Friedrich-Schiller-University Jena

Faculty of Biology and Pharmacy

Institute of Microbiology

The effect of spatial structures on the evolution of cooperative amino acid cross-feeding within bacterial consortia

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Submitted by Daniel Preußger
from Dürrhennersdorf
born 7th March 1987 in Löbau

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Max Planck Institute
for Chemical Ecology

Referees:

Prof. Dr. Erika Kothe

Dr. Christian Kost

ABSTRACT

Mutualisms are interactions between individuals of different species resulting in beneficial effects for interacting partners. Although their fundamental ecological importance is well recognized, the conditions necessary for their evolution and persistence remain poorly understood and scarcely proven. One of the factors suggested to be important in the evolution and maintenance of mutualism is spatial structure. Until now, only a few empirical studies are known, which address the effect of spatial structure on the evolution of mutualism. Bacterial model systems feature the capability to investigate such an ecological parameter. Accordingly, in this study a by-product interaction was synthetically established between the amino acid auxotrophic strains *Escherichia coli ΔtrpB* and *Escherichia coli ΔtyrA*. The aim of this thesis was to perform a long-term coevolution experiment using this bacterial model system. The obligate cross-feeding consortium was subjected to different environmental conditions. Coevolution took place either in a spatially structured environment represented by the surface of agar, or in a liquid reference environment. The effect of spatial structure on the interaction was investigated by comparing productivity and fitness of coevolved consortia in the spatially structured and unstructured environment. Interestingly, productivity of consortia was significantly higher if evolved in the spatially structured environment compared to the spatially unstructured environment.

Moreover, cooperative phenotypes were isolated predominantly in agar-evolved consortia. Analysis of released amino acids by coevolved populations revealed that true cooperation did evolve in one out of four populations on agar surface. Overall, spatially structured populations released significantly increased amounts of amino acids than cocultures in liquid medium. Hence, a mutualistic interaction evolved between partners of the same species, which is termed cooperation.

In conclusion, the presented work supplies the theoretically predicted positive effect of spatial structure to facilitate the evolution of cooperation.

ZUSAMMENFASSUNG

Mutualismen stellen Interaktionen zwischen Vertretern verschiedener Arten dar, welche sich positiv auf beide Partner auswirken. Obwohl die fundamentale ökologische Bedeutung von Mutualismen bekannt ist, sind die Parameter, welche sowohl deren Evolution als auch deren Beständigkeit begünstigen, nach wie vor Theorie oder nur ansatzweise empirisch erforscht. Von räumlichen Strukturen wird angenommen, dass sie einen solchen Parameter repräsentieren. Bisher wurden nur wenige experimentelle Studien publiziert, welche die Effekte von räumlichen Strukturen auf Mutualismen demonstrieren. Zur Aufklärung des Einflusses eines solchen ökologischen Faktors eignen sich bakterielle Modellsysteme. Aus diesem Grund wurden im Rahmen dieser Arbeit Interaktionen zwischen *Escherichia coli* Stämmen synthetisch etabliert, welche auf dem Austausch von Aminosäuren basieren. Die Aminosäure-auxotrophen Organismen *E. coli ΔtrpB* und *E. coli ΔtyrA* coevoluierten anschließend innerhalb eines Langzeit-Experimentes mit verschiedenen Umwelt-bedingungen. Das Konsortium wurde entweder in einer räumlich strukturierten Umgebung, und zwar auf der Oberfläche von Agar, oder einer flüssigen unstrukturierten Referenzumgebung über mehrere Wochen inkubiert. Der positive Effekt der räumlichen Struktur auf die Evolution des Konsortiums wurde durch den Vergleich von Produktivität und Fitness zwischen Umgebungen quantifiziert. Die Produktivität der Cokulturen war im Vergleich signifikant höher, wenn diese auf Agar evolviert sind.

Darüber hinaus würden kooperative Phänotypen überwiegend in Konsortien entdeckt, welche auf der Oberfläche von Agar inkubiert wurden. Die Analyse von Aminosäuren in Kulturüberständen von coevoluierten Populationen, welche zuvor auf Agar interagierten, identifizierte echte kooperierende Partner in einem von vier untersuchten Konsortien. Zusammengefasst schieden die Agar-evolvierten Populationen signifikant höhere Mengen an Aminosäuren aus als die Cokulturen in Flüssigmedium. Folglich wurde die Entwicklung einer mutualistischen Interaktion zwischen Vertretern der gleichen Art beobachtet, was mit dem Begriff Kooperation beschrieben wird. Die vorliegenden Ergebnisse unterstützen somit die theoretisch vorhergesagte positive Bedeutung einer räumlichen Struktur auf die Evolution von Kooperation.

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1 INTRODUCTION

1.1 SYMBIOSIS

The historical development of life on Earth is fundamentally a history of species interactions [1, 2]. Those interactions, summarized in the term symbiosis, comprise every facette between highly positive (mutualism) and strongly negative effects (parasitism) that species of different kingdoms have on each other. Different major forms of symbiosis are summarized in Table 1-1. In parasitism one organism, the parasite, is living continuously or temporarily on or even in its host, to the disadvantage of the host and the benefit of the parasite. In most of the cases the host is not killed by the feeding process of the parasite, but suffers a decrease in fitness. Interestingly, one third of all species exhibits a parasitic lifestyle that is therefore involved in manifold biodiversity on earth [3]. Commensalism is another form of coexistence of species, where negative effects are absent. In fact, one organism has a benefit from the behaviour or excretion of another one, whereas the other organism is not effected at all [4]. A further major relationship between species is mutualism. Two partners have a benefit in this positive interaction. They invest energy into a metabolite or behaviour that is provided for the respective partner by paying costs, but receive a benefit due to the partners good thereby increasing fitness. If such a positive interaction is present between partners of the same species, the term cooperation is used instead.

Table 1-1: Symbiotic relationships and impact on organisms [5].

Relationship	Self	Opponent
Amensalism	Neutral	Harm
Commensalism	Benefit	Neutral
Competition	Harm	Harm
Mutualism	Benefit	Benefit
Parasitism	Benefit	Harm
Predation	Benefit	Harm

Mutualistic interactions can be classified by the kind of shared goods, and by relationship or dependency of partners. The relationship can be either facultative or obligate [6]. Obligate mutualisms, for example, comprise leaf-cutting ants and their fungus garden, the root nodule-legume association, or lichen, whereas the latter for instance include oxpeckers and ants "farming" aphids. Shared goods, however, can be represented by metabolites like nutrients, or services. A more detailed way to categorize mutualism is to discriminate between shared goods and shared services [6]. Such classification would lead to following three categories of mutualistic interaction: 1) Both partners exchange resources, 2) Both partners provide services and 3) One partner shares a common good, while the other provides a service. Examples would be mycorrhiza, sea anemones with anemone fish, and ants protecting aphids for honeydew, respectively [4]. Despite those interactions mentioned above there are also other special forms, especially in macroorganisms (e.g. hyperparasitism and metabiosis). However, microorganisms also exhibit many of such interactions. Synergism, related to mutualism, is widely distributed in the microbial world [4].

