synthase activity for phenol activation were studied, and it was concluded that hydrogen sulfide severely inhibited these enzyme activities. For improving the growth of *D. anilini* with aniline, the effects of metal ions and hydrogen sulfide were examined. Growth could not be stimulated by increasing the concentrations of metal ions. Hydrogen sulfide was found to inhibit growth of *D. anilini* regardless whether the strain was grown with aniline or phenol, and complete inhibition was observed at 20 mM hydrogen sulfide. The sulfide-consuming phototrophic bacterium *Thiocapsa roseopersicina* was co-cultured with *D. anilini* in a co-cultivation device to continuously remove hydrogen sulfide from the *D. anilini* culture. The doubling time of *D. anilini* was 15 days in the co-cultivation device, as compared to 26 days in the absence of a sulfide-oxidizing partner.

3.2. Importance

Desulfatiglans anilini is the only known sulfate-reducing bacterium that can degrade toxic aniline under completely anoxic conditions. Elucidation of the aniline activation mechanism and strengthening the ability of utilizing aniline are crucial for employing *D. anilini* in bioremediation of aniline-contaminated anoxic sites. Our work provides an activation mechanism for degradation of aniline, i. e. carboxylation, with CO_2 as a co-substrate. Studies on the influence of metal ions and hydrogen sulfide on enzyme activity and growth rate present a more comprehensive understanding of the enzyme and growth characteristics. We have also established a co-cultivation device in which the rate of aniline consumption by *D. anilini* was effectively increased.

3.3. Key words

Aniline, *Desulfatiglans anilini,* sulfide, carboxylation, *Thiocapsa roseopersicina*

3.4. Introduction

Aniline is a toxic organic compound and mainly used in the production of a variety of important chemicals such as dyes, additives to rubbers, photographic chemicals, pharmaceuticals, pesticides, and explosives [92]. Extensive use of aniline in industry and agriculture has led to serious pollution in waters and soils, making it necessary to remediate aniline-contaminated sites. Compared to physicochemical methods, bioremediation is an attractive treatment strategy for removing environmental contaminations as it can completely mineralize organic pollutants and can be applied in *in situ* processes [49]. In the past few decades, many studies have paid attention to the biodegradation of aromatic compounds. Efforts spent on the optimization of biodegradation conditions, elucidation of biodegradation pathways of aromatic compounds and characterization of degradation in pure or enrichment cultures enabled the removal of aromatic compounds from contaminated sites by bioremediation [93]. Some toxic aromatic compounds can be eliminated by novel bioremediation techniques. For example, mixtures of benzene, toluene, and p-xylene can be removed at high and sustained rates by a constructed microbial consortium immobilized on a biofilter [94], while phenol and 4-chlorophenol can be bio-transformed by *Pseudomonas putida* immobilized on polystyrene beads or hollow fiber [95, 96].

Analogously, microorganisms capable of degrading aniline would be useful for developing bioremediation techniques to be applied to aniline-contaminated sites. Thus far, it has been well documented that aniline can be removed efficiently by few aerobic aniline degraders [97-102]. An aerobic aniline-degrading bacterium, strain HY99, was found to degrade aniline under both aerobic and anaerobic nitrate-reducing conditions [73]. *Desulfatiglans anilini* is the only bacterium known to degrade aniline under strictly anoxic sulfate-reducing conditions [31, 74] which are typical of carbon metabolism in marine sediments.

Nonetheless, there are some limitations to application of bacteria like *D. anilini* in bioremediation processes, as the cells grow slowly and the metabolic pathway of aniline degradation is only insufficiently understood. Benzoyl-CoA is the most common central intermediate in the anaerobic degradation of aromatic compounds such as p-cresol, phenol, benzyl alcohol, toluene, ethylbenzene, and others [34]. The strategies that are used by anaerobic bacteria to transform aromatic compounds via the peripheral pathways to benzoyl-CoA differ based on the ring substituents or redox potentials of the electron acceptors supplied [35]. Modification reactions comprise, among others, carboxylation of phenolic compounds [36, 37], fumarate addition [38, 39], or oxidation of methyl substituents [40, 41]. Nitrate-reducing bacteria often use strategies different from those used by sulfate-reducing, iron-reducing or fermenting bacteria in general, since the average standard redox potential of nitrate/N_2 (+750 mV) is close to that of O_2/H_2O (+810 mV) and thus much higher than that of SO_4^{2-}/HS^- (-0.218 mV) or $Fe(OH)_3/Fe^{2+}$ (+100 mV, pH 7.0) [35].

Aniline was proposed to be carboxylated to 4-aminobenzoate, followed by activation of 4-aminobenzoate to 4-aminobenzoyl-CoA which is reductively deaminated to benzoyl-CoA. Activities of 4-aminobenzoyl-CoA synthase and benzoyl-CoA synthase could be measured in cell-free extracts of *D. anilini* [74]. However, no strong evidence exists to prove the conversion of aniline to 4-aminobenzoate. Carboxylation as an initial step in degradation of aromatic compounds has so far been observed in phenol degradation by nitrate-reducing bacteria [19, 36, 64, 66, 77], an iron-reducing bacterium [69], and the sulfate-reducing bacterium *D. anilini* [103]. Carboxylation of phenol requires the expenditure of two ATP equivalents which can easily be afforded by nitrate-reducing bacteria. The β phosphate is covalently bound to phenol, and the γ phosphate is released as inorganic phosphate [66]. However, oxidation of the aniline carbon skeleton via three acetyl-CoA residues by *D. anilini* proceeds through the Wood-Ljungdahl pathway along with the formation of about one ATP equivalent per acetyl residue [85]. Therefore, sulfate-reducing bacteria can hardly spend two ATP equivalents on aniline activation. In a study on acetone degradation by sulfate-reducing bacteria, CO or formate was found to be the co-substrate for activating acetone via carbonylation or formylation [86, 104], thus providing a novel mechanism for the activation of organic compounds in sulfate-reducing bacteria.

Investigation of the metabolism of *D. anilini* during degradation of aniline or other aromatic compounds like phenol, 4-aminobenzoate or benzoate is rendered difficult as growth and metabolic turnover of aromatic compounds with this strain are slow. Some factors that may affect growth of bacteria are generally the pH of medium, growth factors like metal ions, incubation temperature, and growth inhibitors. In sulfate-reducing bacteria, sulfate is reduced to hydrogen sulfide, which was found to inhibit growth at elevated concentrations [105-107]. Hence, the objectives of this study are to identify the ideal co-substrate for the activation of aniline as well as studying the effect of sulfide on the activity of aniline-activating enzymes and the growth of *D. anilini*. The latter was accomplished by co-cultivating *D. anilini* with the sulfide-oxidizing purple sulfur bacterium *Thiocapsa roseopersicina*.

3.5. Results

3.5.1. Co-substrate for aniline activation

To investigate whether CO_2 is a co-substrate for aniline activation, the activity of aniline utilization was tested in cell-free extracts of aniline-grown *D. anilini* cells by measuring the decrease of aniline in the presence of ATP and CO_2 (Fig. 1). Figure 1 shows that in the presence of CO_2 as a co-substrate, the rate of aniline degradation is 0.009 µmol/ (min*mg) within 2 hours of incubation, and nearly no aniline was degraded in the absence of CO_2. ATP is an essential co-factor for aniline utilization. Formate was also tested as a co-substrate to replace CO_2, but no aniline was activated in the presence of formate (data not shown). No product was detected by HPLC or LC-MS in the reaction system.

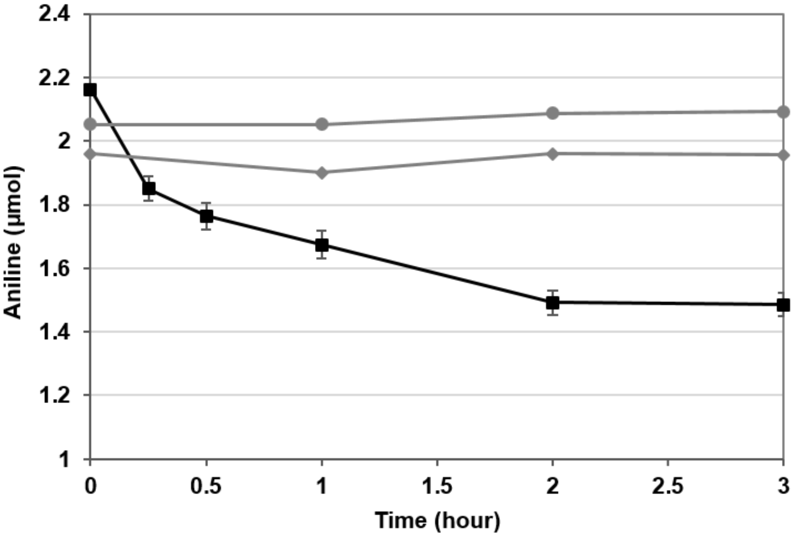

Fig. 1. Aniline consumption by cell-free extracts of *D. anilini* with CO_2 as co-substrate. For black circles, black squares and grey circles, values are means ± standard deviation (n = 3). Standard assay with CO_2 (black squares); standard assay with N_2 (grey circles); control assay without ATP with CO_2 (rhombus).

3.5.2. Effect of sulfide on aniline carboxylation and phenylphosphate synthase

In pure cultures of *D. anilini*, approximately 4 mM sulfide was formed by sulfate reduction if 1 mM aniline was oxidized to carbon dioxide according to the reaction stoichiometry [31]. In the present work, the influence of sulfide on the activity of aniline carboxylation in *D. anilini* was tested. The in vitro enzyme activity was reduced by half if 5 mM sodium sulfide was added to the assay system (Table 1). The pH of the enzyme assay system was 7.5, 7.7 or 7.9 in the presence of 0 mM, 2 mM or 5 mM sodium sulfide, respectively, showing no significant differences in pH by addition of sodium sulfide, and thus indicating that the sulfide rather than the pH is the factor that influences enzyme activity.

To test if the effect of sulfide on enzyme activity is specific for the aniline-carboxylating enzyme system, the influence of sulfide on the activity of phenylphosphate synthase, the enzyme catalyzing the conversion of phenol to phenylphosphate in the presence of ATP, was also measured. The activity of phenylphosphate synthase was measured with phenol and ATP as substrates in cell-free extracts of phenol-grown *D. anilini* cells [103]. The activity of phenylphosphate synthase is documented as the rate of phenol consumption (Table 1). 2 mM or 5 mM sodium sulfide was added to the enzyme assay system to measure the effect of sodium sulfide on the activity of phenylphosphate synthase. Table 1 shows that the activity of phenylphosphate synthase decreased by 60% after addition of 2 mM sodium sulfide, and nearly 90% of phenylphosphate synthase activity was inhibited by 5 mM sodium sulfide.

Table 1. Effect of sulfide on the activity of aniline-carboxylating enzyme system and phenylphosphate synthase

	Aniline-carboxylating enzyme system			Phenylphosphate synthase		
Sodium sulfide (mM)	0	2	5	0	2	5
Activity (µmol/min*mg)	0.013	0.011	0.007	0.025	0.007	0.0025
Percent (%)	100	84.6	53.8	100	28	10

3.5.3. Effect of metal ions and sulfide on the growth rate

To optimize the growth conditions, the effects of six metal ions (Co^{2+}, Mn^{2+}, Zn^{2+}, Rh^{3+}, Pd^{2+} or Rb^{+}) on the growth of *D. anilini* were studied. Growth experiments were performed in basal medium with 7 treatments in triplicates as follows: one pure culture as control and 6 pure cultures with one additional trace element in each medium. Optical densities were analyzed in the mid-exponential growth phase 48 days after inoculation (Fig. S1). The growth responses of *D. anilini* to a higher concentration of one trace element differed among the trace elements (Fig. S1). The single addition of Pd^{2+}, Rb^{+} or Mn^{2+} reduced the growth rate of *D. anilini* compared to the pure culture. Addition of Rh^{3+} or Co^{2+} to the pure culture had no effect on the growth rate. When Zn^{2+} was added, the growth rate of *D. anilini* increased slightly by 7.5%. Neither one of the above six metal ions caused a significant increase of the growth rate of *D. anilini*. Similar results were observed if lower concentrations (5 μM) of the metal ions were employed.

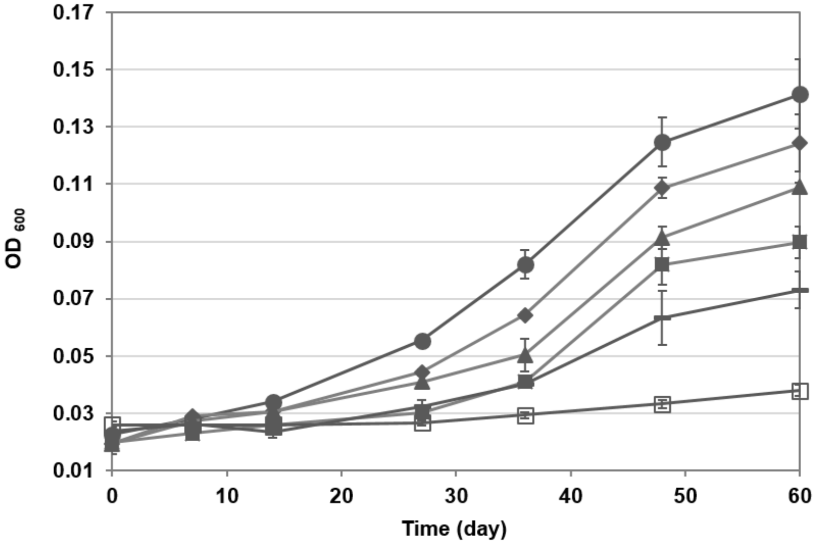

Fig. 2. Effect of sodium sulfide on the growth of *D. anilini* with aniline (1 mM) and sulfate (4 mM). Sodium sulfide (in addition to the sulfide present in the medium as a reducing agent) was added to the medium before inoculation as follows: none (solid circle); 2 mM sodium sulfide (solid rhombus); 5 mM sodium sulfide (solid triangle); 10 mM sodium sulfide (solid square); 15 mM sodium sulfide (short line); 20 mM sodium sulfide (empty square).

To investigate which impact sulfide has on the growth of *D. anilini*, growth of *D. anilini* was monitored via OD_{600} measurements in six cultures (with 1 mM aniline as substrate) in triplicates with various concentrations of sodium sulfide. The results shown in Fig. 2 indicate an inhibition of growth in the presence of additional sodium sulfide. The growth rate decreased with increasing sodium sulfide concentrations, and complete inhibition was observed at a concentration of 20 mM sodium sulfide. If 5 mM sodium sulfide was added, growth of *D. anilini* decreased by 26.8%. Similar results were obtained when using 2 mM phenol as substrate (Fig. S3).

3.5.4. Growth in co-culture

Since sulfide inhibited the growth of *D. anilini*, a co-culture with a sulfide-consuming bacterium, *Thiocapsa roseopersicina*, was established. *T. roseopersicina* is a purple sulfur bacterium belonging to the family of *Chromatiaceae* and capable of photolithoautotrophic growth under anoxic conditions in the light with sulfide as electron donor [108]. Growth curves of the cultures and aniline concentrations in pure and co-cultures are shown in Fig. 3. The pure culture of *D. anilini* grew very slowly with a doubling time of 20 days. The pure culture of *T. roseopersicina* stopped growing when the electron donor sulfide added as a reductant was consumed. *T. roseopersicina* in the co-culture grew continually with the sulfide provided by *D. anilini*. The rate of aniline consumption by *D. anilini* in co-culture was much higher with 0.016 mM per day compared to the pure culture of *D. anilini* with only 0.006 mM per day. Since *T. roseopersicina* is unable to utilize aniline (Fig. 3), the higher aniline consumption rate in the co-culture must result from an increased activity of *D. anilini*.

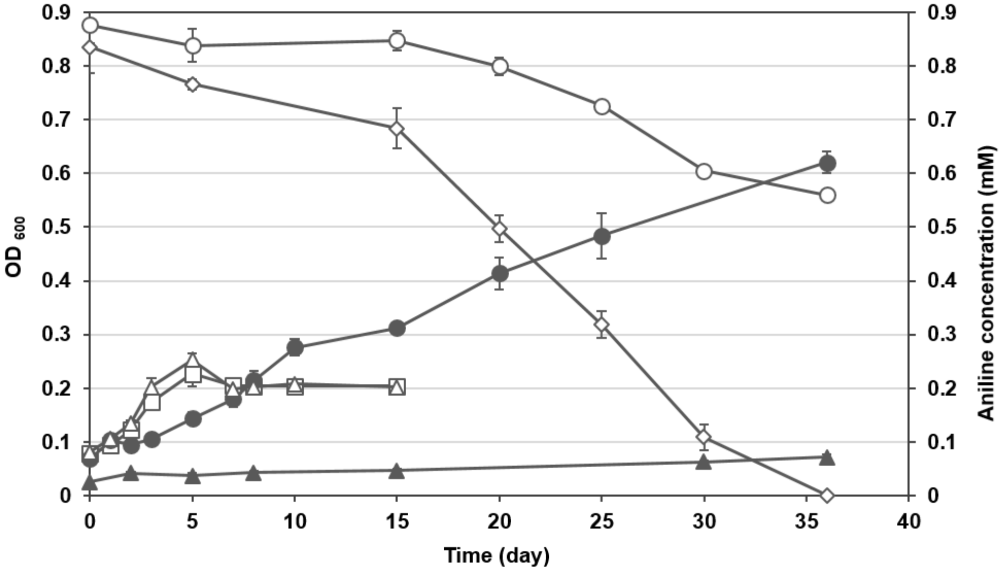

Fig. 3. Growth and aniline degradation in pure cultures (*D. anilini* or *T. roseopersicina*) and co-culture (*D. anilini* plus *T. roseopersicina*). Growth of *D. anilini* (solid triangle); Growth of *T. roseopersicina* (empty triangle); Growth of *T. roseopersicina* with 1 mM aniline (empty square); Growth of co-culture including *D. anilini* and *T. roseopersicina* (solid circle); Aniline degradation in co-culture (empty rhombus); Aniline degradation in pure culture of *D. anilini* (empty circle). Values are means ± standard deviation (n=3).