Since ecosystems are filled with different forms of life, it can be posed that there is the minority of species on earth existing isolated, interacting exclusively with its own kind for decades. In fact, interspecies interactions are suspected to "have played a central role in the diversification and organization of life" [7]. Organisms in an ecosystem are therefore connected in a complex web of dependencies, equilibria and dynamics on several ecological dimensions [8], thereby being changed in coevolutionary processes. During development of life, novel interactions were involved in major evolutionary transitions. Emerging mutualisms, for example, had a great impact on several of those transitions [9], in particular on all eukaryotes, which would otherwise not exist in their present form. Important examples therefor are mitochondria as well as chloroplasts representing prerequisites of eukaryotic life, furthermore lichens, mycorrhizae, and rhizobia, which enabled terrestrial colonisation, and finally gut symbionts focal for many animals digestion [7]. Taken together, these statements indicate the following: Mutualistic interactions are ubiquitous in every ecosystem and their influence at all levels of biological organization is undisputable [9-11]. Although their fundamental, ecological importance is well known, the conditions, which are necessary for their evolution and stability, remain rudimentary proven and theoretical approaches [12].

1.2 Evolution and Maintenance of Mutualism

Commonly, members of different species interact in mutualisms by sharing goods or services, thereby benefitting each other [13]. These interactions are stable over extremely long time scales. However, this phenomenon is in direct contradictory to darwinian priciples. Natural selection would always favour the fittest representatives of a species, thus the most selfish ones. Non-cooperating individuals would theoretically not start to pay a cost, without having an advantage. Otherwise it would be selected against those phenotypes, because of decreased fitness. Hence, cooperative interactions should be especially vulnerable to evolving cheating phenotypes [14, 15]. This is also in accordance with the modern selfish gene view [16]. The term “tragedy of the commons”, introduced by Hardin in 1968 [17], was originally used in another context, but decribes the abovementioned conflict: In order to gain higher benefit, inividuals destroy those resources on which the total society depends due to selfish overexploitation. This finally would lead to extinction. Morality, responsibility, negotiation, or even mutual coersion might prevent this in human societies. But animals and especially prokaryotes tend not to “know” such mechanisms. Moreover, individual-level selection should favour self-interested behaviours in natural mutualistic interactions [18]. Mechanisms on that account must exist, which support the evolution and maintenance of mutualisms. In the following paragraph theoretical and empirical insights regarding the evolution of mutualism are presented.

The dilemma, which individuals face in the early steps towards a mutualistic interaction, was first introduced in this context by Trivers, 1971 [19]. The model was further extended to the “Iterated Prisoner’s dilemma” [14]. If an individual offers costly goods or services to another species, this in the first turn altruistic behaviour is only favoured by natural selection, when benefits are returned to individuals of the same species [20]. The iterated Prisoner’s dilemma is a game theoretical approach and adresses this assumption. In this game model players interact repeatedly and have to decide, wheather to cooperate to the benefit of both, or to defect. In addition, a strategy was added for the players, which is called TIT FOR TAT [14]. In this strategy the decision of participants to cooperate depends on the partners last decision. Cooperative behaviour will therefore be rewarded and defection will be

punished. Cooperation can emerge under these conditions to the mutual benefit of both. In fact, reciprocation and repeated interactions between two partners are theoretically important for cooperation to evolve, but also to be maintained. These conditions were summarized in the term partner fidelity and proposed to support mutualists in another study [21]. The iterated Prisoner's Dilemma model of Axelrod & Hamilton [14] was created to explore dynamics in intraspecies interactions, and therefore cannot be applied directly to interspecies interactions like mutualism. In another model the Prisoner's Dilemma was extended to interspecies interactions [12]. The outcome of this study was that reciprocation should correlate with increasing investments. As another conclusion, a spatially structured environment is required in the presence of competition for mutualisms to evolve. Hence, initial by-product interactions and reciprocation are assumed to be prerequisites for cooperation to emerge.

In general, there are ecological factors suggested to be involved in the evolution and maintenance of mutualisms:

Genotypic uniformity describes the genetic background of each partner to exhibit high relatedness. This leads to reduced conflict within a population. The benefit is thereby increased in cooperating populations and may cause higher specificity of the interaction, which might decrease the chances of exploitation [9, 10]. Strassmann *et al.* 2011 point out that relatedness is important for positive interactions among microbes and give several examples [2]. Hamilton's rule moreover describes how kin selection works and introduced the term inclusive fitness [22, 23]. The latter concept explains actors, which pay a cost by benefitting related individuals, thereby increasing the average fitness. The former term, kin selection, would be also capable to act as a driving force to generate high relatedness [2]. Growth of microbes in spatially structured environments could also cause neighbors to be clonemates [2], due to simple colony formation from a single originated cell growing on a substrate.

High partner fidelity comprises the interaction of partners in close association lasting long enough to respond to a shared good [21]. This enables them to enter a positive feedback-loop. The more one partner invests into the interaction, the higher benefit is received in return. The fitness of cooperators is therefore connected. This can be established via vertical transmission [24]. Partners encounter each other in every following generation, they coevolve and can intensify their interaction. The opposite would be horizontal transmission, in which the offspring of cooperators encounter different partners, theoretically leading to a scenario comparable to the defector types in the Prisoner's Dilemma model.

Spatial structure could permit high partner fidelity and is defined as a local structure, which gains the interaction of two (or more) partners over several generations in close proximity. Increased interactions between clones in a bacterial population could also gain the evolution of cooperation, which is again an effect of a spatially structured environment [22]. Additionally, spatial arrangements in cooperating populations are expected to enable mutualists to persist cheaters [16, 24, 25]. In the microbial world, spatial structure can for example be represented by solid substrates [2]. Another relevant example are biofilms, mostly consisting of different bacterial species [26]. They are suggested to represent the dominant life-style and the most natural form of growth in bacteria [27]. Moreover, microorganisms create spatial arrangements in populations even in spatially non-structured environments, simply by adherence [26] and excretion of viscous polymers [28].

There are theoretical as well as experimental studies, which adress the relevance of spatial structure in mutualistic interactions. Frank 1994 concluded from a model that spatial genetic correlations of altruistic aids of two partners enhance the spread of altruism and might be important for the evolution and maintenance of mutualism [29]. This is also connected to genotypic uniformity of partners. In parallel, genetic population structure is predicted to support evolving cooperation and solid substrates in turn support long-term high relatedness [2]. Foster and Wenseleers concluded from a model, that high within species relatedness and high between species fidelity are important for mutualisms to evolve [30]. In a computer simulation and an analytical approach, it was already demonstrated that cooperation, based on the use of external energy resources, may evolve in a spatially structured environment [31, 32]. Another computer simulation indicated that spatial arrangements might decrease interactions with non-cooperators [33]. Empirical studies yet

investigated the effect of spatially structured environments on evolving bacterial populations [34, 35], intraspecies cooperation [36] as well as interspecies interaction [37] and interspecies cooperation [38, 39]. These examples reveal that microorganisms are very useful tools to study evolutionary mechanisms. More reasons for bacterial model systems to be utilized in experimental evolution are enumerated in the next chapter.

1.3 BACTERIAL MODEL SYSTEMS

There are multiple reasons, why bacterial model systems are very appropriate for studying evolutionary mechanisms. Bacteria exhibit rapid generation times and reproduce asexually resulting in increased precision of experimental replication [1]. In favorable conditions for example, *Escherichia coli* needs only 30 minutes for one cell division. This enables evolutionary biologists to observe changes in the phenotype as well as genotype of evolving subpopulations within relatively short time spans. Otherwise this would take hundrets of years in higher eukaryotes as model organisms. One particular evolution experiment with *Escherichia coli* even reached 20,000 generations [40]. Another advantage of microorganisms is the extremely high number of individuals, whose cultivation is comparatively cost-efficient and simple, even for long-term experiments. Large population sizes and high numbers of generations create scores of beneficial as well as deleterious mutations. These mutations can easily be analysed due to small genome sizes and completely annotated genomes in the case of several popular bacterial model organisms. For those strains, extensive tools for genetic modification are available. This allows the precise alteration of the genetic background in the model organism for the proposed experiment. Replicated populations, found by the same ancestor, ensure identical clones and can be propagated under constant well-defined conditions. Cryo-cultures in glycerol-stocks are available anytime for further analysis and can be stored indefinitely. In direct competition experiments, the relative fitness of evolved phenotypes to their ancestor is determined [1], which is an important tool to quantify changes in fitness during the adaptation to the biotic and abiotic environment.

Bacterial populations were already used in a variety of long-term evolution experiments [37, 39, 41, 42]. Moreover, utilizing microorganisms to study positive intraspecies interactions seemed to be appropriate [43-45]. In the most rapid example, *Pseudomonas fluorescens* evolved a cooperating phenotype within 3 days incubation in a liquid stable environment [46]. Overall, microbial model systems are relevant instruments to study evolution, and might also allow implications for more complex systems in nature [1, 47]. Additionally, it was previously noted that there are indeed analogs between social cooperation in higher eukaryotes and prokaryotes [48].

On that account two microbial strains interacting and sharing goods in a spatially structured environment for generations can be used as a model system to determine the effect of spatial structures on the evolution and maintenance of mutualisms.

1.4 Amino Acid Cross-Feeding

The principal of cross feeding is the exchange of goods between microorganisms [49], and is generally based to the mechanism of invested benefits [6, 50]. Cooperative interactions in the world of microorganisms are common and diverse [50, 51]. Among these cooperative traits, cross-feeding is the act of exchanging metabolites and can be classified as a form of reciprocal altruism [52]. The majority of known cross-feeding based mutualistic interactions of prokaryotes exists in association with eukaryotes, whereas much less such interactions are known among microbes [53]. This is why unculturable bacteria are suggested to exhibit much specified cooperative interactions with other organisms that are difficult to mimic in the lab [53]. Moreover, bacterial strains were recently shown to loose traits much more rapidly than assumed, which is then compensated via the interaction with other species [54]. The most prevalent example of partners, sharing metabolites for the mutual benefit of both, is legumes in association with *Rhizobia*. The legume provides nutrients for the bacterial endosymbiont and supports microaerophilic conditions. The bacteria in the nodules fix nitrogen and release amino acids, which are metabolized by the host, thereby increasing its fitness. In many cases of cooperative interactions in the microbial world, partners complement each other in the degradation process of a substance. Usually, one metabolite is released as a degradation product, which is further degraded by another bacterial strain.

By removing this metabolite, conditions are kept thermodynamically favourable for the degradation process of the first strain [3]. This type of cooperative interaction is termed syntrophy. In contrast to mutualistic cross-feeding, classical syntrophy describes only one partner "feeding" another by excreting a metabolic waste product without paying a cost. The second partner instead provides a service in manipulating the environment. One example would be the association of methanogens and ethanol fermenters [55].

Cross feeding of amino acids has been observed in mutualistic interactions. Bacterial symbionts provide amino acids for their insect-host in many cases [56]. Analysis of soil bacteria revealed that up to 15% of culturable bacteria are auxotrophic for amino acids, predominantly from the class *Bacilli* (Holger Merker, unpublished data). Lactic acid bacteria were also found to exhibit amino acid auxotrophies [57]. In addition, amino acid auxotrophic *Pseudomonas aeruginosa* phenotypes were isolated from cystic fibrosis patients [58]. Hence, the release and uptake of amino acids is a natural process, which is capable to be utilized for the construction of a bacterial model system. This was first shown by Shou *et al.* 2007, where they engineered cross-feeding of the amino acid lysine and the nucleobase adenine within the yeast *Saccharomyces cerevisiae* [45]. One Terminal amino acid synthesis genes can also be removed from the genome of *Escherichia coli* to establish specific auxotrophies for amino acids. Auxotrophy cannot be established for every proteinogenic amino acid. This is because some of them are degradation products of other amino acids (aspartate, glycine, serine), or the biosynthesis pathways are connected (glutamine and asparagine; valine and isoleucine) (Felix Bertels, Diploma thesis). That is why several gene knockouts would generate double auxotrophs. The amino acids, auxotrophies were established for, are given in Figure 1-1. Combined in a coculture in minimal medium, amino acid auxotrophic *E. coli* strains represent a synthetically designed by-product interaction. Such a consortium can be utilized for approaches addressing questions in evolutionary biology. The designed interaction is obligate since the extinction of one strain would remove the focal amino acid source for the respective partner, and based on by-products as both strains naturally release amino acids in their environment. When constructed amino acid overproducers are combined in such a consortium, even the *E. coli* wild type is outcompeted in a head-to-head fitness assay (Pande *et al.* 2013, submitted). This demonstrates the potential of a by-product interaction to evolve towards an efficient cooperating consortium. Evolved cooperation will

be characterized by increased amounts of a shared metabolite, relative to ancestors. This release of elevated amino acid concentrations is costly for each partner, because the metabolic anabolism of amino acids in minimal medium is energy consuming. Thereby, another requirement to describe such a designed consortium to be cooperative is fulfilled: Paying costs. Several studies already demonstrated the potential of such a model system [38, 45, 59].