To investigate the individual growth rate of *D. anilini* and *T. roseopersicina* and the turnover rate of sulfide, sulfate and aniline in each pure culture, a co-cultivation device (Fig. S2) was designed which connects the headspaces of the pure cultures of *D. anilini* and *T. roseopersicina* to allow hydrogen sulfide exchange through the gas phase. As shown in Fig. 4, 1.01 mM aniline was completely consumed in 33 days along with 3.89 mM sulfate reduced to sulfide, which did not accumulate in the *D. anilini* culture but was taken up by *T. roseopersicina* leading to the formation of sulfate and growth of the *T. roseopersicina* culture. Most of the 6 mM sulfide (2 mM was present in the medium as a reductant and 4 mM was formed by the *D. anilini* culture) consumed by *T. roseopersicina* was oxidized to elemental sulfur as judged by the milky appearance of the culture as well as the presence of bright refractive round particles visible in the cells by phase contrast microscopy. The sulfide concentration dissolved in the *D. anilini* culture was constantly in the range of 2 mM during the co-cultivation process, which is lower than in the pure culture (4.52 mM dissolved sulfide in the pure *D. anilini* culture in stationary phase). The doubling time of *D. anilini* in the co-culture decreased significantly to 15 days. Obviously, *D. anilini* benefitted from the lower toxicity of hydrogen sulfide on the co-culture compared to the pure culture.

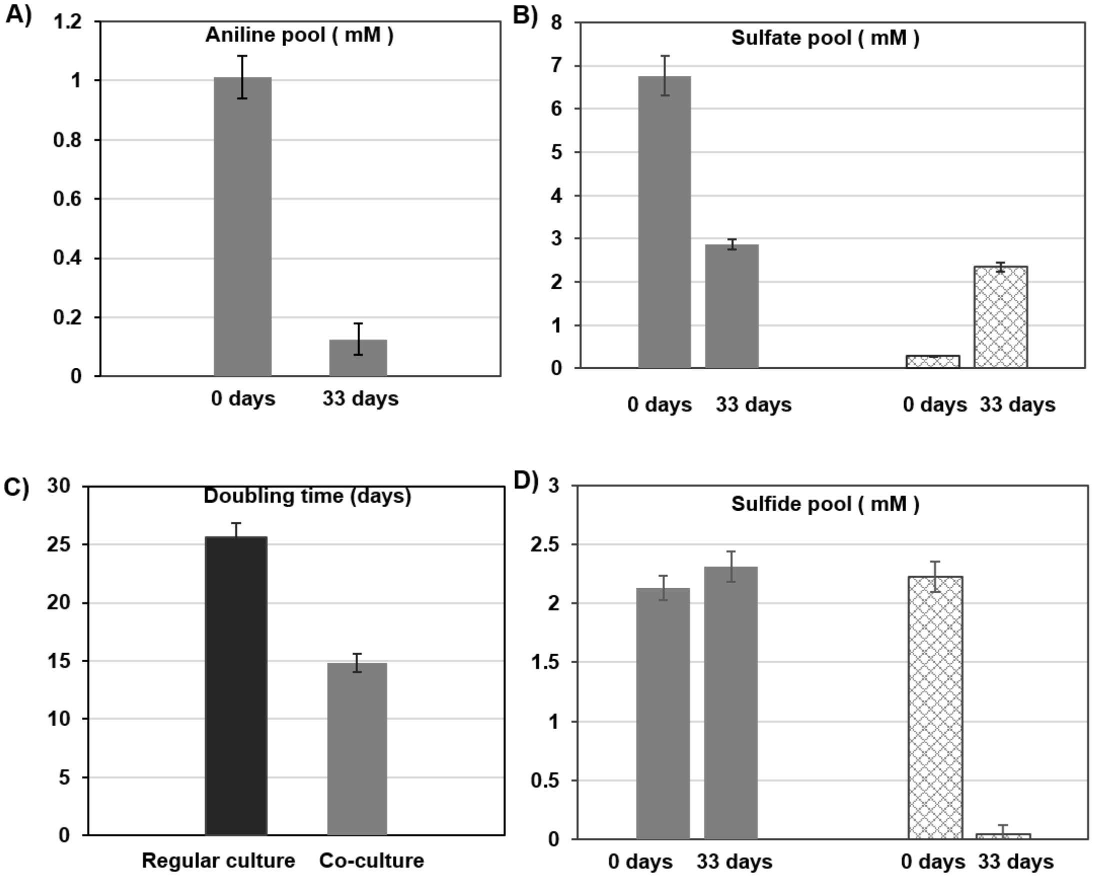

Fig. 4. Changes of aniline, sulfate, and sulfide in co-cultivation setup after 33 days. A) Aniline concentration in the bottle with *D. anilini* culture; B) Sulfate concentration in both cultures; C) Comparison of the doubling time of *D. anilini* in the co-cultivation setup and pure culture; D) Sulfide concentration in both cultures. *Desulfatiglans anilini* in co-culture (solid grey); *Desulfatiglans anilini* in pure culture (solid dark grey); *Thiocapsa roseopersicina* (pattern fill). Values are means ± standard deviation (n=3).

3.6. Discussion

In the present study, degradation of aniline under sulfate-reducing conditions was investigated with *Desulfatiglans anilini*. Our results show that CO_2 is needed as a co-substrate to activate aniline. In this reaction, ATP is required and most likely hydrolyzed to ADP or AMP and inorganic phosphate, such that a phosphate-aniline ligation product can be formed as an intermediate, analogous to the formation of phenylphosphate as an intermediate in phenol degradation. This intermediate could then be carboxylated to form 4-aminobenzoate. The effects of sulfide on aniline carboxylation and on phenylphosphate synthetase were investigated. In the presence of sulfide, the activities of the aniline-carboxylating enzyme system and phenylphosphate synthase were inhibited in cell-free extracts of *D. anilini*. Hydrogen sulfide is known to inhibit enzyme reactions by modifying the catalytic centers of metalloproteins, e. g. in angiotensin-converting enzyme. This enzyme containing zinc, and hydrogen sulfide may react with zinc and thus inhibit the enzyme activity [109]. In a recent study investigating phenol degradation in *D. anilini*, it was reported that phenylphosphate synthase activity depended on manganese, magnesium and potassium ions as co-factors, and the most likely candidate genes responsible are annotated as pyruvate-water dikinase [30]. The genes coding for enzymes involved in aniline activation by anaerobic bacteria were not yet identified, and the metal co-factors of aniline-carboxylating enzyme system are unknown. We therefore analyzed growth of the strain in the presence of various metal ions to identify a responsible metal co-factor that is inhibited by sulfide.

The addition of trace elements including cobalt, manganese, zinc, rhodium, palladium or rubidium to the basal medium could not enhance the growth of *D. anilini*, indicating that these trace elements are not crucial limitation factors for growth of *D. anilini* and that the co-factor responsible for activation or carboxylation of aniline is possibly different from those trace elements tested, or can be exchanged with other metals.

Hydrogen sulfide is formed by sulfate reduction in the sulfate-reducing strain *D. anilini* and besides its strong effect on the activity of aniline-activating enzymes (Table 1), it also inhibits growth of *D. anilini* (Fig. 2), which is in accordance with previous studies on the inhibition of growth by hydrogen sulfide in sulfate-reducing bacteria (SRB) [105-107]. Inhibition of SRB due to hydrogen sulfide was found to be direct and reversible [107]. By co-culturing with *Thiocapsa roseopersicina*, a phototrophic sulfide consuming bacterium, a significant increase in the growth rate of *D. anilini* could be achieved, as well as an increase in the aniline consumption rate of *D. anilini*. During the course of co-cultivation in a co-cultivation device, the concentration of sulfide in the liquid phase was continuously quantitatively recorded. Compared to a previous growth study performed at pH 6.2 and pH 6.6 [107], more gaseous H_2S converts to water-soluble HS^- in our culture (pH 7.2), according to a sulfide solubility chart at varying pH [110]. At the same time, a certain amount of sulfide was always left in the liquid phase and could not be removed efficiently by *T. roseopersicina.* By lowering the cultivation pH further, the equilibrium of hydrogen sulfide concentrations between the gaseous and liquid phase could be shifted towards gaseous hydrogen sulfide release. However, at more acidic pH *D. anilini* does not grow anymore, as the pH range of growth lies between 6.0 and 8.0 [31]. In the co-cultivation device used in the present study, the gaseous H_2S in the cultivation vessel of *D. anilini* was driven into the vessel of *T. roseopersicina* due to the increased partial pressure generated by continuous hydrogen sulfide formation by *D. anilini* and the low partial pressure resulting from H_2S consumption by *T. roseopersicina*. This experimental setup was chosen to be able to properly assess the contributions of both strains to biomass production and aniline degradation. The system could be improved further by cultivating both strains directly in one cultivation vessel, such that communicating gas phases are not necessary anymore and the sulfide concentrations during growth can be kept at lower levels. Yet, aniline degrading SRBs like *D. anilini* would still need a certain amount of sulfide left in the cultivation media to maintain a sufficiently low redox potential. We have attempted to replace sulfide as reducing agent by titanium-NTA, however, growth of *D. anilini* did not improve under these conditions (data not shown). Testing other potential reducing agents such as palladium/hydrogen or thiol-compounds that are less toxic compared to sulfide (dithiotreitol, thioglycolate) will be subject of further investigations. We propose that anaerobic aniline degradation for bioremediation purposes could be improved by employing consortia consisting of aniline degrading SRBs and phototrophic purple sulfur bacteria. The fact that phenylphosphate synthase is also sensitive to elevated concentrations of sulfide indicates that anaerobic phenol degradation by *D. anilini* could also be improved by maintaining low sulfide concentrations. While aerobic microorganisms (e. g. *Pseudomonas putida*) are more efficient in the degradation of aromatic pollutants, anaerobic aniline or phenol degraders could be of importance at contaminated sub-surface sites, where pump-and-treat procedures cannot be employed [111].

Being a rather artificial system in this study, it is questionable on the other hand whether such consortia of aniline-degrading SRBs and phototrophic purple sulfur bacteria also occur in nature. *D. anilini* was isolated from marine sediments of the North Sea coast, but also grows well in brackish water medium [31]. Marine sediments, especially in coastal areas, are known for increased activities of SRBs and thus for their high sulfide concentrations [112]. *Thiocapsa* species are also known to be present in marine systems [113, 114] and consortia of the type described in this study could therefore possibly also occur in natural environments. On the other hand, aniline as a mainly man-made aromatic compound is not present in non-contaminated environmental systems, which suggests that co-operations between SRB and phototrophic purple sulfur bacteria may play a significant role mainly during breakdown of organic and naturally occurring aromatic compounds.

3.7. Materials and methods

3.7.1. Bacterial strains and culture media

Desulfatiglans anilini DSM 4660 was isolated from marine sediments as a sulfate-reducing bacterium oxidizing aniline [31]. *Thiocapsa roseopersicina* (DSM 217) and *D. anilini* (DSM 4660) were both purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *D. anilini* was grown in bicarbonate-buffered (30 mM) and sulfide-reduced (2 mM) brackish water medium [87] with sulfate as the electron acceptor as described before [31]. The basal medium was autoclaved at 121°C and 1 bar overpressure and cooled to room temperature under a stream of N_2/CO_2 (80% N_2, 20% CO_2). Then, 30 mM $NaHCO_3$, 1 mL selenite-tungstate solution/L and 1 mL 7 vitamins solution/L [88] were added to the medium. A stock solution of a trace element mixture (SL 13) [89] was prepared, sterilized by autoclaving, and added to the basal medium (1:1000 v/v). Resazurin (0.4 mg L^{-1}) was added as redox indicator. Sodium sulfide was added from anoxic stock solutions sterilized by autoclaving at a final concentration of 2 mM. The pH of the medium was adjusted to 7.2. *T. roseopersicina* was cultured in the same bicarbonate-buffered and sulfide-reduced brackish water medium. The medium used for the co-culture experiments of *D. anilini* and *T. roseopersicina* was the same bicarbonate-buffered and sulfide-reduced brackish water medium as described above.

3.7.2. Cultivation conditions

Pure cultures of *D. anilini* were prepared in triplicate by inoculating approximately 0.5 mL of a pre-culture (exponential phase) into 10 mL medium in rubber-stoppered Hungate tubes with

N_2/CO_2 in the headspace. 1 mM aniline (or 2 mM phenol) and 5 mM (or 8 mM) sodium sulfate were added from 1.0 M sterile stock solutions. To study the influence of sulfide and metal ions on growth, various concentrations of sodium sulfide (0 mM, 2 mM, 5 mM, 10 mM, 15 mM or 20 mM) or different metal ions (50 µM $RhCl_3$, 50 µM $PdCl_2$, 50 µM RbCl, 50 µM $ZnCl_2$, 50 µM $MnCl_2$ or 25 µM $CoCl_2$) were added to the basal cultures of *D. anilini* from individual sterile stock solutions. Cultures were incubated at 30℃ in the dark.

Pure cultures of *T. roseopersicina* were prepared in triplicate by inoculating approximately 0.5 mL of a pre-culture (exponential phase) into 10 mL basal brackish medium in rubber-stopped Hungate tubes with N_2/CO_2 in the headspace. Co-cultures were prepared by inoculation of *D. anilini* to the pure culture of *T. roseopersicina* with additional 1 mM aniline and 5 mM sodium sulfate. Pure cultures of *T. roseopersicina* and co-cultures were incubated at 30℃ under constant illumination at a distance of 300 mm from a 25 Watt tungsten bulb. Growth in the test tubes was monitored at 600 nm by using a tube spectrophotometer (Camspec M107 visible spectrophotometer, Leeds, LS25 1DX, UK).

The co-cultivation device for allowing the exchange of H_2S between the pure cultures of *D. anilini* and *T. roseopersicina* was made from regular lab bottles with a GL45 opening and a glass outlet at the bottom and were purchased from Ochs Glasgerätebau (Bovenden-Lenglern, Germany). Butyl rubber stoppers for GL45 openings were from the same source. The glass outlets of the bottles were connected with butyl-rubber tubing (isoversinic) and an outlet valve to allow for pressure compensation during filling the bottles with media. A glass tube containing cotton wool as a sterile filter was added to the tubing connection. Rubber-stoppered 1 L bottles were flushed with a mixture of N_2/CO_2 (80%/20%) and autoclaved. Each bottle in this device was filled with 500 mL medium by syringes.

3.7.3. Preparation of cell-free extracts

Cells grown with aniline were harvested in the late exponential growth phase at an optical density of 0.2 at 600 nm (OD_{600}). All experiments with cell-free extracts were done under strictly anoxic conditions inside an anoxic glove box (Coy Laboratory Products, Michigan, USA). Cells were centrifuged at 20,300 x g at 4°C for 30 min (Dupont Sorvall, Midland, Canada). The pellet was washed at least twice with 50 mM potassium phosphate (KPP) buffer, pH 7.5, supplemented with 3 mM dithiothreitol (DTT) as reducing agent. Cell-free extracts were prepared in the same KPP buffer, pH 7.5, with 3 mM DTT and 0.5 mg DNase I/mL, by passaging the cells through a French pressure cell (SLM Aminco, Cat. No. FA003, Urbana, Illinois, USA) three times at a pressure of 137 MPa, followed by centrifugation (30 min, 30,300 x g, 4°C, Optima^{TM} TL Ultracentrifuge, Beckman Coulter, Brea, California, USA) to remove cell debris.

3.7.4. Co-substrate assay

Aniline degradation activity was measured in a 2 mL assay mixture containing KPP buffer (3 mM DTT, pH 7.5), 0.5-0.6 mg protein, 0.5 mM aniline, 2 mM ATP, 2 mM $MgCl_2$, and 30 mM $NaHCO_3$ at 30°C. Control assays did not contain ATP. The gas phase was N_2. Samples (200 µL) were taken at different time intervals during 3 hours. 200 µL acetonitrile was added to the samples, followed by centrifugation at 11,700 x g for 10 min to stop the enzyme reaction and remove proteins. The supernatant was used for determination of aniline concentrations by HPLC (see analytical methods).

3.7.5. The effect of sulfide on enzyme activity

For detecting the effect of sulfide on enzymatic activity of aniline-carboxylating enzyme system, 2 mM or 5 mM sodium sulfide from a 500 mM sodium sulfide stock solution were added to the above-mentioned standard enzyme assay mixture. To detect the effect of sulfide on enzymatic activity of phenylphosphate synthase for activating phenol in cell free extracts of phenol-grown cells, 2 mM or 5 mM sodium sulfide from a 500 mM sodium sulfide stock solution was added to the standard phenylphosphate synthase assay mixture as described previously [103]. Samples (200 µL) were taken at different time intervals during several hours. 200 µL acetonitrile was added to the samples, followed by centrifugation at 11,700 x g for 5 min to stop the enzyme reaction. The supernatant was used for determination of aniline or phenol concentrations by HPLC (see analytical methods).

3.7.6. Analytical methods

Samples were taken periodically using sterile syringes and needles. For the determination of growth and of concentrations of aniline, sulfate, and sulfide in the co-cultivation device, three samples were taken at the same time: one sample was used immediately to measure the optical density at 600 nm by using a cuvette spectrophotometer (6300 visible spectrophotometer, Jenway, Staffordshire, UK). The second sample was centrifuged at 11,700 x g for 10 min and the supernatant was used for aniline and sulfate measurement. The third sample was used immediately to measure sulfide concentrations (see below).

Concentrations of aniline, phenol or 4-aminobenzoate were measured with a Shimadzu UPLC (Shimadzu, Kyoto, Japan) system equipped with a UV-visible diode array detector and a reversed-phase 4 µm Max-RP 80Å LC column (250*4.6 mm, Synergi, Phenomenex, Torrance, California, USA). Eluents were prepared with filtered ultrapure water with 0.1 % H_3PO_4 (buffer B), and acetonitrile with 0.1 % H_3PO_4 (buffer A). A gradient of buffer B decreasing from 70% to 50% was used at a flow rate of 1 mL min^{-1}. Samples of 50 µL each were injected into the column. Peaks were identified by comparison of retention times and UV spectra to standard samples. Sulfate was analyzed with the barium chloride method [115], and sulfide in the liquid phase was quantified photometrically by the methylene blue method [116]. For higher sulfide concentrations, samples were diluted by adding 0.1 M NaOH.

3.7.7. Chemicals

All standard chemicals were of analytical quality and were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) or Sigma (St. Louis, USA). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

3.8. Acknowledgements

X. X. is grateful to the China Scholarship Council (CSC) for providing a PhD scholarship. The authors thank Bernhard Schink for critically reading the manuscript, and Antje Wiese for preparing growth media.

3.9. Supplementary information

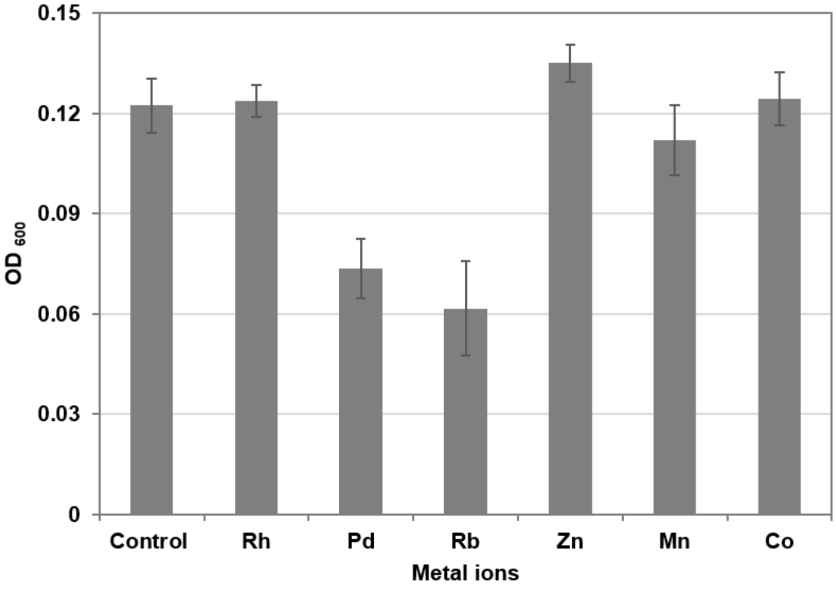

Fig. S1. Effect of the individual addition of metal ions (Rh, Pd, Rb, Zn, Mn or Co) on the growth of *D. anilini*. Values are means ± standard deviation (n = 3). Control is the regular culture without further additions (see method section).

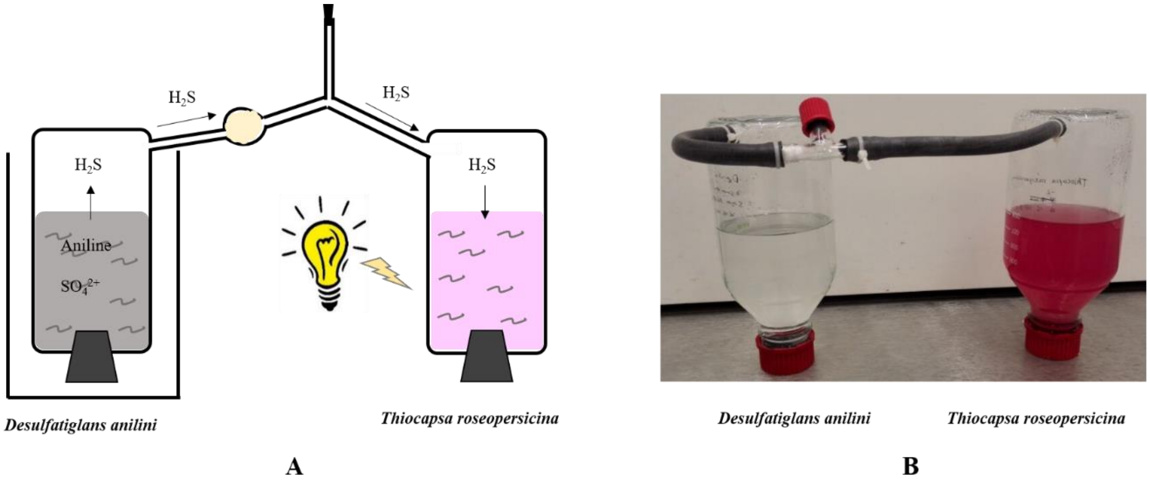

Fig. S2. A: Sketch of the co-culture device. B: Co-culture setup with *D. anilini* and *T. roseopersicina*. The two bottles were connected via oxygen-tight butyl rubber tubing and were incubated under constant illumination at a distance of 300 mm from a 25 Watt tungsten bulb. During cultivation, the bottle with the *D. anilini* culture was shielded from light by covering with aluminum foil.

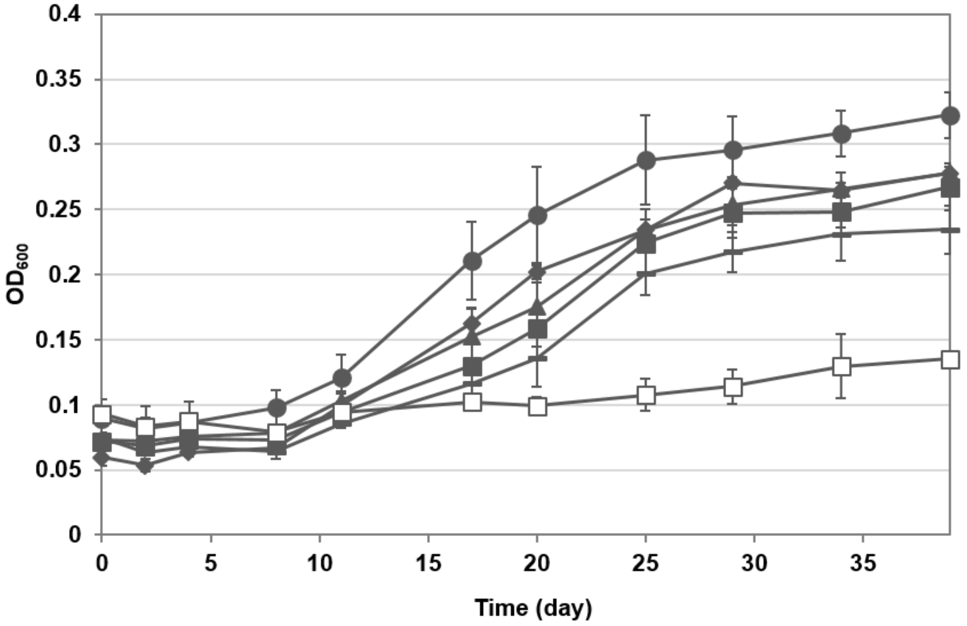

Fig. S3. Effect of sodium sulfide on the growth of *D. anilini* with phenol (2 mM) and sulfate (8 mM). Sodium sulfide (in addition to the sulfide present in the medium as a reducing agent) was added to the medium before inoculation as follows: none (solid circle); 2 mM sodium sulfide (solid rhombus); 5 mM sodium sulfide (solid triangle); 10 mM sodium sulfide (solid square); 15 mM sodium sulfide (short line); 20 mM sodium sulfide (empty square).

CHAPTER 4

Elucidation of the aniline degradation pathway and enzymes involved in the sulfate-reducing bacterium *Desulfatiglans anilini*

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To be submitted

4.1. Abstract

The pathway of anaerobic aniline degradation was studied in the sulfate-reducing bacterium *Desulfatiglans anilini*. Aniline-induced gene clusters were identified by comparison of the proteome of *D. anilini* grown with different substrates (aniline, 4-aminobenzoate, phenol and benzoate). The genes of the aniline-induced gene clusters were highly induced only in aniline- or 4-aminobenzoate-grown *D. anilini* but not in phenol- or benzoate-grown cells. The transcription of this gene cluster did not show any differences in aniline- or benzoate-grown cells. Four candidate genes (H567DRAFT_03868, H567DRAFT_03871, H567DRAFT_03872 and H567DRAFT_02059) were cloned and overexpressed in *E. coli*. The refolded recombinant proteins H567DRAFT_03872 and H567DRAFT_02059 converted phenylphosphoamidate to 4-aminobenzoate with Mn^{2+}, K^+ and FMN as cofactors. A combined reaction with the proteins H567DRAFT_03868, H567DRAFT_03871, H567DRAFT_03872 and H567DRAFT_02059 converts aniline to 4-aminobenzoate via the intermediate phenylphosphoamidate. It is concluded that activation of aniline for degradation in *D. anilini* requiresphosphorylation of aniline with ATP and that phenylphosphoamidate is an intermediate generated by this phosphorylation.

4.2. Introduction

Aniline can be degraded by some aerobic bacteria to the central intermediate catechol with oxygenase in an oxygen-dependent reaction. Three enzymes are involved in the conversion of aniline to catechol, a glutamine synthetase-like enzyme, an aniline dioxygenase and a glutamine aminotransferase [50]. The function of the key enzymes required in the pathway of aerobic aniline degradation were characterized by cloning and purifying these enzymes in *E.coli* [51]. Catechol is further degraded via *meta-cleavage* or *ortho-cleavage* depending on the type of bacterium [54-59].

Anaerobic bacteria use completely different strategies to degrade aniline because of the absence of O_2 in anaerobic systems. So far only few bacteria were reported to be able to degrade aniline under anaerobic conditions. *Desulfatiglans anilini* is a Gram-negative, sulfate-reducing bacterium capable of utilizing aniline as carbon and electron source under strictly anaerobic conditions [31], while on the other hand strain HY99, a close relative to *Delftia acidovorans* was found to be able to metabolize aniline under aerobic or anaerobic conditions linked to nitrate reduction [73]. For most aromatic compounds, different degradation strategies are used by different types of bacteria, depending on the electron acceptor available [35]. For instance, 4-aminobenzoate was identified as an intermediate in aniline metabolism in strain HY99 during nitrate reduction [73]. With sulfate as electron acceptor, aniline was proposed to be carboxylated to 4-aminobenzoate too, followed by activation of 4-aminobenzoate to 4-aminobenzoyl-CoA which is then reductively deaminated to benzoyl-CoA. Activities of 4-aminobenzoyl-CoA synthase and benzoyl-CoA synthase could be measured in cell-free extracts of *D. anilini*, yet the mechanism of carboxylation of aniline is poorly understood [74]. Hints of how carboxylation of aniline could occur come from the degradation mechanism of phenol.

Phenol is activated by phenylphosphate synthase and phenylphosphate carboxylase regardless of the terminal electron acceptor (nitrate-, iron- or sulfate-reducing bacteria) [36, 66, 69, 103], indicating that the pathway of anaerobic phenol degradation in nitrate-, iron- or sulfate-reducing bacteria are analogous, nevertheless, different catalytic mechanisms of phenylphosphate carboxylase were adopted by energy-limited bacteria (iron- and sulfate-reducing bacteria) because of the lack of the gene coding for phenylphosphate carboxylase subunit γ in the genome of *Geobacter metallireducens* GS-15 or *D. anilini* [69, 103]. In any of the above-mentioned cases, phenylphosphate is an intermediate of phenol degradation and is generated by phosphorylation of phenol with ATP. During this reaction in *D. anilini*, a phosphate residue is covalently linked to the OH-group of phenol which requires hydrolysis of ATP to AMP and inorganic phosphate and thus two energy-rich phosphodiester [16]. We postulate an analogous mechanism of aniline activation which would generate phenylphosphoamidate as an intermediate.

In the present study, the catalytic enzymes that were specifically induced by aniline in *D. anilini* cells were identified by total proteome analysis. We then cloned, heterologously expressed, purified and characterized these enzymes in *E. coli* to elucidate the functions of these enzymes in aniline degradation by *D. anilini*. Phenylphosphoamidate was then synthesized as a reference substance and substrate for in-vitro enzyme assays to prove the postulated mechanism of aniline activation.

4.3. Results

4.3.1. Total proteomics analysis

The total proteomes of *D. anilini* grown on four different substrates (phenol, benzoate, aniline and 4-aminobenzoate) were compared semi-quantitatively. The abundance of the proteins encoded by gene cluster A (locus tag from H567DRAFT_03866 to H567DRAFT_03876) and gene cluster B (locus tag from H567DRAFT_03202 to H567DRAFT_03204) were highly induced in aniline- and 4-aminobenzoate grown cells, but not in phenol- or benzoate –grown cells (Fig. 1). There is no obvious difference for all the proteins in cell-free extracts of aniline-grown cells, compared to 4-aminobenzoate-grown cells. For gene cluster A (locus tags from H567DRAFT_03866 to H567DRAFT_03876), the organization is shown in Fig. S2. Among the enzymes encoded by gene cluster A, pyruvate water dikinase (H567DRAFT_03868) and phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) displayed high identities to phenylphosphate synthase subunit β (41%) and phenylphosphate synthase subunit α (28%) in the nitrate-reducing strain *Thauera aromatica* individually, which are suspected putative enzymes for activating aniline to phenylphosphoamidate in *D. anilini*. Phenylphosphate carboxylase beta subunit (H567DRAFT_03872) is a putative enzyme for carboxylating phenylphosphoamidate to 4-aminobenzoate. The other enzymes in gene cluster A and gene cluster B are potentially involved in the downstream degradation pathway of 4-aminobenzoate in *D. anilini*.

The abundance of enzymes involved in the phenol degradation pathway in *D. anilini* were also compared in aniline-, 4-aminobenzoate-, benzoate- and phenol-grown cells. According to our previous results, the enzymes encoded in a gene cluster (from H567DRAFT_02049 to H567DRAFT_02059) are highly induced in phenol-grown cells, but not in benzoate-grown cells [103]. Fig. S3 shows that the enzymes in this gene cluster were expressed at a low level in aniline and 4-aminobenzoate-grown cells except for H567DRAFT_02059, indicating that *D. anilini* uses different gene clusters to degrade phenol and aniline (or 4-aminobenzoate). Hence the genes coding for pyruvate water dikinase (H567DRAFT_03868), phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) and phenylphosphate carboxylase beta subunit (H567DRAFT_03872 and H567DRAFT_02059) were cloned and overexpressed in *E. coli* in the following experiments.

4.3.2. Reverse-transcription PCR

The transcription of genes (from H567DRAFT_03866 to H567DRAFT_03872) were studied by reverse-transcription PCR with mRNA extracted from aniline- or benzoate-grown cells. The primers for the genes from H567DRAFT_03866 to H567DRAFT_03872 were tested by using genomic DNA of *D. anilini* as template. Figure 2A shows that all expected bands of these genes were amplified by the respective primers. Transcription of all genes (from H567DRAFT_03866 to H567DRAFT_03872) was induced in both aniline- and benzoate-grown cells (Fig. 2B). No significant differences were obtained at the transcriptional level in aniline-grown cells compared to benzoate-grown cells.

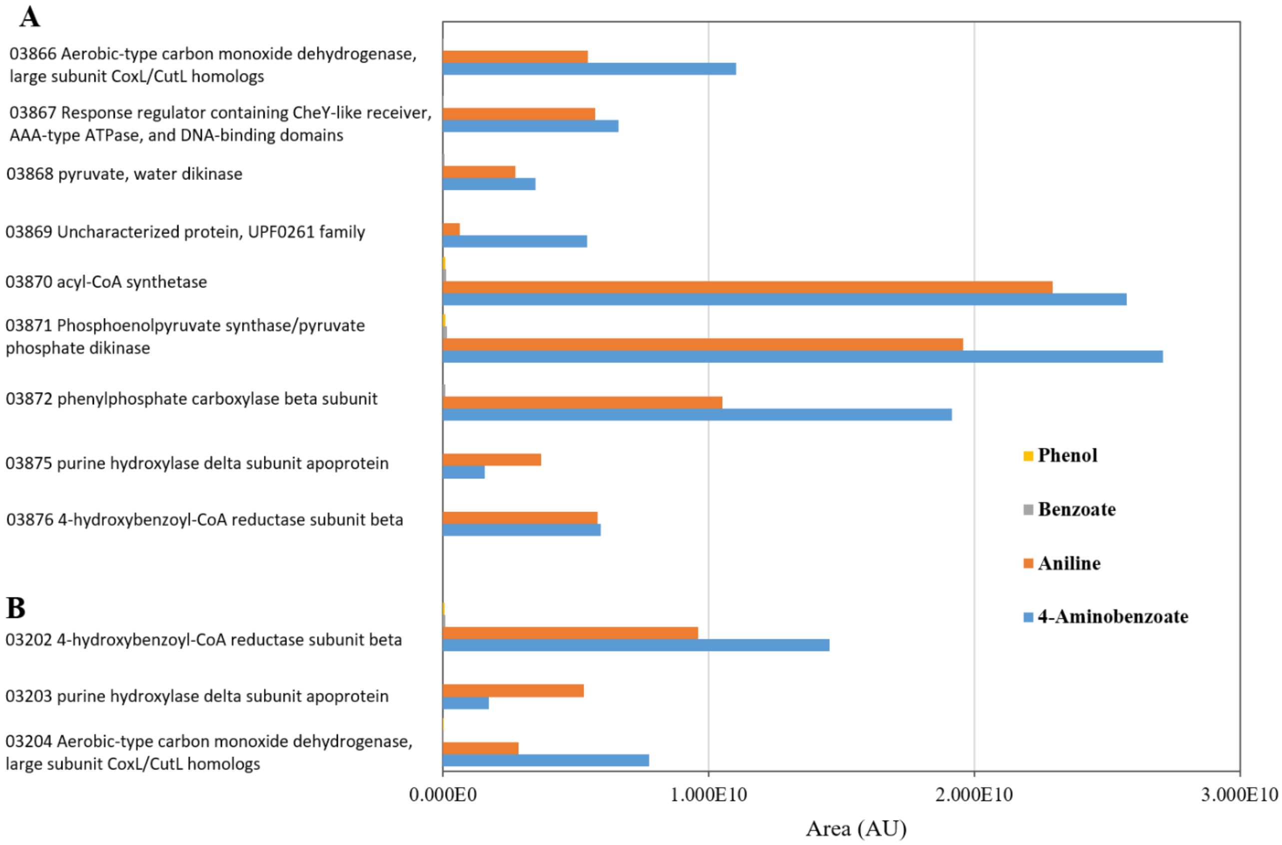


Figure 1. Total proteome analysis of cell-free extracts of cells of *D. anilini* grown on phenol (yellow bars), benzoate (grey bars), aniline (orange bars) and 4-aminobenzoate (blue bars). **(A)** HPLC area of the enzymes encoded by gene cluster A (locus tag 03866-03876). **(B)** HPLC area of the enzymes encoded by gene cluster B (locus tag 03202-03204).