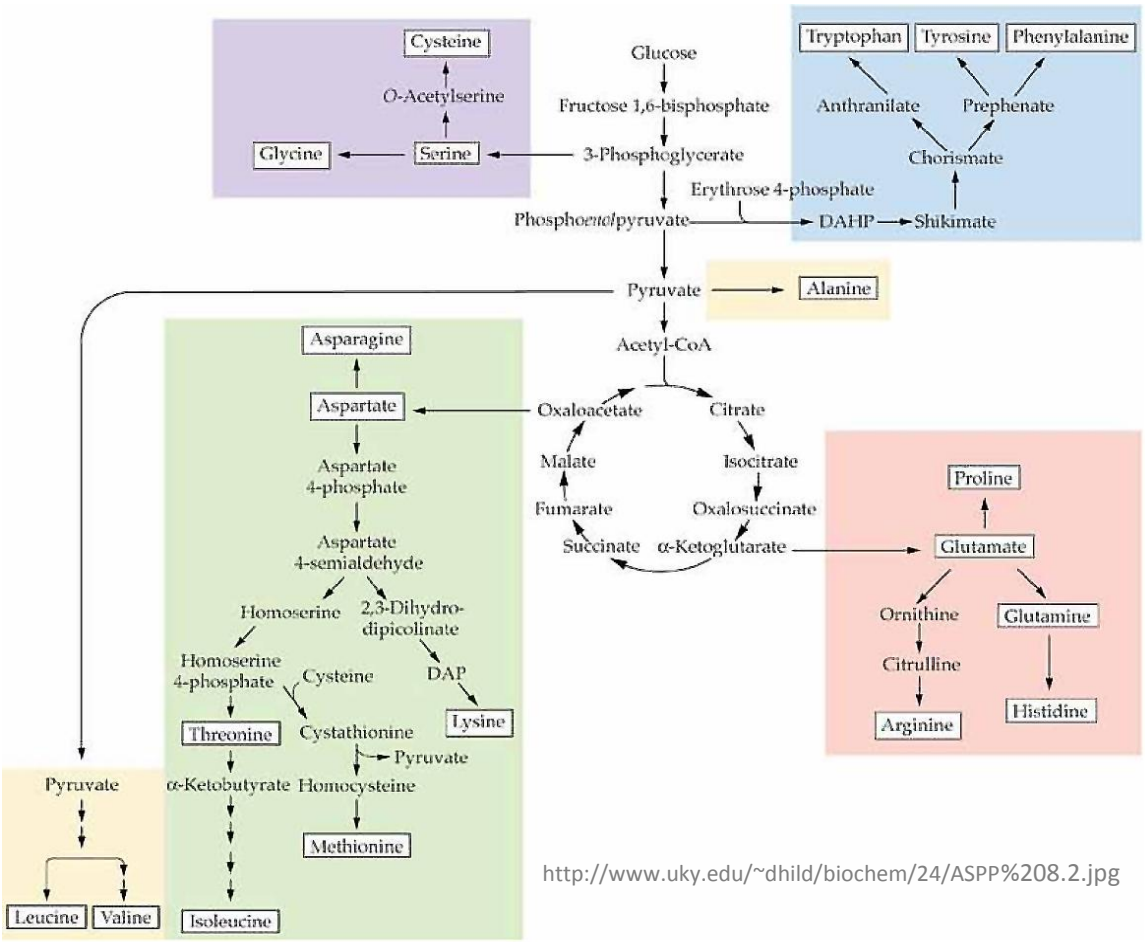


Figure 1-1: Amino acid biosynthesis families. The synthesis of following amino acids can be blocked with a gene-knockout: Arginine, histidine, proline, lysine, methionine, threonine, leucine, isoleucine, tryptophan, tyrosine, and phenylalanine.

The term “essential” is used in a related context, describing an organism to be dependent on the external supply of one or more metabolites. However, this term was not allowed in the manuscript of a recently transmitted publication (Pande *et al.* 2013) to define the demand of an auxotroph on a specific amino acid. Therefore, the term “focal” will be used instead in this work.

1.5 DESIGN OF A LONG-TERM EXPERIMENT

In this work, spatial structures are of special interest. To verify a positive influence of this ecological factor on the evolution of cooperation, the experimental design has to be adjusted for this purpose. Biofilms, common in the world of microorganisms, fulfil the conditions of a spatially structured environment. However, biofilms are complicated to characterize. The productivity and total number of cells are difficult to analyse. In addition, biofilms are unsuitable for experiments with serial transfers, because destruction of the biofilm under harsh conditions is necessary to propagate a representative amount of every subpopulation into the new generation (i.e. into fresh medium). This treatment could alter the outcome of an experiment in a sensitive way and limit the reproducibility. Due to have a more predictable system, agar will be utilized as an artificial spatially structured environment and a consortium of *Escherichia coli* will interact on its surface. Since there will be utilized an interaction between partners of the same species, this cannot be termed “mutualism” but “cooperation”.

The long-term experiment will include one group with non-shaken spatially structured cultures and one reference group with shaken spatially non-structured cultures. Both treatments can be compared concerning growth and cooperative aids during a coevolution experiment. Observed differences between the environments will be assumed as positive effects of the spatial structure. The basic idea behind the long-term experiment is to transfer interacting populations regularly for a time span of several weeks. At every transfer, the cultures are homogenized and an aliquot is used to inoculate fresh medium to found the next cycle. Thereby, the optical density is measured and the consortia are plated on agar to determine total cell numbers and the ratio of partners. At the same time, a sample will be taken for cryo-stocks to enable further genetic analysis and comparison with the ancestor. Meanwhile, the productivity as well as dynamics in the population is monitored.

At the end of the long-term experiment cooperating phenotypes and, as an optional target, cheaters will be isolated. Coevolved strains and ancestors will be compared for further conclusion, which includes fitness as a quantitative parameter for productivity [1]. For this purpose, the growth of the coevolved partners, relative to the corresponding ancestors, will

be determined. Coevolved *E. coli* consortia are expected to exhibit higher productivity than the ancestor, what can be explained by overproducing and sharing goods.

Increased productivity is assumed to be established either via elevated efficiency in amino acid utilization, or increased amounts of released amino acids as an indicator of true cooperation. Therefore, amino acid analysis will be performed with the ancestor as well as the evolved strains. Detected concentrations are expected to reflect a similar outcome as the comparison of productivity between coevolved consortia. Cocultures evolved on the agar surface should release significantly higher amounts of amino acid than the liquid-evolved cocultures, which represent evolved cooperation.