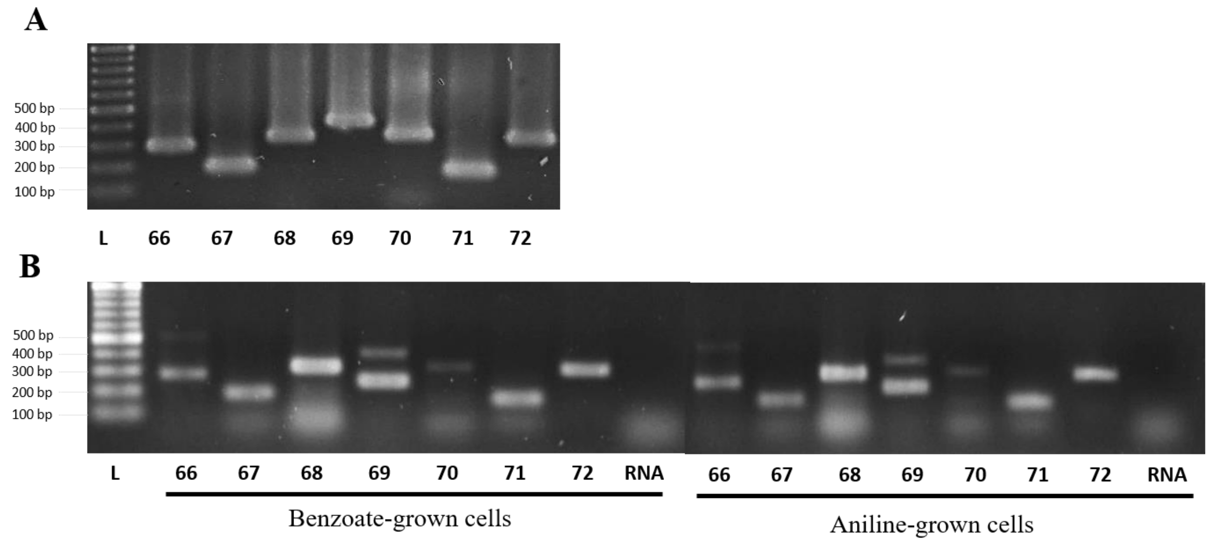


Figure 2. Reverse transcriptase PCR of genes in gene cluster A (locus tag from H567DRAFT_03866 to H567DRAFT_03872) in *D. anilini*. (A) Reverse transcriptase PCR products using genomic DNA of *D. anilini* as template. (B) Reverse transcriptase products using the cDNA from benzoate-grown cells and aniline-grown cells. L: Marker. 66: gene with locus tag 03866. 67: gene with locus tag 03867. 68: gene with locus tag 03868. 69: gene with locus tag 03869. 70: gene with locus tag 03870. 71: gene with locus tag 03871. 72: gene with locus tag 03872. RNA: Reverse transcriptase PCR products using RNA of *D. anilini* as template.

4.3.3. Heterologous overexpression and refolding of proteins 03871, 03868, 03872 and 02059

The overexpression of proteins 03871, 03868, 03872 and 02059 was performed in *E. coli* Rosetta 2 DE3 cells. The heterologously overexpressed proteins were not soluble when cells were grown in LB medium aerobically at 37℃ and induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Figure S4). Anoxic medium (see materials and methods) and addition of ethanol were tried to get soluble heterologous overexpressed proteins, but unfortunately, no soluble desired proteins were obtained and all the overexpressed proteins were present in inclusion bodies. Proteins in inclusion bodies were therefore solubilized with Triton X-100 and urea and refolded (see methods section). Fig. 3 shows the refolded proteins 03868, 03871, 03872 and 02059 in SDS-PAGE gel with a molecular mass of approximately 39 KDa, 63 KDa, 53 kDa and 52 KDa.

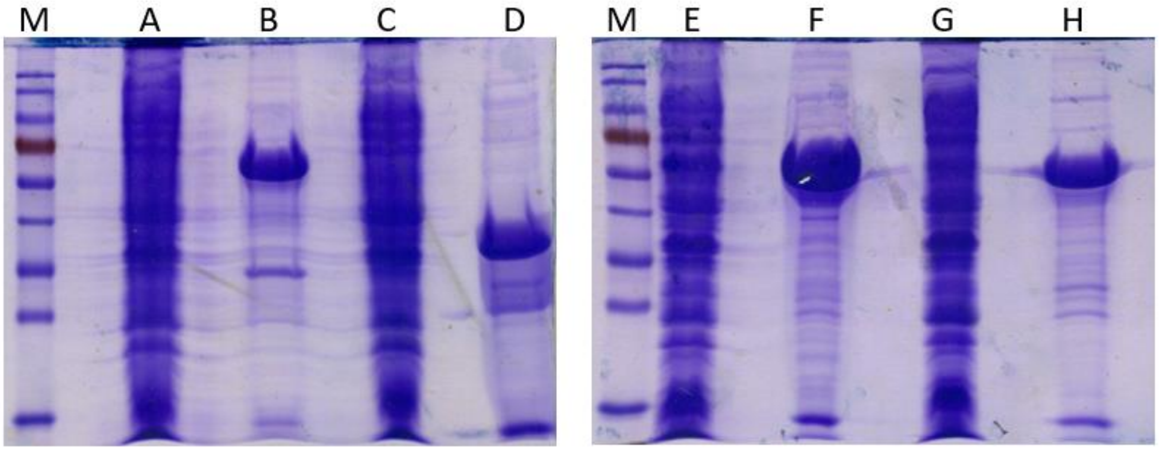


Figure 3. SDS-PAGE gels of total proteins in *E. coli* and refolded proteins. M: Molecular mass-marker. A: Cell-free extracts of *E. coli* cells containing vector pXX5. B: Refolded protein 03871. C: Cell-free extracts of *E. coli* cells containing vector pXX6. D: refolded protein 03868. E: Cell-free extracts of *E. coli* cells containing vector pFL 1. F: Refolded protein 03872. G: Cell-free extracts of *E. coli* cells containing vector pSS 1. H: refolded protein 02059.

4.3.4. Enzyme activity of proteins 03868 and 03871

The proteins encoded by H567DRAFT_03868 and H567DRAFT_03871 were predicted to be pyruvate water dikinase and pyruvate phosphate dikinase, indicating that the proposed function of these two proteins in aniline metabolism in *D. anilini* are to add a phosphate group to the amino group of aniline to form phenylphosphoamidate, which is analogous to the phosphorylation of phenol to phenylphosphate by phenylphosphate synthase. The enzyme activities of proteins 03868 and 03871 were tested by measuring the conversion of aniline to phenylphosphoamidate by HPLC and LC-MS. Unfortunately, no enzyme activity could be obtained (data not shown).

4.3.5. Enzyme activity of proteins 03872 and 02059

The proteins encoded by H567DRAFT_03872 and H567DRAFT_02059 were predicted to be phenylphosphate carboxylase beta subunits, indicating a potential carboxylation of phenylphosphoamidate to 4-aminobenzoate in *D. anilini* which is analogous to the carboxylation of phenylphosphate to 4-aminobenzoate by phenylphosphate carboxylase during phenol degradation. Figure 4A and 4B show the consumption of phenylphosphoamidate and the formation of 4-aminobenzoate in the presence of proteins 03872 and 02059, phenylphosphoamidate, $NaHCO_3$, FMN, and the co-factors Mn^{2+} and K^+ in the assay system. No activity could be measured in the absence of the co-substrate $NaHCO_3$. Carbon monoxide (CO) was also tested as a co-substrate in the enzyme assay system, but no enzyme activity could be detected (data not shown). The enzyme activity is dependent on Mn^{2+} and K^{+}-ions.

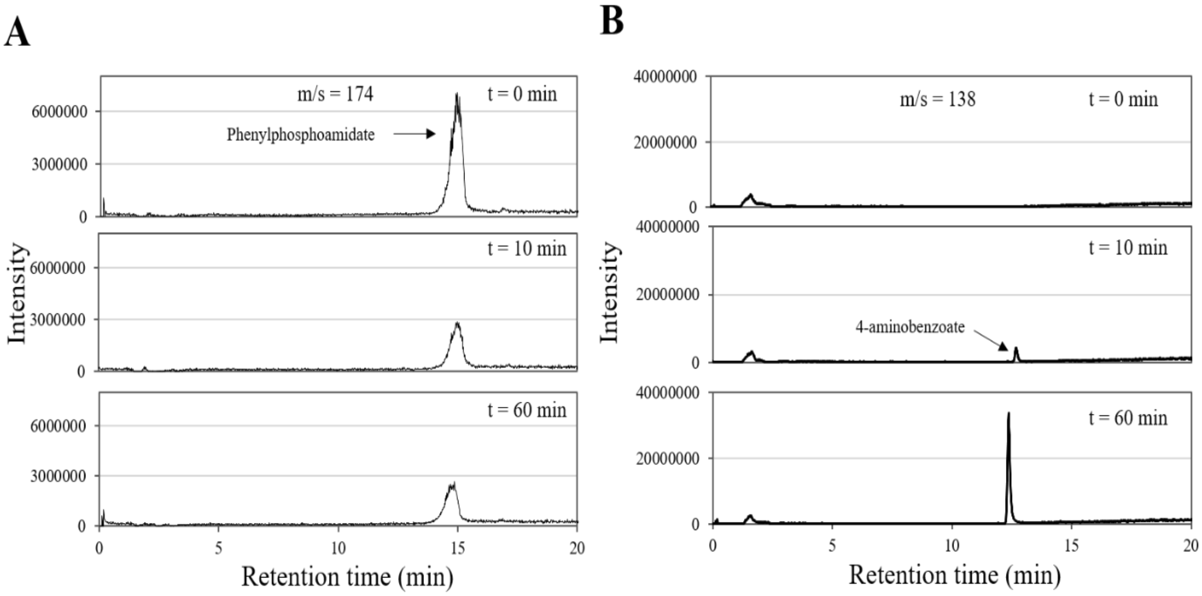


Figure 4. Time course of enzymatic phenylphosphoamidate consumption (A) and 4-aminobenzoate formation (B) discontinuously assayed by LC-MS with recombinant putative phenylphosphoamidate carboxylase.

4.3.6. Enzyme activity by combining proteins 03871, 03868, 03872 and 02059

Since no enzyme activity was detected for phosphorylation of aniline in the enzyme assays with proteins 03868 and 03871, a combined assay reaction (aniline phosphorylation and phenyplhosphoamidate carboxylation) with proteins 03871, 03868, 03872 and 02059 was performed with aniline and $NaHCO_3$ as substrates and ATP, Mg^{2+}, Mn^{2+}, K^{+} and FMN as co-factors. The formation of 4-aminobenzoate (Figure 5B) concomitant with degradation of aniline (Figure 5A) was observed by LC-MS after 30 min after starting the reaction. $NaHCO_3$ could not be replaced by CO. No intermediate accumulation of phenylphosphoamidate was observed, neither by HPLC by LC-MS measurement.

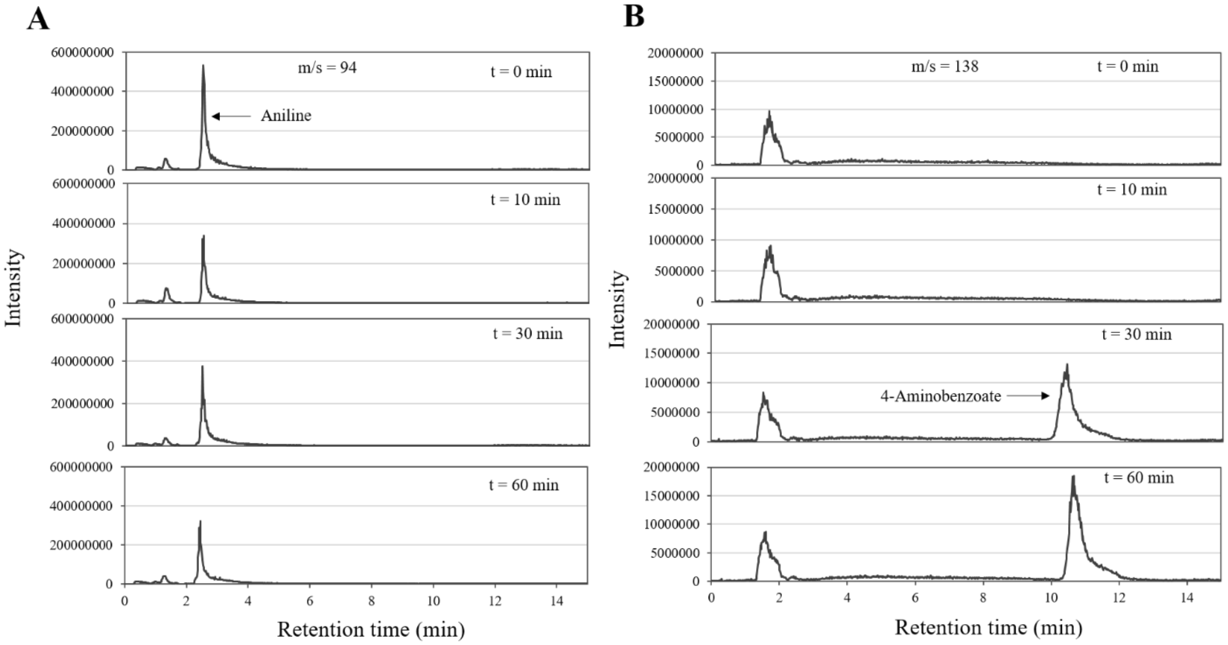


Figure 5. Time course of enzymatic aniline consumption (A) and 4-aminobenzoate formation (B) discontinuously assayed by LC-MS with heterologously expressed putative phenylphosphoamidate synthase and phenylphosphoamidate carboxylase.

4.4. Discussion

In this study, we investigated the degradation pathway of aniline in the sulfate-reducing strain *D. anilini* and the enzymes involved in the initial activation steps of the pathway. The proteome of *D. anilini* was analyzed in aniline-, 4-aminobenzoate-, phenol- and benzoate-grown cells, and two aniline- (or 4-aminobenzoate-) induced gene clusters (gene cluster A and gene cluster B) were identified which are different from the phenol-induced gene cluster [103] but contain similar enzymes such as pyruvate water dikinase (H567DRAFT_03868), phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) and phenylphosphate carboxylase beta subunit (H567DRAFT_03872). The proteome of aniline-grown cells is basically the same as the proteome of 4-aminobenzoate-grown cells, which suggests that the genes in gene cluster A are co-regulated as not all of the genes in gene cluster A are involved in 4-aminobenzoate degradation in *D. anilini*. In addition, 4-aminobenzoate might be an intermediate in the aniline degradation pathway in *D. anilini*. The transcription level of gene cluster A as determined by RT-PCR showed no difference in aniline- or benzoate-grown cells, but the translation of some genes in gene cluster

A were not found in benzoate-grown cells, suggesting the presence of posttranscriptional regulatory elements.

According to the proteome of aniline-grown *D. anilini* (Figure 1) and annotations of aniline-induced proteins from IMG, pyruvate water dikinase (H567DRAFT_03868) and phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) are putative enzymes to activate aniline to phenylphosphoamidate. Phenylphosphate carboxylase beta subunit (H567DRAFT_03872) and phenylphosphate carboxylase beta subunit (H56DRAFT_02059) are putative enzymes to carboxylate phenylphosphoamidate. These four enzymes were successfully cloned and overexpressed in *E. coli*. The target proteins accumulated in inclusion bodies and were solubilized and refolded correctly as judged by enzyme activity measurements. The carboxylation activity of the enzymes phenylphosphate carboxylase beta subunit (H567DRAFT_03872) and phenylphosphate carboxylase beta subunit (H56DRAFT_02059) were detected by measuring the conversion of $NaHCO_3$-dependent conversion of phenylphosphoamidate to 4-aminobenzoate. The phosphorylation activity of the enzymes pyruvate water dikinase (H567DRAFT_03868) and phosphoenolpyruvate synthase/pyruvate phosphate dikinase (H567DRAFT_03871) could not be measured by directly detecting the formation of the intermediate phenylphosphoamidate. The possible reason is the instability of phenylphosphoamidate in aqueous solution (Figure S1), and phenylphosphoamidate possibly does not accumulate to detectable concentrations as the equilibrium of the reaction lies on the side of aniline and ATP. Indirect evidence for involvement of phenylphosphoamidate as an intermediate that comes from enzyme assays combining the phosphorylation reaction with the carboxylation reaction by adding all four overexpressed enzymes to the assay mixture. The fact that activity of the phosphorylating enzymes was only detectable only in the latter combined assay system suggests, that the concentration of phenylphosphoamidate has to be kept low by the subsequent carboxylation reaction to shift the reaction equilibrium in favor of 4-aminobenzoate formation.

From our results, the initial activation reactions for aniline degradation in the sulfate-reducing bacterium *D. anilini* are proposed. First,aniline is phosphorylated to phenylphosphoamidate by a phenylphosphoamidate synthase, phenylphosphoamidate is then carboxylated to 4-aminobenzoate by a phenylphosphoamidate carboxylase (Figure 6). Phenylphosphoamidate synthase is similar to phenylphosphate synthase, containing two subunits of pyruvate water dikinase and phosphoenolpyruvate synthase. The catalytic mechanism might be similar to phosphoenolpyruvate synthase [117, 118]. A histidine residue interacts with the β-phosphoryl group of ATP to form a phosphoenzyme intermediate, which proceeds by transferring the additional phosphate unit to the nitrogen atom of aniline to form phenylphosphoamidate through a P-N bond. Compared to phenylphosphate synthase in *Thauera aromatica*, the lack of the γ-subunit in *D. anilini* may explain the lower turnover rate of aniline in *D. anilini* [66]. Phenylphosphoamidate carboxylase contains two subunits showing high similarity with phenylphosphate carboxylase β subunit, which belongs to the family of UbiD-like proteins and requires Mn^{2+} as a cofactor [119]. Further research is needed to study the catalytic mechanism of phenylphosphoamidate carboxylase with two UbiD-like subunits. We hypothesize that the addition of the electrophilic phosphate group to the amino substituent of aniline shifts the electron density of the aromatic ring such that CO_2 can be substituted in para-position. In the subsequent reactions, 4-aminobenzoate is transformed to the central intermediate benzoyl-CoA via 4-aminobenzoyl-CoA. The enzymes involved are 4-aminobenzoyl-CoA ligase and 4-aminobenzoyl-CoA reductase. 4-aminobenzoyl-CoA ligase and 4-aminobenzoyl-CoA reductase are likely encoded by genes with locus tags H567DRAFT_03870 and H567DRAFT_03876 respectively, which are highly induced by aniline or 4-aminobenzoate, and the encoding genes are also located in the aniline- or 4-aminobenzoate-induced gene cluster.

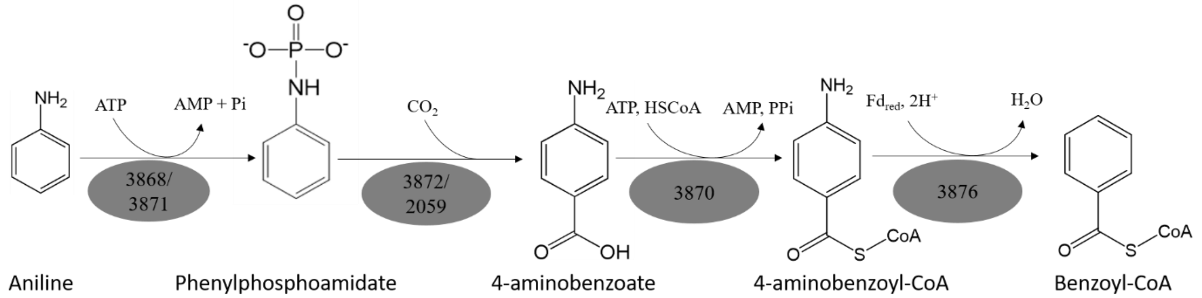


Figure 6. Proposed pathway of anaerobic aniline degradation by *D. anilini*. Numbers in grey ellipses indicate the locus tags of the enzymes.

Based on the results obtained in this study, the aniline degradation pathway in the sulfate-reducing bacterium *D. anilini* and the enzymes involved are proposed in figure 6. Aniline degradation in *D. anilini* occurs analogously to phenol degradation in the nitrate-reducing *T. aromatica*, yet the sulfate-reducing *D. anilini* may use a different catalytic mechanism due to the small energy budget of a sulfate-reducer [35]. For example, the histidine residue of phenylphosphoamidate synthase could interact with the γ-phosphoryl group of ATP instead of the β-phosphoryl group to form phenylphosphoamidate and ADP instead of AMP thus saving one energy-rich phosphodiester bond. ADP could then be reused as a phosphoryl donor for activating another molecule of aniline to phenylphosphoamidate. This hypothetical mechanism is energetically more favorable for the overall degradation pathway but endergonic from a thermodynamic viewpoint. This would also explain, why intermediary phenylphosphoamidate accumulation was never observed in enzyme assays, as the reaction needs to be driven by a low concentration of phenylphosphoamidate supplied by the subsequent carboxylation reaction.

4.5. Materials and Methods

4.5.1. Bacterial growth conditions

Desulfatiglans anilini was grown in anoxic bicarbonate-buffered (30 mM) and sulfide-reduced (2 mM) brackish water medium as described before [103]. Aniline, 4-aminobenzoate, benzoate or phenol were added at a concentration of 1 mM respectively as sole source of carbon and energy. Sodium sulfate was added as electron acceptor at a concentration of 5 mM. The cultures were incubated at 30°C in anoxic cultivation vessels with butyl-rubber stoppers. For cloning, *Escherichia coli* strains NovaBlue and Rosetta™ 2(DE3) were grown aerobically in LB medium ((10 g l^{-1} peptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) at 37°C under shaking conditions (200 rpm/min) with individual antibiotics (see plasmid construction and overexpression). For overexpression, *Escherichia coli* strains Rosetta™ 2 (DE3) was grown in anoxic medium, which was prepared from freshwater medium [120]. Instead of adding sodium sulfide, 2 mM cysteine was added as reducing agent. Further, the anoxic medium for growing *Escherichia coli* strains Rosetta™ 2 (DE3) was amended with yeast extract (0.1 w/v), glucose (0.4% w/v), $NaNO_3$ (50 mM), and the respective antibiotics.

4.5.2. RT-PCR

For extracting RNA of *D. anilini*, 20 mL of aniline- or benzoate-grown cells in log-phase were collected by centrifuging at 11,700 x g for 20 minutes. RNA was extracted and cleaned according to the method described before [103]. PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad, Hercules, California, USA). The standard PCR mixture had a volume of 25 µl and contained 2.5 µl of 10 × PCR buffer, 5 nmol dNTPs, 50 pmol of each primer (Microsynth, Balgach, Switzerland), 4 nmol $MgCl_2$, 0.2 µl Tag-polymerase (5 U/µl, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 2 µl cDNA or 10 to 50 ng genomic DNA as template. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 31 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. Primer pairs used to amplify DNA or cDNA fragments of genes are listed in Table 1. The quality of PCR products was analyzed by electrophoresis in a 1.0 % agarose gel at 110 V for 30 minutes and staining with ethidium bromide with a concentration of 0.5 µg/ml for 30 min. The gel was exposed to UV light and the picture of the gel was taken with a gel documentation system (Gel Doc™ XR+ Gel Documentation System, BIO-RAD, California, USA).

4.5.3. Plasmid construction

All primers used for cloning are listed in table 1. Genomic DNA of *D. anilini* was isolated from 10 ml of a dense culture (OD_{600} = 0.16) using the Gentra Puregene Cell Kit (Qiagen) by following the manufacturer's protocol. The gene fragments of 3871, 3868, 3872 and 2059 were amplified by PCR, using the primer-pairs 3871-F & 3871-R, 3868-F &3868-R, 3872-F & 3872-R, 2059-F & 2059-R and genomic DNA of *D. anilini* as template. The PCR mixture had a volume of 50 µl and contained 10 µl Phusion High Fidelity Polymerase buffer (New England Biolabs GmbH, Frankfurt am Main, Germany), 5 nmol dNTPs, 50 pmol of each primers, 10 to 50 ng genomic DNA and 0.5 µl Phusion High Fidelity Polymerase (2 U/ µl). PCR program was the same with that described in the RT-PCR section. Primers were ordered from Microsynth (Balgach, Switzerland). The DNA fragment generated by PCR using primers 3871-F and 3871-R was digested with *Sac I* and *Xho I*, and inserted into the same sites of pET 28a, resulting in pXX 5. The DNA fragment generated by PCR using primers 3868-F and 3868-R was digested with *Sal I* and *Nde I*, and inserted into the same sites of pET 28a, resulting in pXX 6. The plasmid pcrscript/Mss I was digested completely with *Mss I* and ligated with the PCR product of 3872 or 2059, resulting in pXX 7 or pXX 8 respectively. Plasmid pXX 7 or pXX 8 were digested with *Nde I* and *Sal I* yielding an approximately 1500 bp fragment, which was inserted into the same sites of pET 28a, resulting in pFL 1 or pSS 1 individually. T4 DNA ligase (5 U/µl, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used in the ligation systems and ligation reactions were performed according to the protocol of T4 DNA ligase.

4.5.4. Plasmid transformation and identification

All ligation products were transformed chemically into *Escherichia coli strains* NovaBlue competent cells to identify and store positive colonies. The chemical transformation protocol is as follows [121]: Put the frozen competent cells of NovaBlue of 50 µl from -80°C on ice for 5 min to allow cells to thaw. Add 10 µl ligation mixture or 1 µl plasmid. Quickly flick the tube several times. Immediately place tubes on ice for 5 minutes. Heat shock the cells for 30 seconds in a water bath at exactly 42°C. Immediately place tubes on ice for 2 minutes. Add 950 µl of room temperature SOC medium and incubate for 50 min at 37°C with shaking at 200 rpm. Centrifuge the cells at 1000 x g for 3 min to get the pellet. Resuspend the cells with 200 µl SOC medium and plate onto antibiotic plates. Place plates in the 37°C incubator and grow overnight 14-18 hours. Positive colonies were identified by doing colony PCR. The plasmids from positive colonies were purified by using the QIAprep Spin mini kit (QIAGEN, Venlo, Netherlands) and sequenced by Microsynth, (Balgach, Switzerland) to confirm the correct plasmid.

Table 1. Primers used in this study

	Primers’ name[a]	Primers’ sequence (5’-3’)[b]	Restriction enzymes
Primers for cloning	3871-F	CGGAGCTCATGCATTACGGAAGAA	*Sac I*
	3871-R	CGGCTCGAGTTAGCTAATTTTATATACACGC	*Xho I*
	3868-F	CCCATATGATGCTTGAGACAAGACCA	*Nde I*
	3868-R	GCCGTCGACCTATTCATTAACTGGAAA	*Sal I*
	3872-F	GCCCATATGATGAATGATCTTCGTTCA	*Nde I*
	3872-R	GGCCGTCGACTTAAAATTTTGGTTCA	*Sal I*
	2059-F	GCCCATATGATGAAAAGCATGAGAGATT	*Nde I*
	2059-R	GCGTCGACTTAGAAACCCAGTTCAGA	*Sal I*
Primers for RT-PCR	3866for	AGAAGGTTTATGTGGCGGGG	
	3866rev	GGGTTGATAGGGAAACCGCA	
	3867for	GCATCAGAGTCCTTCTCGGG	
	3867rev	CCCATGGGTGTGCAAAAAGG	
	3868for	CGTGCCAATTGAAGATGCGT	
	3868rev	TGGATTGGCGGTAAAACCGA	
	3869for	CGGGACTGGAGGGACTCTTA	
	3869rev	CATTGCAGCATCACCTACGC	
	3870for	GGTTCAACCCATTCGTTGGC	
	3870rev	AAAACCGCCTCACCTCTAGC	
	3871for	ATGACGCCTTTGTACTGCGA	
	3871rev	CGCGCCGGAATTTCATCTTT	
	3872for	TTCCCATGCCGAGATTGTCC	
	3872rev	ACTGCCCAAAATCGTCCACT	

[a] F or for, forward primer; R or rev, reverse primer.

[b] Restriction enzyme sites added in the primers were underlined and listed.

Table 2. Plasmids used in this study

Plasmids	Derivation and relevant characteristics[c]	Reference or source
pET 28a	Km^r, pET 28a, carrying an N-terminal His Tag/thrombin/T7 Tag configuration plus an optional C-terminal His Tag sequence, can be used for expression of recombinant proteins in *E. coli*.	Merck Millipore
pcrscript/MssI	Ap^r, pcrscript/MssI is a cloning vector carrying an ampicillin-resistance gene and multiple cloning site (MCS), which is modified to include *Mss I* site in MCS.	Provided by Prof. Dr. Peter Kroth
pXX 5	Km^r, pET 28a derivative containing gene 3871	This study
pXX 6	Km^r, pET 28a derivative containing gene 3868	This study
pXX 7	Ap^r, pcrscript/MssI derivative containing gene 3872	This study
pXX 8	Ap^r, pcrscript/MssI derivative containing gene 2059	This study
pFL 1	Km^r, pET 28a derivative containing gene 3872	This study
pSS 1	Km^r, pET 28a derivative containing gene 2059	This study

[c] Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

4.5.5. Overexpression

E. coli Rosetta 2 (DE3) cells (Chloramphenicol resistance; Merck KGaA, Germany) were used to overexpress the recombinant proteins. Purified DNA plasmids (pXX 5, pXX 6, pSS 1 and pFL 1) were transformed chemically into *E. coli* Rosetta 2 (DE3) cells. Cells were grown in LB medium containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol over night at 37°C under shaking conditions (200 rpm). For aerobic overexpression, overnight cultures of *E. coli* containing the respective plasmid were inoculated into fresh LB medium containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol at an initial OD_{600} of 0.05. When the OD_{600} reached 0.4 - 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a concentration of 0.5 mM and the culture was further incubated at 37°C under shaking conditions (200 rpm) to induce protein expression. Samples were taken aerobically at time intervals (0h, 2h, and 4h) to monitor the overexpression of proteins under oxic conditions. For anoxic overexpression, an overnight culture of *E. coli* containing the respective plasmid was inoculated into anoxic freshwater medium with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol at an initial OD_{600} of 0.05. IPTG (0.2 mM) or 3% (v/v) of ethanol were added to the culture when the OD_{600} reached 0.4 - 0.5 while growing at 37°C under shaking conditions (200 rpm). The anoxic cultures were incubated at 15°C overnight to produce heterologous proteins. Samples were taken anaerobically at different time points to monitor the overexpression of proteins.

4.5.6. Preparation of cell-free extracts

Cells of *Desulfatiglans anilini* were harvested by centrifuging at 8000 rpm for 30 min. Pellets were washed with 50 mM Tris-HCl buffer twice, followed by passing the cells through a French pressure cell (SLM Aminco, Cat. No. FA003, Urbana, Illinois, USA) three times at a pressure of 137 MPa to disrupt the cells. Then, cell debris was removed by centrifugation (30 min, 30,300 x g, 4°C, Optima^{TM} TL Ultracentrifuge, Beckman Coulter, Brea, California, USA). The supernatant was used for total proteome analysis. To obtain purified proteins overexpressed in *E.coli* as active enzymes, all operations were performed under strictly anoxic conditions in an anoxic tent (Coy, Ann Arbor, USA). Cells of *E. coli* were harvested by centrifugation at 7000 rpm for 30 min in a centrifuge (Dupont Sorvall, Midland, Canada). Pellets were washed with 50 mM Tris-HCl buffer twice and finally resuspended in a 5 mL 50 mM Tris-HCl buffer, followed by passing the cells through a French pressure cell (SLM Aminco, Cat. No. FA003, Urbana, Illinois, USA) three times at a pressure of 137 MPa for cell-disruption in anoxic serum vials sealed with butyl-rubber stoppers. After centrifugation to remove cell debris, the supernatant was further used for refolding proteins.

4.5.7. Total proteome analysis and database search

The cell-free extracts containing soluble proteins of *D. anilini* grown with different substrates (aniline, 4-aminobenzoate, benzoate and phenol) were submitted to the Proteomics Core Facility of the University of Konstanz for total proteome analysis. Samples were analyzed by using a LTQ Orbitrap Discovery with an Eksigent 2D-nano HPLC ((Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Mascot search engine [Matrix Science, London, UK] was used to match each peptide fingerprint against the protein database of the IMG annotated genome of *D. anilini*. Relative protein abundances were expressed with the area of each protein measured by the Eksigent 2D-nano HPLC.

4.5.8. Refolding of proteins

The preparation of inclusion bodies and refolding of proteins followed the procedures described by Schmeling et al [66]. Inclusion bodies were obtained from the cell-free extracts (see 4.6.6. Preparation of cell-free extracts) by centrifugation at 5000 x g for 10 min at 4°C. Then the pellets containing inclusion bodies were washed sequentially with two washing buffers, washing buffer 1 (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1% (vol/vol) Triton X-100) and washing buffer 2 (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.5 M urea). The inclusion bodies were solubilized in urea solution (100 mM Tris-Cl, pH 8.5, 8 M urea, 50 mM mercaptoethanol) by stirring for 1 h on ice. The soluble proteins were present in the supernatant after centrifugation at 40,000 g for 30 min. Afterwards, the supernatant was added dropwise into cold refolding solution (15% (vol/vol) glycerol, 50 mM Tris-Cl, pH 8.5, 10 mM mercaptoethanol) while stirring on ice. All operations were performed in an anoxic tent (Coy, Ann Arbor, USA). After these treatments, the final concentration of the refolded proteins was approximately 3 mg/ml. The protein concentration was measured with the Bradford assay using bovine serum albumin as protein standard [90]. The refolded proteins were then used to run one-dimensional denaturing polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the purity of the proteins and were also used for enzyme assays.

4.5.9. Identification of protein expression

Samples of each 1 ml of cultures of *E.coli* for protein overexpression taken during induction with IPTG were treated with two methods. To obtain total proteins, samples were centrifuged at 14,000 rpm for 10 min and pellets were resuspended in 60 µl polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and cells opened by heating at 99°C for 10 min. Total protein was used for SDS-PAGE analysis. To obtain soluble proteins, 1 ml samples of *E.coli* cultures were harvested by centrifugation at 14,000 rpm for 10 min, resuspended in 30 µl 50 mM Tris-HCl buffer with 5 µg/ml lysozyme and incubated at 37°C for 1 hour. Then, non-lysed cells and cell debris was removed by centrifugation (14,000 rpm, 10 min), and 30 µl SDS-PAGE loading buffer was added to the supernatant and used for SDS-PAGE analysis.