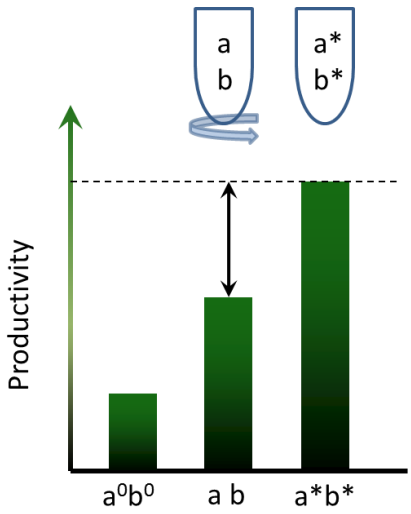


Figure 1-2: Proposed experimental design and expected fitness (productivity) derived in the long-term experiment. a^0 and b^0 represent the ancestor-strains, whose productivity should be less than that of cocultures "a b" and "a*b*", caused by adaptation neither to the abiotic environment, nor to a partner. "a*b*", agar-evolved, is expected to perform better than "a b", liquid-evolved. The coevolved partners a*b* are suggested to perform significantly better and to exhibit improved cooperation. The significant difference in productivity between spatially structured and non-structured cultures (black arrow) will be exclusively caused by the spatial structure.

The derived interaction between the *Escherichia coli* strains should fulfil following criteria to be considered as a mutualism:

The coevolved strains on agar invest a shared costly good, each beneficial to the respective partner [29]. Strains from the liquid environment are suggested to release amino acids similar to the wild type and must have a significant lower fitness than agar-evolved populations. Accordingly, cooperators excreting amino acids to benefit an absent partner would sustain a fitness disadvantage in the combinations **a*b$^{\lozenge}$** and **a$^{\lozenge}$b***. As cooperating partners pay a certain cost, they have to benefit in an exceeding magnitude.

1.6 OBJECTIVES

During the long-term coevolution experiment, the performance of an amino acid cross-feeding consortium will be observed. A spatially structured environment, represented by an agar-surface will be compared to a liquid, spatially non-structured reference environment. In general, the structured environment is theoretically assumed to support the evolution of a cooperative interaction. The liquid environment should not support the evolution of a cooperative interaction or at least in significant difference to the agar-surface.

1) The growth of the consortium improves over time. Total productivity represented by optical density as well as total counts of colony forming units (cfu) of both partners increase.

2) The ratio of partners is not stable but exhibits dynamics and fluctuation.

3) Cooperating phenotypes, which release increased amounts of amino acid, evolve in both strains. Concentrations of released amino acids are higher in the evolved strains as compared to the ancestors.

4) Cooperators pay a cost for releasing increased amounts of amino acid.

5) The fitness of evolved consortia is higher than in the ancestor and at the highest level in the environment, where the long-term experiment took place.

Following hypothesis ideally account for the spatial structure and should by contrast either not apply to the liquid environment, and be significantly different: 1), 3), 4), 5)

2 MATERIAL AND METHODS

2.1 MEDIA AND SOLUTIONS

Media and solutions were autoclaved for 20 min at 121°C, if not announced different.

M9 minimal salts (Sigma-Aldrich, St. Louis, USA)

Following quantities of salts were dissolved in 1 l bi-distilled water:

6.8 g/l	Disodium phosphate (Na_2HPO_4)
3 g/l	Monopotassium phosphate (KH_2PO_4)
0.5 g/l	Sodium chloride (NaCl)
1 g/l	Ammonium chloride (NH_4Cl)

To 1 l of autoclaved M9 minimal medium, the following sterile supplements were added:

2 ml	1 M Magnesium sulfate ($MgSO_4 \bullet 7H_2O$) in H_2O bidest
0.1 ml	1 M Calcium chloride ($CaCl_2$) in H_2Obidest
5 g/l	Glucose (25 ml 20% Glucose solution)

LB broth (Carl-Roth, Karlsruhe, Germany)

Composition:

10 g/l	Tryptone
5 g/l	Yeast extract
5 g/l	Sodium chloride (NaCl)

Minimal medium for Azospirillum brasiliense (MMAB)

The composition of MMAB-medium according to Vanstockem *et al.* 1987 [60]:

3 g/l	K_2HPO_4
0.15 g/l	KCl
1 g/l	NaH_2PO_4
1 g/l	NH_4Cl
50 mg/l	Na_2MoO_4

The pH was adjusted to 6.85 – 7.0 before autoclavation.

Added sterile-filtrated solutions after autoclavation:

5 g/l	Fructose
5 ml/l	$MgSO_4$ (60 g/l)
0.5 ml/l	$CaCl_2$ (20 g/l)
0.25 ml	$FeSO_4$-EDTA (12.62 g/l $FeSO_4 \bullet 7H_2O$; 11.84 g/l EDTA)

TA-Agar

10 g/l	Tryptone
1 g/l	Yeast extract
5 g/l	NaCl
16 g/l	Kobe agar

Added sterile-filtrated solutions after autoclavation:

50 ml/l	20% L(+)Arabinose-stock
1 ml/l	5%-TTC-stock (Tetrazoliumchloride; Sigma)

Amino acid stock solutions

For each amino acid, the corresponding mass was dissolved in 10 ml of bidistilled water to receive concentrations of 150 µM. If an amino acid did not dissolve at room temperature, HCl-solution (32%) was added. Subsequently, stock solutions were filter sterilized.

2.2 STRAINS

Strains from the Keio collection [61] were purchased from the Coli Genetic Stock Center (CGSC), Yale University, USA. Genetic modification regarding gene-deletions and phenotypic labelling was established by Glen D'Souza (D'Souza *et al.* 2013, submitted).

In total, eleven amino acid auxotrophic strains were constructed, where each single amino acid synthesis gene was replaced via transduction and homologous recombination with a cassette, carrying a Kan^R-gene.

Precultures were generally grown O/N between 16-18 hours at 30°C and 225 rpm, if not announced different.

2.3 METHODS

2.3.1 PREPARATION OF GLYCEROL STOCKS

Prior to storage of bacterial strains at - 80°C, 1 ml overnight culture was mixed with 1 ml of 40 % (m/v) glycerol after 16-18 h incubation at 37°C and 225 rpm in 5 ml LB medium. During the evolution experiments, 1 ml was directly taken from cultures for preparation of glycerol stocks.

2.3.2 DETERMINATION OF THE OPTICAL DENSITY IN BACTERIAL CULTURES

The optical density of bacterial cells was measured with a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, USA) using 96-well microtiter-plates. A volume of 200 µl was analysed at a wavelength of 600 nm. Blank reduction was applied in every case. The above-mentioned device was used consistently for preculture analysis.