SDS-PAGE was performed to analyze overexpression, solubilization and purification of proteins. The denaturing polyacrylamide gel consisted of a 4% stacking gel and a 12% resolving gel [122]. Gels were run at a constant current of 20 mV per gel for 1.5 h in running buffer (24.8 mM Tris, 192 mM Glycin and 3.47 mM SDS). Proteins in gels were stained by incubation in colloidal coomassie staining solution containing 2% H_3PO_4, 6% $(NH_4)_2SO_4$, 20% methanol, and 0.08% (w/v) Coomassie Brilliant Blue R-250 overnight [123] and washed in distilled water. Protein concentrations were estimated with the Bradford assay using bovine serum albumin as protein standard [90].

4.5.10. Enzyme assays

The refolded proteins were used in enzyme assays and all enzyme assays were performed in cuvettes sealed with rubber stoppers and previously flushed with 100% nitrogen at 30°C under strictly anoxic conditions. For the enzyme activity of the gene products 03872 and 02059, the standard enzyme assay mixture contained 50 mM KPP buffer (pH 7.5, 0.5 mM DTT), 2 mM $MnCl_2$, 2 mM KCl, 10% CO or 30 mM $NaHCO_3$, 0.2 mM flavin mononucleotide (FMN), 2 mM phenylphosphoamidate and 0.5 mg of each protein (03872 and 02059). For control assays, no CO or $NaHCO_3$ was added to the enzyme assay system. For the enzyme activity of the gene products 03871 and 03868, the standard enzyme assay mixture contained 50 mM KPP buffer (pH 7.5, 0.5 mM DTT), 2 mM ATP, 2 mM $MnCl_2$, 2 mM KCl, 2 mM $MgCl_2$, 1 mM aniline and 0.5 mg of each protein (03871 and 03868).

To analyze the reaction product, 200 µl samples were withdrawn at time intervals and the reaction was stopped by addition of an equal volume of acetonitrile and centrifuged (11,700 x g for 10 min). The supernatant was transferred to 200 µl HPLC vials and analyzed by HPLC.

4.5.11. HPLC analysis

The concentrations of aniline, phenylphosphoamidate and 4-aminobenzoate were determined with a reversed-phase HPLC (Shimadzu, Kyoto, Japan) system equipped with a UV-visible diode array detector and a 4 um Max-RP 80Å LC column (250*4.6 mm, Synergi) (Phenomenex, Torrance, California, USA) at 25°C. Eluents were prepared by mixing ultrapure water with 0.1% H_3PO_4 (buffer B), and acetonitrile with 0.1% H_3PO_4 (buffer A) and filtration through 0.2 µm. A concentration of 90% buffer B was used at a flow rate of 0.8 ml min^{-1}. Samples of 50 µl were injected into the column. The compounds were identified by comparing the retention times and UV-spectra of peaks to the retention time and UV-spectra of the respective standards.

4.5.12. Liquid chromatography-mass spectrometry

Samples of the enzyme assays were analyzed using an Agilent 1100 HPLC system connected to a Thermofisher Exactive Orbitrap high resolution mass spectrometer fitted with a heated electrospray ion source (HESI). The mass spectrometer was operated in positive ionization mode at 50000 resolution with internal calibration to the lock mass 455.12002. For HPLC separation a Macherey Nagel Nucleodur C18 ec column (150 mm x 2 mm, 3 mm) was used. HPLC program: 2 % B 2 min, in 18 min to 100 % B, 5 min 100 % B, in 1 min to 2 % B, 6 min 2 % B, solvent A: H_2O 0.1 % AcOH, solvent B: MeOH 0.1 % AcOH, flow rate: 0.2 ml/min. 1 ul of the samples was injected.

The retention time of aniline: 2.4 min, 94.06545, 3.4 ppm, $C_6H_8N_2$. The retention time of phenylphosphoamidate: 14.4 min, $[M+H]^+$ measured 174.03172, 1.51 ppm, $C_6H_9O_3NP$. The retention time of 4-aminobenzoate: 10.7 min, $[M+H]^+$ measured 138.05506, 0.76 ppm, $C_7H_8O_2N$.

4.5.13. Synthesis of phenylphosphoamidate

Phenylphosphoamidate was synthesized via the intermediates tribenzyl phosphite, dibenzyl *N*-phenylphosphoramidate and triethylammonium *N*-phenylphosphoramidate.

Tribenzyl phosphite [124] (1): A solution of phosphorus trichloride (7.87 g, 57.3 mmol) in diethylether (300 mL) was prepared under inert nitrogen atmosphere. To the stirred solution was added dropwise a solution of triethylamine (18.20 g, 180 mmol) in diethylether (30 mL), followed by benzyl alcohol (18.7 g, 173.1 mmol) in diethylether (30 mL). The reaction mixture was stirred 30 min at -40°C and 48 h at room temperature. The slurry of triethylammonium chloride was filtered through a sintered glass funnel under strict exclusion of oxygen. After solvent removal, tribenzyl phosphite (1) was isolated as slightly yellow oil in 86% yield (17.3g) which was sufficiently pure for the next reaction step. ^{1}H-NMR (400 MHz, $(CD_3)_2SO$): $\delta = 4.88$ (d, $^3J = 8.1$ Hz, 6H, CH_2), 7.25-7.40 (m, 15H, H_{ar}); ^{31}P-NMR (162 MHz, $(CD_3)_2SO$): $\delta = 135.5$.

Dibenzyl N-phenylphosphoramidate [125] (2): A solution of tribenzyl phosphite (1) (17.27 g, 49.0 mmol) in dichloromethane (80 mL) was prepared under nitrogen atmosphere. To the stirred and cooled (0° C) solution was added dropwise bromine (7.48 g, 46.8 mmol) in dichloromethane (60 mL). The mixture was stirred for 1h at 0 °C, then 25 min at room temperature and finally transferred dropwise via canula to a precooled (-40°C) mixture of aniline (6.63 g, 71.2 mmol) dissolved in 120 mL dichloromethane. Stirring was continued for 2 h at -40° C, then for an additional 12 h at room temperature. After solvent removal the residue was taken up in diethylether (500 mL). The solution was consecutively washed with 2 M HCl (2 x 150 mL, 1 x 100 mL), sodium thiosulfate solution (1 x 150 mL) and finally with brine (1 x 150 mL). The organic layer was dried over $MgSO_4$, evaporated to dryness and purified by chromatography on silica gel with a petrol ether/ethyl acetate gradient-system (PE:EE 3:1 →1:1). Amidate 2 was isolated as off-white solid in 78% yield (13.51 g). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.06, 5.16 (2 qAB, $^2J_{HH}$ = 10.7 Hz, $^3J_{HP}$ = 7.5 Hz, 4H, OCH_2-Ph), 6.93-6.99 (m, 1H_{para}, N-C_6H_5), 7.00-7.05(m, 2H_{ortho}, N-C_6H_5), 7.17-7.25 (m, 2H_{meta}, N-C_6H_5), 7.30 (s, 10H, $OCH_2C_6H_5$); ^{13}C-NMR (101 MHz, CDC_{l3}): δ = 68.35 (d, *J* = 4.6 Hz, CH_2), 117.66 (d, *J* = 7.4 Hz, C2-aniline), 121.77 (C4-aniline), 128.01 (C2-Ph), 128.38 (C4-Ph), 128.47 (C3-Ph), 129.23 (C3-aniline), 135.72 (d, *J* = 7.9 Hz, C1-Ph), 139.43 (C1-aniline); ^{31}P-NMR (162 MHz, CDC_{l3}): δ = –0.17.

Triethylammonium N-phenylphosphoramidate [126, 127] (3): Dibenzyl N-phenylphosphoramidate (2) (510 mg, 1.44 mmol) and Pd catalyst (190 mg, 10% Pd/C) were suspended in ethanol (10 mL) containing triethylamine (0.5 g). The flask was evacuated and backfilled with hydrogen (4 x). After 1 h hydrogenation the flask was evacuated and backfilled with nitrogen, the catalyst was removed by filtration over a bed of celite and the solvent removed under vacuum. 3 was isolated in quantitative yield (393 mg) and of sufficient purity for the *in vitro* enzyme assays. ^{1}H-NMR (400 MHz, CD_3OD): δ = 1.29 (t, *J* = 7.2 Hz, 9H, CH_3), 3.12 (q, *J* = 7.2 Hz, 6H, CH_2), 6.71 – 6.79 (m, 1H, aniline), 7.04 – 7.19 (m, 4H, aniline); ^{13}C-NMR (101 MHz, CD_3OD): δ = 9.04 (CH_3), 47.34 (CH_2), 117.84 (d, *J* = 7.0 Hz, C2-aniline), 119.92 (C1-aniline), 129.65 (C3-aniline), 144.94 (C1-aniline) ; ^{31}P-NMR (162 MHz, CD_3OD): δ = –3.73.

The NMR spectras from phenylphosphoamidate synthesis and precursors are shown in Figure S5. The instability of phenylphosphoamidate in distilled water (pH 7.0) or Tris-HCl buffer (pH 8.0) is shown in Figure S1.

4.5.14. Chemicals

All standard chemicals were of analytical quality and were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) or Sigma rather from their German dependence – Sigma and Fluka are the same company –Sigma Aldrich(St. Louis, USA). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

4.6. Acknowledgement

XX is indebted to the Chinese Scholarship Council (CSC) for providing a scholarship. Viktoria Ebel, Sophia Kraft and Carina Jung are gratefully acknowledged for the preparation of starting materials for phenylphosphoamidate. Sandeep Shrestha and Feng Liu are acknowledged for assisting with the cloning experiments.

4.7. Supplementary information:

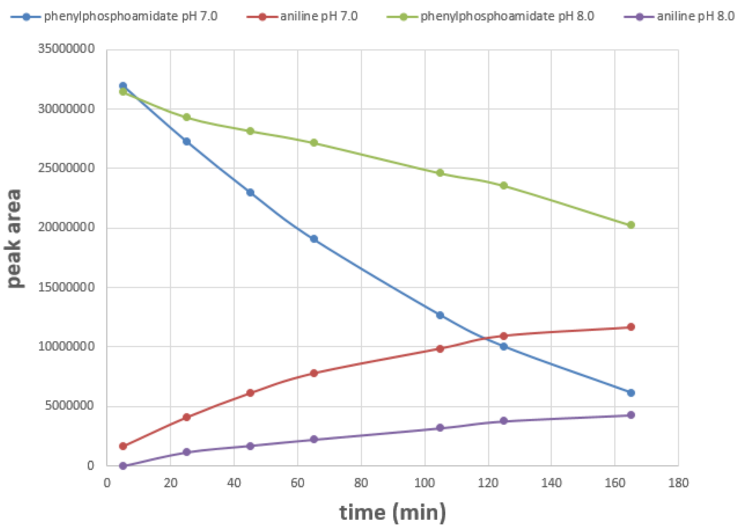


Figure S1. The conversion of phenylphosphoamidate to aniline over time at different pH.

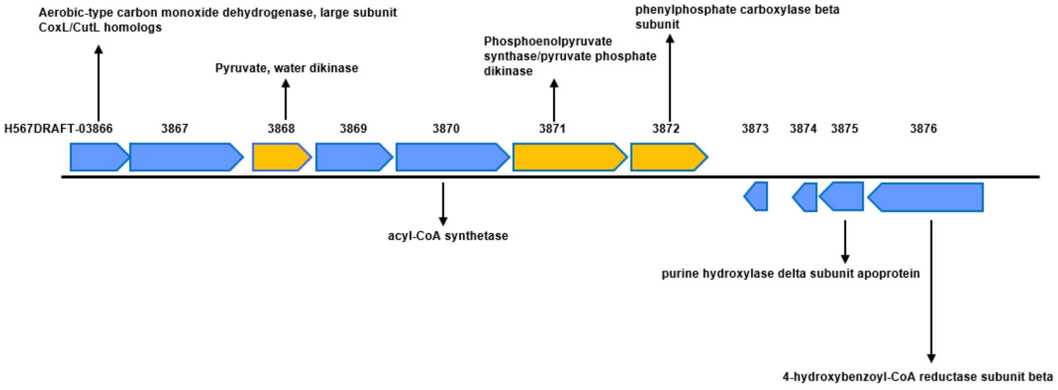


Figure S2. Organization of the genes in the aniline- or 4-aminobenzoate-induced gene cluster with the locus tag from H567DRAFT-03866 to H567DRAFT-03876 in the genome of *D. anilini*. The directions of transcription are indicated by arrows. The genes that were cloned in this study are presented by yellow filling.

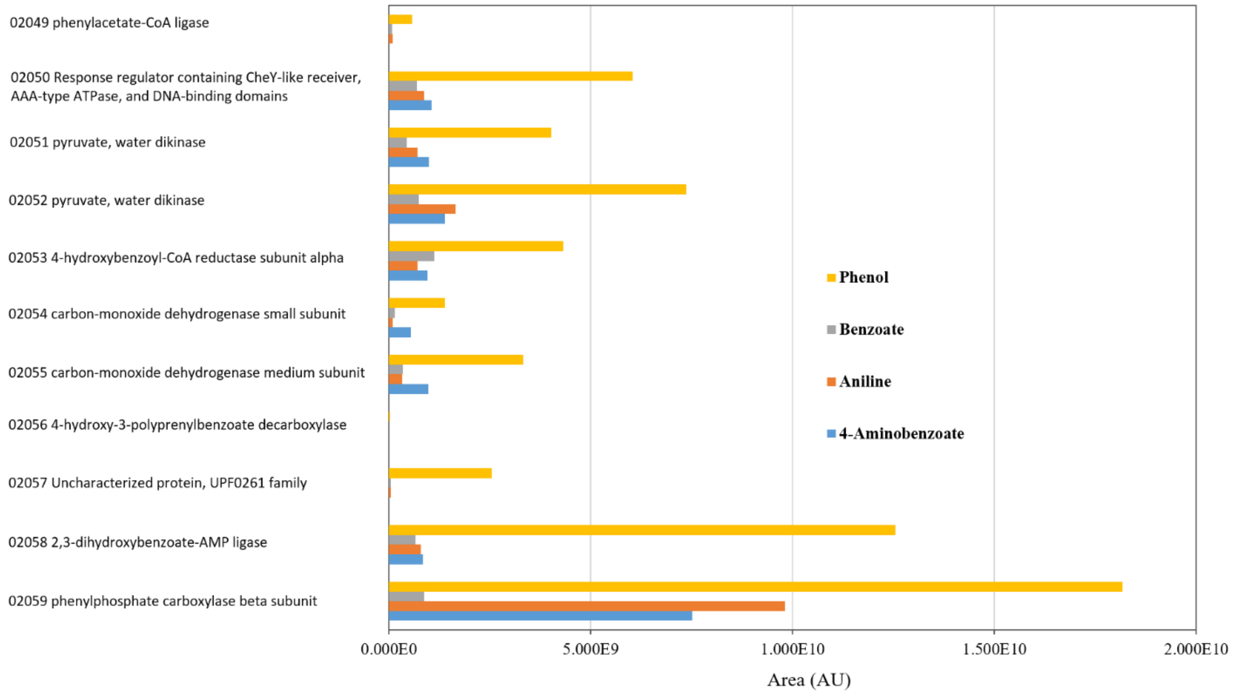


Figure S3. Total proteome analysis of cell-free extracts from the cells of *D. anilini* grown on phenol (yellow bars), benzoate (grey bars), aniline (orange bars) and 4-aminobenzoate (blue bars).

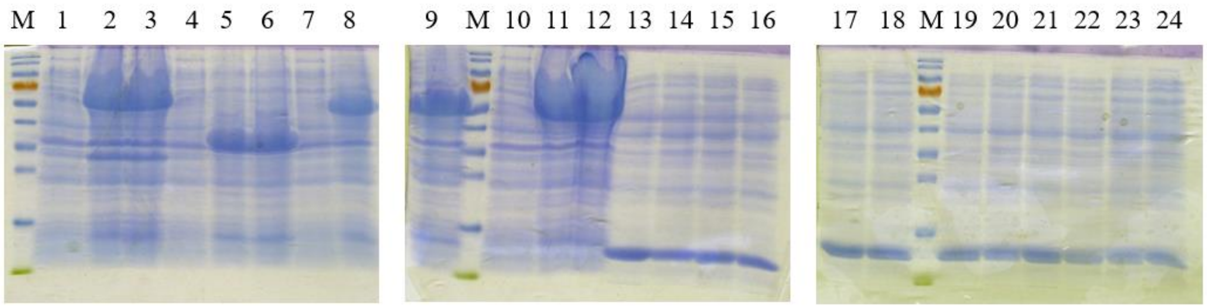
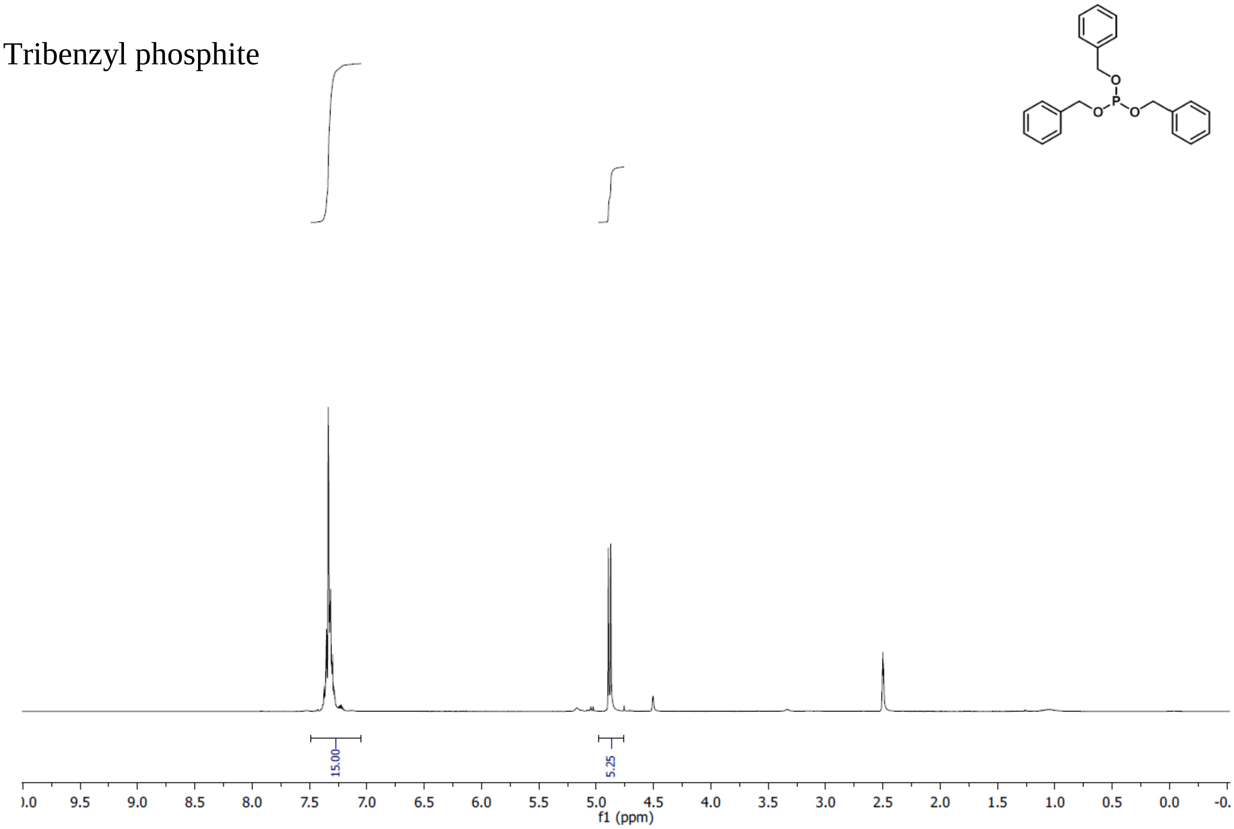