2.3.3 KINETIC MEASUREMENTS

Precultures were grown overnight in M9-medium containing 100 µM focal amino acid. The OD_{600} of precultures was measured in a plate-reader (Spectramax) and set to OD 0.02. Therefore, 50 µl of preculture were centrifuged; subsequently the pellet was resuspended in a respective amount of sterile distilled water or 0.85% NaCl-solution. To inoculate the 96

well plate, 10 µl of OD 0.02 culture were pipetted into 200 µl of M9-medium containing 0.5% glucose. A blank was also added to the 96-well plate before it was sealed with a transparent foil to prevent evaporation of culture during incubation. Initial OD_{600} was therefore 0.001. In general, the following parameters were kept constant in every measurement: incubation temperature of 30°C, wavelength of 600 nm for determination of optical density and cycles of 5 minutes in 24 h measurements. At the beginning of every cycle, cultures were shaken for 180 seconds, followed by measurement of OD_{600}. If not announced different blanks were carried in every experiment and blank reduction was performed before measurements were further analysed.

2.3.3.1 Kinetic Growth with Amino Acid Supplementation

The growth rate and maximum optical density was determined during 24 hours incubation. The medium was supplemented with different concentrations of amino acid (0 µM, 1 µM, 5 µM, 20 µM, 100 µM, and 500 µM). Each eight biological replicates were grown in preculture overnight and 96-well plates were inoculated as previously described (2.3.3). The kinetic measurements were performed in a Tecan F200 (Tecan, Männedorf, Switzerland) using the software iControl (Tecan, Männedorf, Switzerland). Data was collected and μ_{max} as well as OD_{max} were calculated in R, using eight separate time points for analysis.

2.3.3.2 Unsupplemented Auxotrophs in Monoculture – Gain of Function

O/N precultures of each eight biological replicates of all eleven available amino acid auxotrophic strains were incubated with 50 µg/ml kanamycin, to ensure homogeneity of cultures. Precultures were treated, as already described with one exception. OD600 of precultures was set to 1 and 10 µl were used for inoculations. This resulted in a comparably high initial OD of 0.05. This density was chosen, to enhance any current effects of revertant phenotypes. Cycles of OD determination were elongated to 30 minutes, since the total incubation was increased to 72 hours. Therefore, a second period of shaking was introduced in every cycle, to prevent cultures before settling down and attaching to the surface of the 96-well plates. OD_{600} was monitored with Magellan software (Tecan, Männedorf,

Switzerland) on a Tecan M200 plate reader (Tecan, Männedorf, Switzerland). Again, eight times were used for μ_{max} calculation, but only 6 time points for OD_{600} determination. Analysis was performed within in the Magellan software.

2.3.4 COCULTURE EXPERIMENTS

All coculture experiments, comprised in this chapter, were performed in M9-minimal medium, containing 0.5% glucose as carbon source. If not announced different, precultures were prepared for inoculation as mentioned in 2.3.3.

2.3.4.1 COCULTURE

For the coculture experiment, eight precultures for a total of nine amino acid auxotrophic *Escherichia coli* strains were incubated in medium with 50 µg/ml kanamycin (except wild type). Only arabinose negative strains were used for this experiment. To set the ODs of precultures to 1, 4 ml of each culture were centrifuged and the pellet was resuspended in the corresponding amount of M9-medium. Prior to inoculation each 150 µl of OD 1 precultures were mixed to establish the consortia in every combination of used auxotrophs. 50 µl of the consortia, exhibiting an OD_{600} of 1, and 50 µl of single auxotrophs and wild, which were also set to OD_{600} 1, were used to inoculate 1 ml of M9-minimal medium in 96-deep well plates. For every day in total four deep well plates contained in sum eight biological replicates of each coculture, eight biological replicates of monocultures of auxotrophs, and twelve biological replicates of the *E. coli* wild type as well as twelve blanks. Since the coculture experiment was prepared for four days of measurements, 16 deep well plates were inoculated. Cultures were shaken at 225 rpm at 30°C for total 120 hours. Optical density was determined every 24 hours starting after 48 hours growth in four deep well plates per day, which contained all co,- and monocultures mentioned above. Aliquots of 200 µl culture were measured in a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, USA) using 96-well microtiter-plates.

2.3.4.2 PARTNER EQUILIBRIUM IN COCULTURE

Microcosms, as shown in Figure 2-1, were filled with aliquots of 4 ml M9 liquid medium, or 4 ml M9 agar. Each six O/N precultures of *E. coli ΔtrpB* ara+ and *E. coli ΔtyrA* ara- were centrifuged and pellets were resuspended in M9 medium to receive an OD_{600} of 8. Subsequently, parts of these cultures were diluted to prepare bacterial solutions with an optical density of 4 and 2. Identical aliquots of cultures with the same optical density were mixed and finally derived cocultures were used to inoculate each six microcosms with 10 µl of consortia. Final densities were thus 0.02, 0.01, and 0.005 in liquid cultures. In the centre of the agar surface in spatially structured microcosms 10 µl of bacterial suspension was gently placed. Microcosms were not moved until the liquid diffused into the agar and the consortium was thereby fixed on the surface. There had to be incubated six agar microcosms for every day of analysis. Due to a three following days of analysis, in total 18 microcosms were inoculated, because for cfu determination the colony on the agar surface had to be removed by resuspension in medium. This was not the case for liquid microcosms, from which an aliquot was taken every day for analysis. After incubation, cfu and ratios of combined precultures were determined for t_0-values. Therefore, a dilution series was prepared and two dilutions per biological replicate were plated on TA-agar. Liquid microcosms were shaken with 225 rpm at 30°C and agar microcosms were incubated fixed at 30°C. Colony forming units were determined after two, three, and four days of incubation.

2.3.4.3 SPATIAL STRUCTURE

The OD_{600} of each six O/N precultures of *E. coli ΔtrpB* ara+, *E. coli ΔtyrA* ara-, *E. coli ΔpheA* ara+, and *E. coli ΔthrC* ara- was set to 1. After that, *E. coli ΔtrpB* was mixed with *E. coli ΔtyrA* and *E. coli ΔpheA* with *E. coli ΔthrC*. A volume of 10 µl was used to incubate agar microcosms with cocultures. On the agar surface filters of either the material cellulose acetate or polyethersulfone, or nylon membranes were placed. The microcosms were incubated for four days at 30°C. Then the filters or membranes were removed from the agar and the consortium was resuspended. Dilutions were plated on TA-agar to determine the colony forming units of each partner.

2.3.5 Quantification of Released Amino Acids

2.3.5.1 Culture Conditions

The experimental strains were streaked from glycerol-stocks on LB-Agar and incubated at 30°C for 16-18 hours. Colonies of the *E. coli* wild types as well as genetically modified strains were used to inoculate 1 ml M9 liquid medium. In the case of evolved populations and their ancestors in the long-term experiment, cocultures were plated on TA-agar from glycerol stocks. From these plates, predominant phenotypes were picked for precultures, in which 20 colonies were selected in total for every auxotroph per consortium. This means that from every replicated coculture, which was selected for analysis, in total 40 colonies were selected for amino acid analysis. After 16-18 hours incubation at 30°C and 225 rpm the OD_{600} was determined. If there was no growth detected for an evolved phenotype, incubation time was prolonged to 48 hours. After incubation of evolved phenotypes, colony-forming units were determined from a dilution series, which was plated on TA-agar. Thereby, homogeneity of precultures was confirmed.

2.3.5.2 Harvesting of Supernatants and Amino Acid Derivatisation

To remove the cells from the preculture, 400 µl were centrifuged utilizing an AcroPrep™ 96 filter plate (Pall Corporation, USA) with 0.2 µM pore size. During 10 min centrifugation with 3850 rpm, the supernatant was collected in a subjacent NUNC™ 96 well plate. In a second NUNC™ 96 well plate, 100 µl of sodium borate buffer (0.8 M; pH 8) were added to 100 µl supernatant. The borate buffer contained the internal standard amino acid mix of ^{13}C-, ^{15}N-labeled amino acids (algal amino acids ^{13}C, ^{15}N, Isotec, Miamisburg, USA) at a concentration of 20 µg of algal amino acid mix per 1 ml of borate buffer. Subsequently 200 µl of 50 mM fluorenylmethyloxycarbonyl chloride (FMOC-Cl) dissolved in acetonitrile were mixed with the samples and derivatisation with the amino acids was allowed for 5 min. To remove remaining FMOC-Cl, the samples were mixed with 800 µl n-hexane. After separation of the n-hexane from the water-phase, 100 µl of the latter was transferred into another NUNC™ 96 well plate.

2.3.5.3 QUANTIFICATION OF AMINO ACID CONCENTRATIONS

Liquid chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) with a Zorbax Eclipse XDB-C18 column (50x4.6 mm, 1.8 µm, Agilent Technologies, Germany). 10 µl of derivatised sample were injected and amino acids were separated with formic acid (0.05%) in water and acetonitrile as mobile phases A and B respectively. The elution profile at 25°C and 1.1 ml/min flow rate was: 0-1 min, 90%A; 1-4.5 min, 0-90% B in A; 4.51-5 min 100% B and 5.1-8 min 90% A. The eluted fractions were analysed directly after liquid chromatography with a coupled API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source operated in negative ionization mode. Infusion experiments with FMOC-derivatised standards (amino acid standard mix, Fluka, St. Louis, USA) were used to optimize the instrument settings (ionspray voltage: -4500 eV; turbo gas temperature: 700°C; Nebulizing gas: 70 psi; curtain gas: 35 psi; heating gas: 70 psi; collision gas: 2 psi). By multiple reaction monitoring (MRM) analyte parent ions and product ions were detected. For details of MRMs, see Table 2-1. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Finally, the Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. The concentration of the individual amino acids in the ^{13}C-, ^{15}N-labelled amino acids mix had been determined by classical HPLC-fluorescence detection analysis after pre-column derivatisation with ortho-phthaldialdehyde-mercaptoethanol using external standard curves made from standard mixtures (amino acid standard mix, Fluka plus Gln, Asn and Trp; also Fluka). Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N labelled amino acid internal standard. Cysteine, which easily oxidizes to the disulphide cysteine, as well as the stereoisomers L-leucine and L-isoleucine, cannot be distinguished by the applied method.

Table 2-1: Multiple Reaction Monitoring (MRM). Parameters for FMOC-derivatised amino acids on Triple quadrupole mass spectrometer (API3200, Applied Biosystems, Darmstadt, Germany)

Amino acid	Retention time [min]	Declustering potential (DP)	Collision energy [eV]	Analyte ($m/z_{parent\ ion}$ > $m/z_{product\ ion}$)	Internal standard ($m/z_{parent\ ion}$ > $m/z_{product\ ion}$)
Ala-FMOC	4.31	-25	-10	310 > 88	314 > 92
Arg-FMOC	3.27	-45	-18	395 > 173	405 > 183
Asn-FMOC	3.7	-35	-12	353 > 157	359 > 163
Asp-FMOC	3.93	-40	-16	354 > 157.8	359 > 162.8
Gln-FMOC	3.7	-35	-12	367 > 145	374 > 152
Glu-FMOC	3.9	-40	-14	368 > 172	374 > 178
Gly-FMOC	4.14	-30	-10	296 > 74	299 > 77
His-FMOC	3.19	-55	-16	376 > 154	385 > 163
His-FMOC2	5.36	-40	-18	598 > 154	607 > 163
Leu-FMOC	4.88	-30	-10	352 > 130	359 > 137
Lys-FMOC2	5.35	-45	-20	589 > 145	597 > 153
Met-FMOC	4.61	-30	-18	370 > 174	376 > 180
Phe-FMOC	4.84	-30	-10	386 > 164	396 > 174
Pro-FMOC	4.44	-30	-10	336 > 114	342 > 120
Ser-FMOC	3.87	-25	-14	326 > 130	330 > 134
Thr-FMOC	4.01	-35	-14	340 > 144	345 > 149
Trp-FMOC	4.71	-25	-12	425 > 203	*
Tyr-FMOC	4.24	-35	-10	402 > 180	412 > 190
Val-FMOC	4.67	-30	-10	338 > 116	344 > 122

* Quantification with ^{13}C, ^{15}N-Phe applying a response factor of 0.42

2.3.6 Design of Long-Term Experiments

The evolution experiments took place in crew glasses (Figure 2-1). Either 4 ml of completed M9 medium or warm M9 agar were filled into the glasses, which will also be termed "microcosms". During incubation the glasses were tightly closed to ensure the same amount of oxygen in each microcosm and thereby comparability of the different treatments.