Figure S4. SDS-PAGE gels of total cellular protein and soluble protein of *E. coli* after aerobic growth in LB medium and induction with IPTG. M: Molecular mass-marker. 1 to 3: Cell-free extracts of *E. coli* cells containing vector pXX5 after 0, 2, or 4 hours induction with IPTG. 4 to 6: Cell-free extracts of *E. coli* cells containing vector pXX6 after 0, 2, or 4 hours induction with IPTG. 7 to 9: Cell-free extracts of *E. coli* cells containing vector pFL1 after 0, 2 or 4 hours induction with IPTG. 10 to 12: Cell-free extracts of *E. coli* cells containing vector pSS1 after 0, 2 or 4 hours induction with IPTG.13 to 15: Soluble proteins of *E. coli* containing vector pXX5 after 0, 2 or 4 hours induction with IPTG. 16 to 18: Soluble proteins of *E. coli* containing vector pXX6 after 0, 2 or 4 hours induction with IPTG. 19 to 21: Soluble proteins of *E. coli* containing vector pFL1 after 0, 2 or 4 hours induction with IPTG. 22 to 24: Soluble proteins of *E. coli* containing vector pSS1 after 0, 2 or 4 hours induction with IPTG.

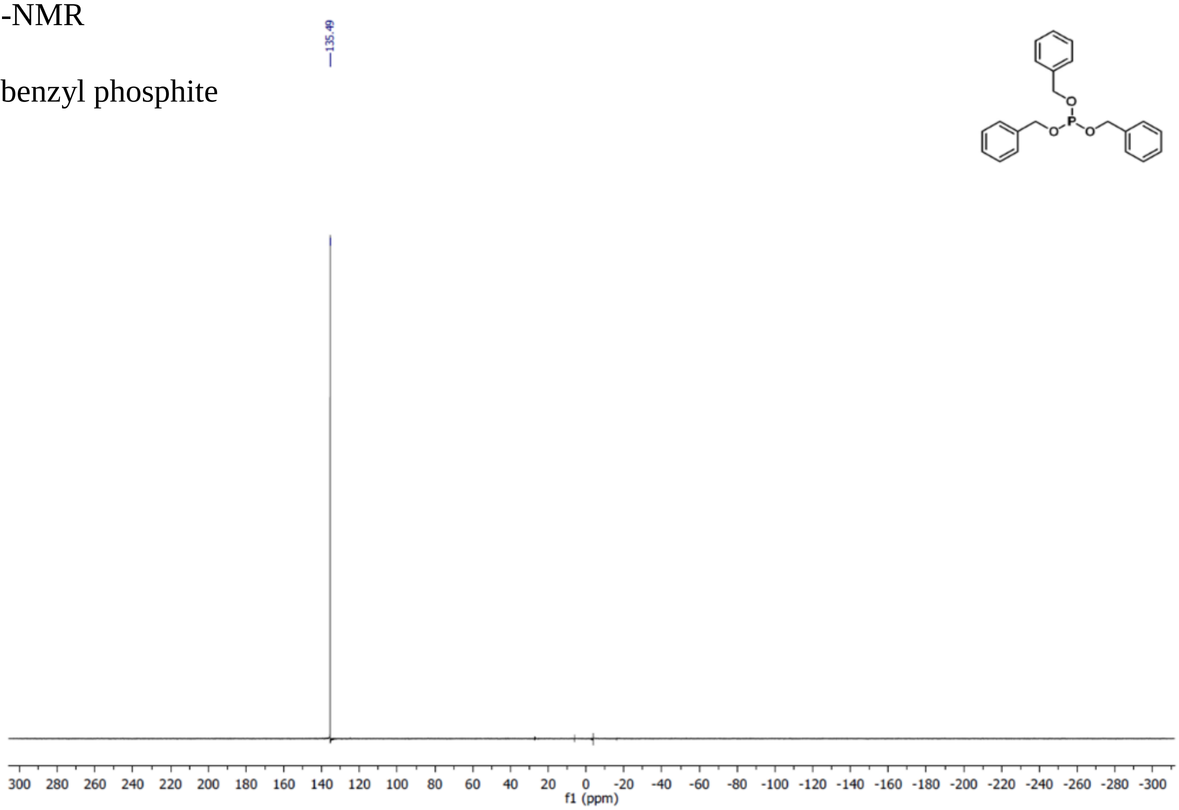
^{1}H-NMR

Tribenzyl phosphite



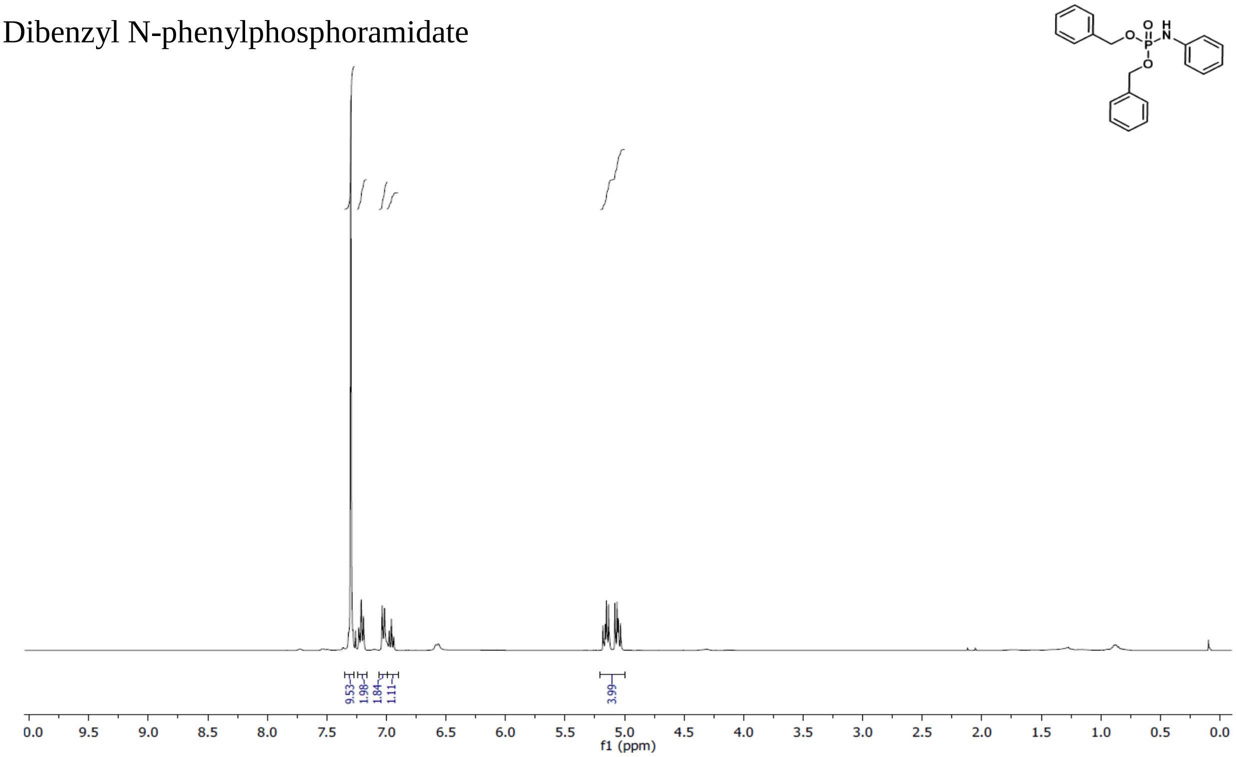
^{31}P-NMR

Tribenzyl phosphite



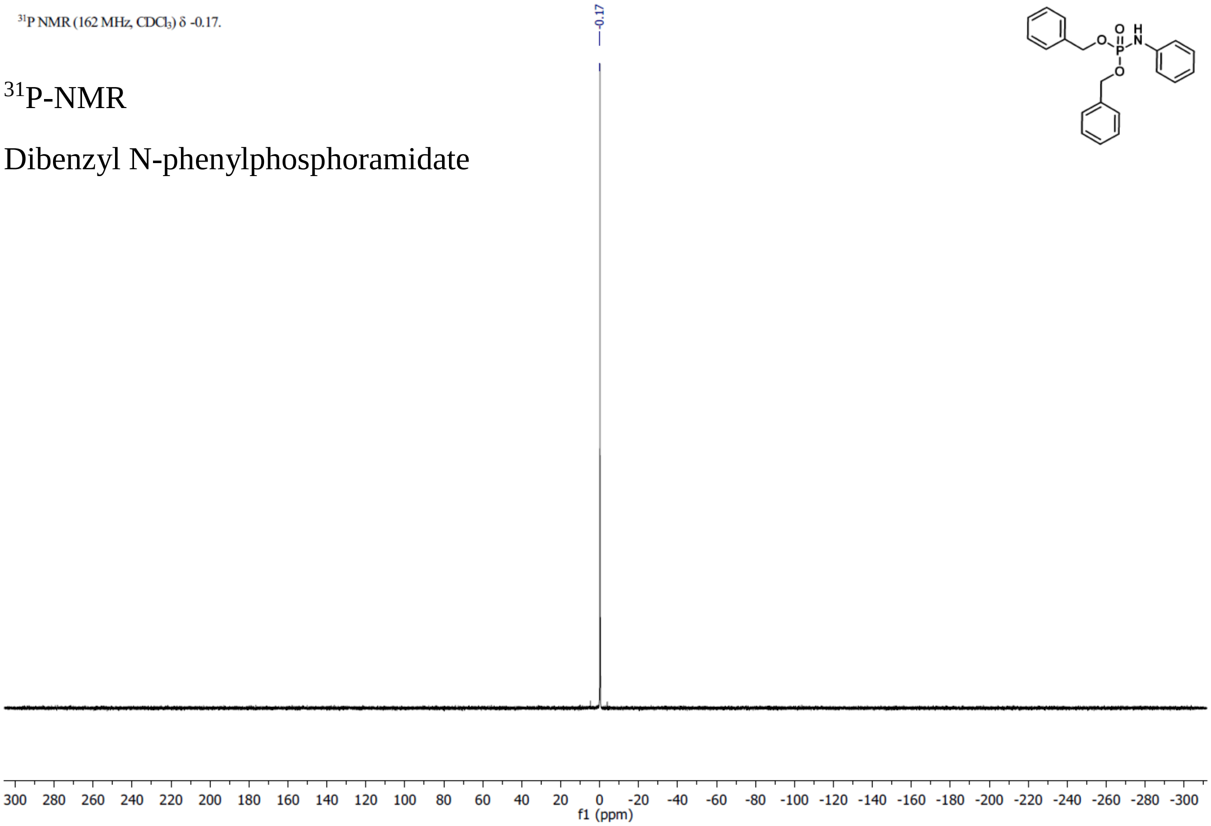
^{1}H-NMR

Dibenzyl N-phenylphosphoramidate



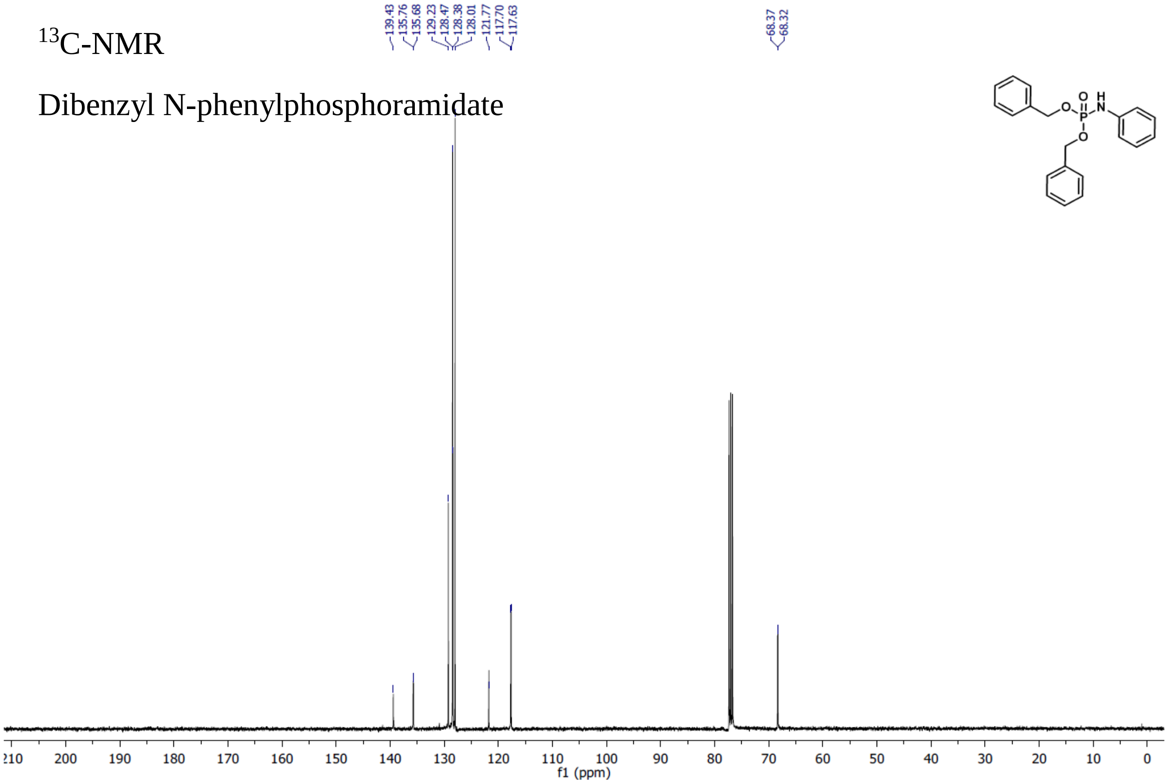
^{31}P-NMR

Dibenzyl N-phenylphosphoramidate



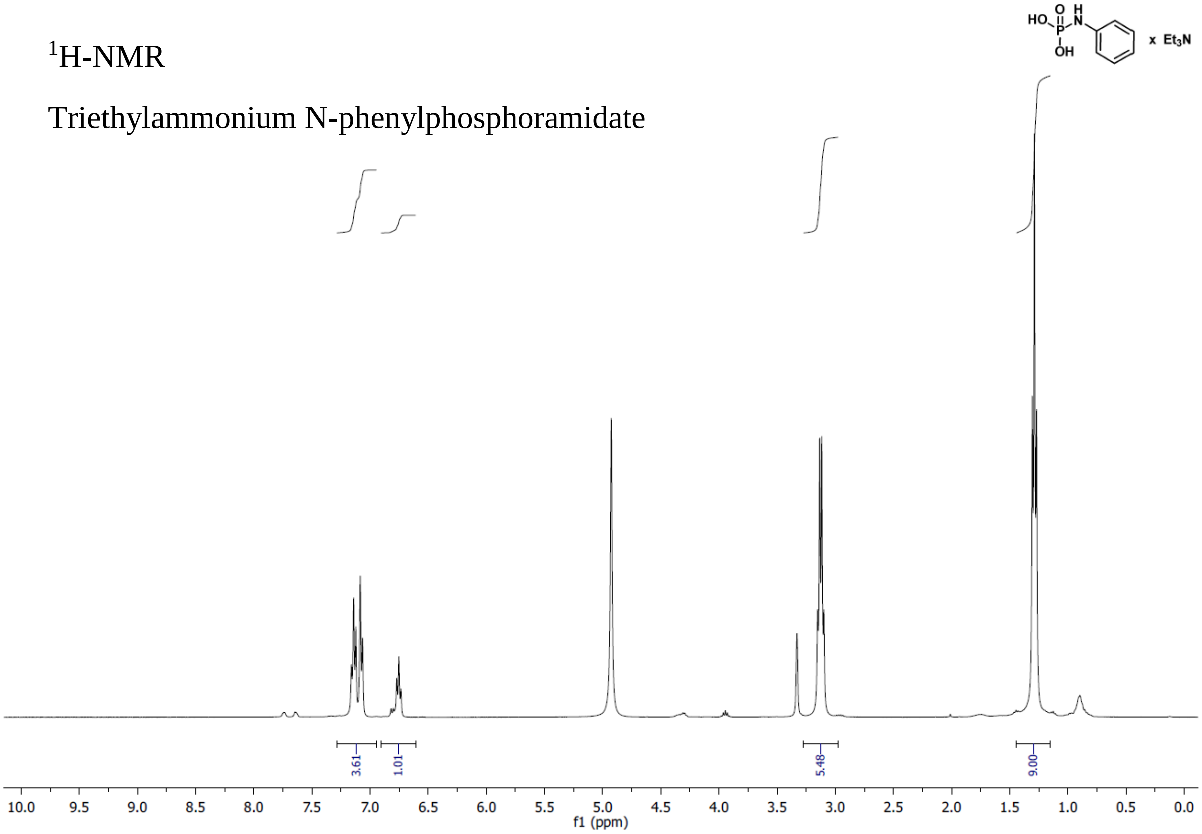
^{13}C-NMR

Dibenzyl N-phenylphosphoramidate



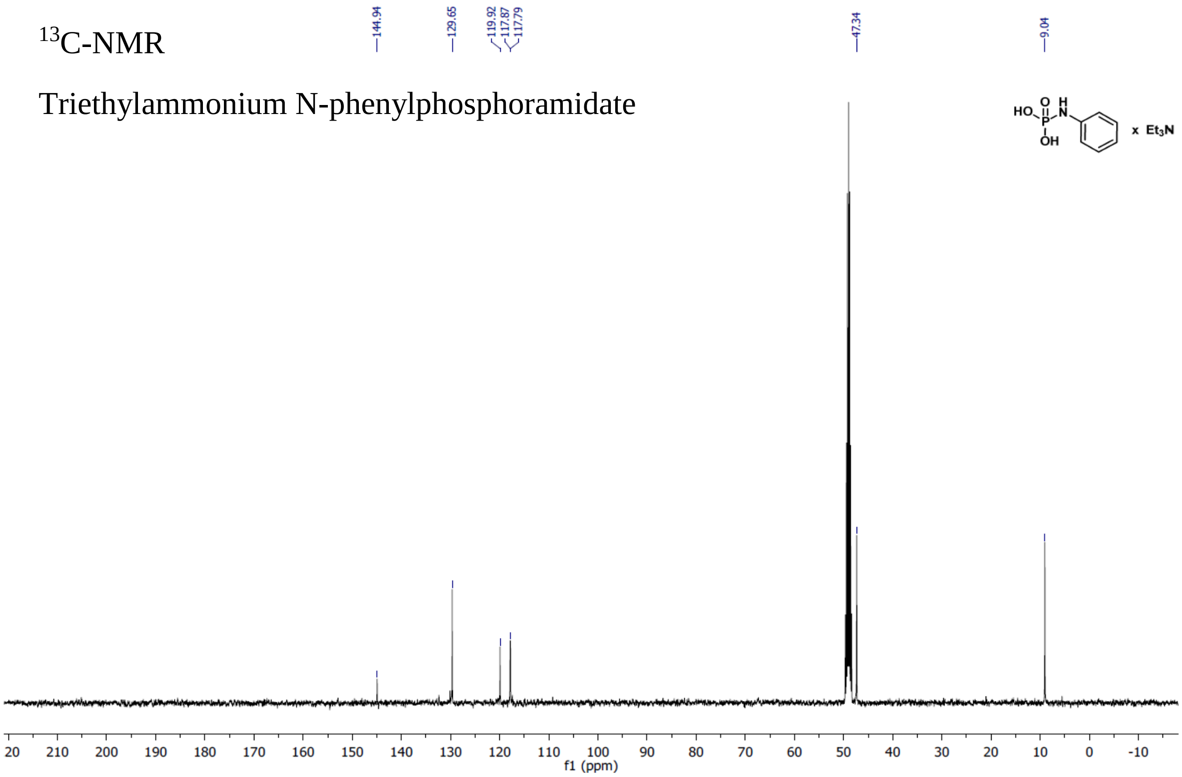
^{1}H-NMR

Triethylammonium N-phenylphosphoramidate



^{13}C-NMR

Triethylammonium N-phenylphosphoramidate



^{31}P-NMR

Triethylammonium N-phenylphosphoramidate

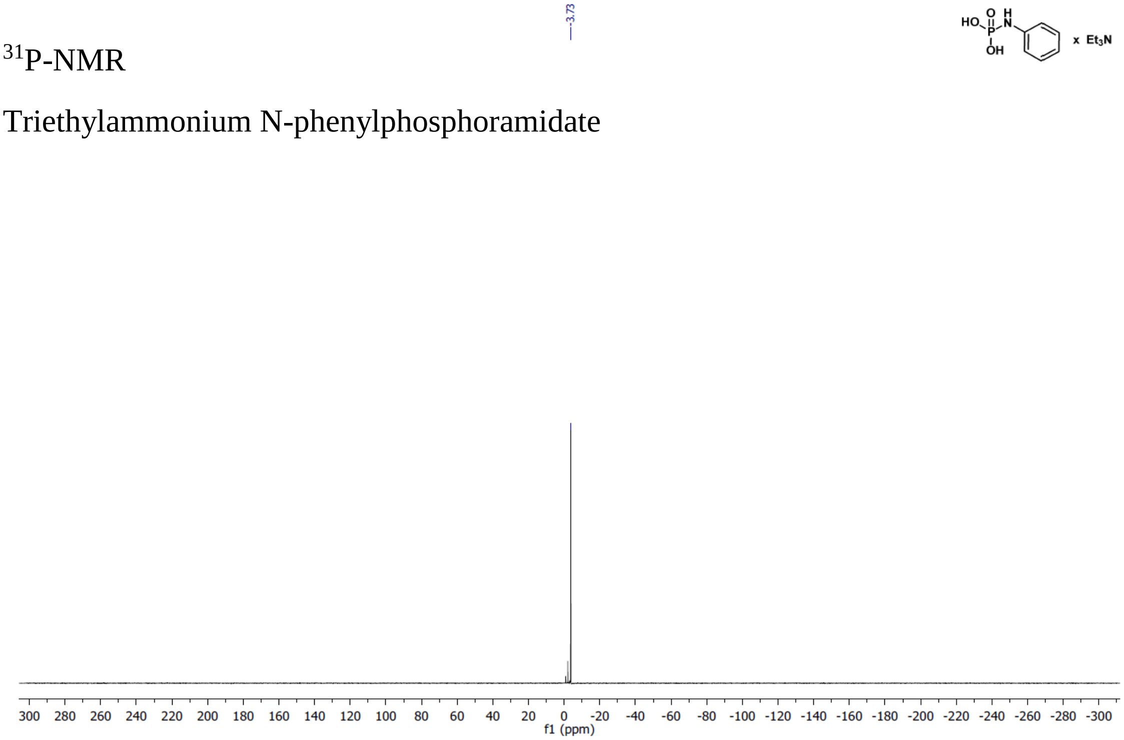


Figure S5. The NMR spectras from phenylphosphoamidate synthesis and precursors.

CHAPTER 5

General discussion

To date, little is known about the pathway of anaerobic aniline and phenol degradation in sulfate-reducing bacteria. *Desulfatiglans anilini* is the only described anaerobic bacterium that is able to use both aniline and phenol as sole electron donors and carbon sources under strictly anaerobic, sulfate-reducing conditions [31]. Furthermore, the availability of the genome sequence of *D. anilini* provides new ideas and methods for investigating the activation reactions and enzymes involved in anaerobic degradation of aniline and phenol by *D. anilini*.

The studies on phenol degradation in the nitrate-reducing bacterium *Thauera aromatica* showed that phenol is converted to 4-hydroxybenzoate via phenylphosphate at the cost of two ATP equivalents [64, 66, 76]. In chapter 2 of this study, phenylphosphate was proven also to be an intermediate for phenol degradation in the sulfate-reducing bacterium *D. anilini*, in which phenol is initially phosphorylated to phenylphosphate and then carboxylated to 4-aminobenzoate [103]. Sulfate-reducing bacteria and nitrate-reducing bacteria apparently use the same strategy to activate phenol, yet there are some differences concerning the genes coding for enzymes involved in phenol degradation. In nitrate-reducing bacteria, phenylphosphate synthase (pps) consists of three subunits (α, β, γ) [66, 77], phenylphosphate carboxylase (ppc) contains four subunits (α, β, γ, δ) [36, 77], and the encoding genes for phenylphosphate synthase and phenylphosphate carboxylase are located adjacent to each other in one operon. In sulfate-reducing bacteria, the gene coding for ppsγ subunit is not located in the same gene cluster with pps α and pps β, and the protein of ppsγ was not induced by phenol in *D. anilini* (see chapter 2, Figure 4), which might be the reason for the lower enzyme activity of phenylphosphate synthase in *D. anilini* compared to *Thauera aromatica*, as the function of ppsγ is to stimulate the phosphorylation of phenol [66]. For phenylphosphate carboxylase in *D. anilini*, only the gene coding for ppc β exists in the phenol-induced gene cluster and the expression of ppcβ was also highly induced by phenol. No indications of the gene coding for ppc δ can be found in the genome of *D. anilini*, whose function is to catalyze a very slow hydrolysis of phenylphosphate [36]. This indicates that the hydrolysis of phenylphosphate in *D. anilini* is not catalyzed by ppc δ-homologues. Instead, phenylphosphate is assumed to be hydrolyzed by a non-specific phosphatase or hydrolase in *D. anilini* [128, 129].

Analogous to the phenol activation reaction, the study in chapter 4 showed that also aniline is activated by a phosphorylation reaction to form phenylphosphoamidate in the sulfate-reducing strain *D. anilini*. The reaction mechanisms of phenol phosphorylation and aniline phosphorylation are likely similar to phenylphosphate synthase [66] and PEP synthase [117, 118]: the substituents of phenol (-OH group) and aniline (-NH_2 group) can attack the β-phosphoryl group of the universal phosphoryl donor ATP, resulting in the transfer of a phosphate group to the substituent of the aromatic ring, forming phenylphosphate and phenylphosphoamidate, respectively, through a P-O bond and P-N bond. Comparing to the P-O bond, the P-N bonds is intrinsically instable and not easily accessed, but a similar reaction occurs in the conversion of azidophenylalanine into a phosphotyrosine analogue in a protein through the Staudinger-phosphite reaction (Figure 1) [130]. The initial iminophosphorane adduct formed between the phosphite and azide undergoes a reaction to provide the N-linked phosphoramide [131]. Both of the enzymes that catalyze the phenol and aniline phosphorylation contain a histidine residue which can interact with the β-phosphoryl group of ATP. Mg^{2+} is required for the phosphorylation reaction to chelate the γ- and β- phosphoryl groups to lower the threshold for the phosphoryl transfer [132]. The instability of the P-N bond forces *D. anilini* to overcome even more difficulties to activate aniline, which explains the weaker growth of *D. anilini* on aniline than phenol and benzoate. In this hypothetical mechanism of phosphorylation, AMP and inorganic phosphate are released. Another hypothetical mechanism of aniline phosphorylation could be the interaction of the histidine residue of the enzyme with the γ-phosphoryl group of ATP instead of the β-phosphoryl group to form ADP. ADP can be reused as a phosphoryl donor. It is not clear yet which mechanism is used by phenylphosphoamidate synthase, further studies should therefore focus on investigating the formation of AMP and ADP or the possibility of using ADP as a co-factor for phenylphosphate or phenylphosphoamidate synthase to unravel the mechanism of phosphorylation.

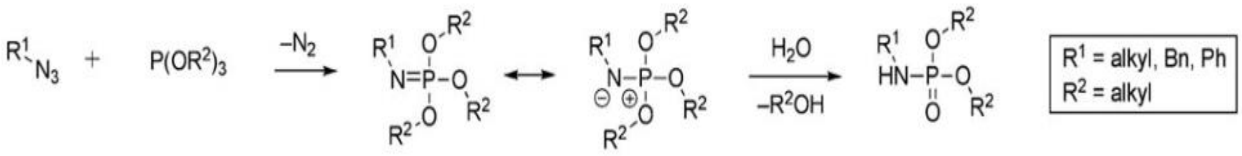


Figure 1. The staudinger-phosphite reaction is followed by the hydrolysis of resulting phosphorimidates to produce phosphoramidates, adapted from Serwa R. et al [130].

The second step in aniline activation is the carboxylation of phenylphosphoamidate to 4-aminobenzoate by a carboxylase, which is analogous to phenylphosphate carboxylation in the phenol degradation pathway of *D. anilini*. The carboxylase for carboxylating phenylphosphoamidate is ATP-independent, thus suggesting a reaction mechanism different from the ATP-and biotin-dependent carboxylase [133, 134]. Mn^{2+} is required for carboxylating phenylphosphoamidate by a carboxylase, analogous to UbiD [135]. Two UbiD-like subunits are required in the *Thauera aromatica* phenylphosphate carboxylase complex to carboxylate phenylphosphate [36, 77]. Similar to the phenylphosphate carboxylase complex in *Thauera aromatica*, the phenylphosphoamidate carboxylase complex in *D. anilini* also contains two UbiD-like subunits but without γ- or δ-subunit. In the UbiX-UbiD system, UbiX acts as the flavin prenyltransferase to produce prenylated FMN with FMN. Prenylated FMN serves as the cofactor for the carboxylase UbiD [119]. It is mysterious that FMN is still required for the two UbiD-like subunits of phenylphosphoamidate carboxylase, even though the UbiX-like subunit is missing in *D. anilini*. Overall, the activation of aniline by *D. anilini* faces the same problem as phenol activation in the same organism, as aniline also seems to be activated through a pathway under high energy consumption. It is not well understood how sulfate-reducing bacteria can maintain an overall positive ATP balance under these conditions. Studying the catalytic mechanism of phenylphosphoamidate synthase and phenylphosphoamidate carboxylase or investigating novel more efficient energy-conserving mechanisms in sulfate-reducing bacteria may help to solve this puzzle.

In chapter 3 of this study, sulfide was found to have a negative influence on the activity of the aniline-carboxylating enzyme system and phenylphosphate synthase in *D. anilini*. This also explains the inhibition of growth of *D. anilini* with aniline or phenol by sulfide. The enzymes involved in phenol and aniline phosphorylation are pyruvate water dikinase-like proteins and pyruvate water dikinase was reported to be inhibited by sulfhydryl groups [136]. It is assumed that the S-atom of the sulfhydryl group interacts with the active site of pyruvate water dikinase to compete with the substrate of the enzyme, which was inspired by the study on inhibition of cytochrome *c* oxidase (CcO) by sulfide. Sulfide is a classic CcO inhibitor, as H_2S can act as a competitor for the substrate of CcO, O_2, to bind to Fe^{II} in the reduced active site of CcO. However, this inhibition is reversible as O_2 can easily replace H_2S bound to a reduced Fe^{II} site [137]. Since the inhibition of H_2S is not deadly to the bacteria, the growth of bacteria can be recovered by removing sulfide from the culture.

The attempt to remove sulfide from cultures of *D. anilini* was accomplished by co-culturing *D. anilini* with a phototrophic sulfide-consuming bacterium, *Thiocapsa roseopersicina*. The growth rate of *D. anilini* with aniline and the aniline turnover rate increased significantly after removing sulfide. More biomass of *D. anilini* was produced by culturing *D. anilini* in this artificial system compared to a regular axenic batch culture (Chapter 3, Figure 4), which is beneficial for studying the metabolism of *D. anilini*. Yet, the cultivating system is still far from being efficient enough to be applied in technological applications such as bioremediation and could be improved by optimizing the co-cultivating method. Co-culture systems and technologies were reviewed by Goers L. et al [138]. For instance, the artificial co-culture system in this study can be further improved by using a porous membrane [139] to partition the *D. anilini* and *T. roseopersicina* cultures, which would still allow physical and biochemical crosstalk between the two cultures and increase the contact area, so that sulfide can be transferred to the *T. roseopersicina* culture more efficiently. Moreover, growth and aniline degradation rates could be improved by genetically engineering the pyruvate water dikinase to be less sensitive to sulfide inhibition or exhibiting higher turnover rates of phosphorylation. This could be accomplished, for instance, by increasing the expression level of phenylphosphate or phenylphosphoamidate synthase by increasing the copy numbers of the *pps* gene. Extensive research was done in the past few years on developing methods of genetic manipulation of the genus *Desulfovibrio* [140, 141]. Until now, however, unfortunately no genetic engineering system for *D. anilini* exists. As the genome sequence of *D. anilini* is now available, and as the genes of aniline and phenol degradation were identified, genetically engineering the strain would be possible. This could help to modify *D. anilini* to exhibit the desired degradation features as well as to further deepen our knowledge on the reaction mechanisms of the degradation of aromatic compounds in sulfate-reducing bacteria.

Author contributions

Chapter 2

Enzymes involved in the anaerobic degradation of phenol by the sulfate-reducing bacterium *Desulfatiglans anilini*.

Xiaoman Xie, Nicolai Müller*

XX and NM designed experiments, analyzed the data and wrote the manuscript. Experiments were conducted by XX. All authors read and approved the final manuscript.

Chapter 3

Sulfide inhibits activation of aniline in *Desulfatiglans anilini*

Xiaoman Xie, Nicolai Müller*

XX and NM designed experiments, analyzed the data and wrote the manuscript. Experiments were conducted by XX. All authors read and approved the final manuscript.

Chapter 4

Elucidation of the aniline degradation pathway and enzymes involved in the sulfate-reducing bacterium *Desulfatiglans anilini*

Xiaoman Xie, Dieter Spiteller, Thomas Huhn, Bernhard Schink, Nicolai Müller*

XX did experiments designed by XX, NM, BS, and DS. Phenylphosphoamidate was synthesized by TH. Cloning was done by XX, Sandeep Shrestha and Feng Liu. LC-MS measurement was done by DS. All authors have read and approved the final version of the manuscript.

List of publications

Xie, Xiaoman, and Müller, Nicolai. Enzymes involved in the anaerobic degradation of phenol by the sulfate-reducing bacterium *Desulfatiglans anilini*, BMC Microbiology, 2018, 18, 93.

Xie, **Xiaoman,** and Müller, Nicolai. Sulfide inhibits activation of aniline in *Desulfatiglans anilini*. In preparation.

Xie, Xiaoman, Spiteller, Dieter, Huhn, Thomas, Schink, Bernhard, and Müller, Nicolai. Elucidation of the aniline degradation pathway and enzymes involved in the sulfate-reducing bacterium *Desulfatiglans anilini*. In preparation.

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