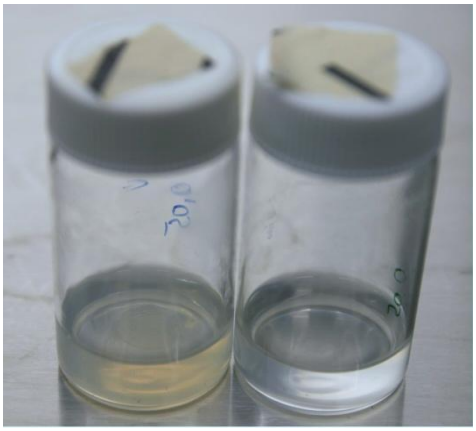


Figure 2-1: Small screw glasses as used for the evolution experiment. Left bottle contains agar; right bottle contains liquid medium. (WHEATON, Millville, NJ, USA)

2.3.6.1 *E. coli* Wild Type

For the *E. coli* wild type evolution experiment, the arabinose positive and the arabinose negative phenotype were used. Each one colony of both phenotypes was picked from LB agar plates and 5 ml of LB broth were inoculated. Overnight cultures were set to OD_{600} of 0.4. For the experiment, three kinds of environments were chosen: liquid shaken medium (225 rpm), liquid non-shaken medium, and agar surface. Each treatment had eight replicates, out of which four were inoculated with 10 µl preculture of each *E. coli* phenotype. This amount of bacterial suspension was gently placed on the agar surfaces and let diffuse into the agar, before the microcosms were placed in the incubator. MMAB medium was chosen for the experiment, supplemented with 0.5% fructose. Microcosms were incubated at 30°C. Every 24 hours OD_{600} was determined and 20 µl of the liquid cultures were propagated into 4 ml of fresh medium in another microcosm. The colonies on agar were resuspended with 2 ml of MMAB medium prior to propagation of 10 µl culture on fresh agar.

Glycerol stocks were prepared weekly. The total duration of the evolution experiment was 86 days.

After 52 days incubation, which reflects approximately 400 generations, a screening for evolved auxotrophs was performed. Two microcosms of each treatment were therefore randomly selected and dilution series were plated on TA-agar. From these agar plates, 500 colonies were transferred with pipette tips in 96 well microtiterplates, containing MMAB-medium, and on plates with MMAB-agar with yeast extract. The former medium would only let prototrophic colonies grow, whereas the latter agar represented a full medium as a backup for eventually detected auxotrophs. In total, 3000 colonies were tested. Optical density in 96 well microtiterplates was measured after 24 hours incubation at 30°C.

2.3.6.2 Coevolution of Amino Acid Auxotrophs

In preparation of the coevolution experiment, precultures of each six biological replicates of *E. coli ΔtrpB* ara+, *E. coli ΔtrpB* ara-, *E. coli ΔtyrA* ara+ and *E. coli ΔtyrA* ara- were incubated O/N. The OD_{600} of precultures was set to 2. Thereafter each 150 µl of complementary phenotypes were mixed, to receive twelve cocultures consisting of each two auxotrophic strains with different arabinose activity. For inoculation 10 µl of each coculture were used to inoculate two microcosms, containing either 4 ml liquid M9-medium, or 4 ml M9 agar. Hence, the initial OD_{600} in the microcosms was 0.005. The procedure of inoculation and further incubation was similar to the wild type evolution experiment. In parallel, dilution series were prepared, which were plated on TA-agar (dilutions 10^{-6} and 10^{-7}) for determination of exact initial ratio and colony forming units of strains. Cocultures were incubated for seven days at 30°C, either shaken for liquid cultures or fixed for agar.

Cultures were transferred weekly. In 1:4 treatment 1 ml and in 1:20 treatment 200 µl were transferred into fresh medium, or on fresh agar. In treatment with OD_{600} of 0.005, bacterial suspension was set to OD_{600} of 2, after colony was washed from agar with 4 ml 0.85% NaCl-solution. Dilutions consortia were plated on TA-agar for cfu and ratio determination. OD_{600} was measured and glycerol stocks were prepared.

Within this thesis, the arabinose activity in cocultures will be given as the abbreviation "+-" or "-+", in which the first activity accounts for *E. coli ΔtrpB* and the second for *E. coli ΔtyrA*. (see Figure 3-12 and Figure 3-13)

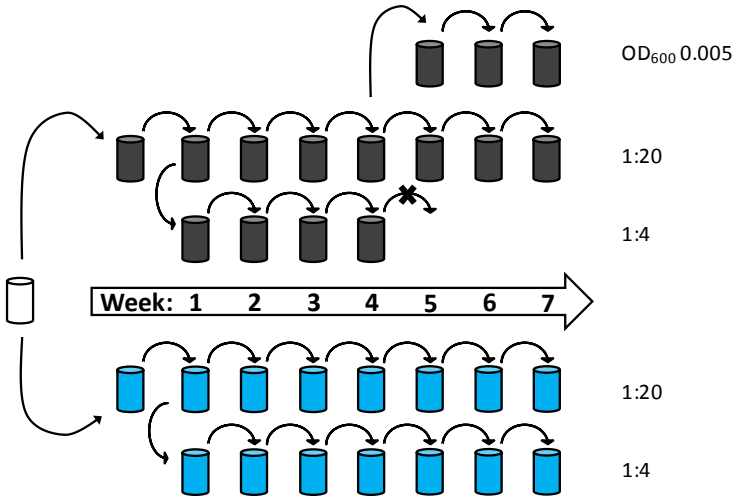


Figure 2-2: Propagation model during the coevolution experiment. Scheme demonstrates dilutions and number of transfers in each treatment. Grey represents agar microcosms and blue represents liquid shaken microcosms. One combination of biological replicates of precultures was used to inoculate one microcosm in each treatment. A parallel setup of microcosms was found after the first week and was further propagated with weekly 1:4 dilution. In agar microcosms, this 1:4 dilution setup was excluded after four weeks coevolution. After four weeks, another parallel series was found from agar microcosms, which was set to initial OD_{600} of 0.005 at every transfer.

2.3.7 Statistical Analysis

For statistics, data was categorized and prepared for analysis in the program SPSS. Data was first tested for skewness and kurtosis. After this the Levene-test for homogeneity of variances was performed. If this test was significant for inhomogeneity of variances or the allowed level of skewness and/or kurtosis was exceeded, data transformation was applied. If the transformation did achieve acceptable levels in skewness and kurtosis, as well as homogeneity of variances, an appropriate statistical test was applied. If the transformation did not result in proper quality of the distribution of values, linear mixed effects models were utilized in R.

3 RESULTS

The results section is devided in two major chapters: The part of characterization includes all the preliminary experiments. These experiments were necessary to establish and characterize an amino acid cross-feeding consortium that exhibits cooperative growth; and the part of performing a long-term coevolution experiment with further analysis.

This first section contains the verification of genetically modified *Escherichia coli* strains to exhibit one specific amino acid auxotrophy. Therefore, these strains grow amino acid concentration-dependent. Released proteinogenic amino acids, analysed in culture-supernatants are also shown, representing a premise for cooperative amino acid cross-feeding in cocultures of such auxotrophs. Furthermore several cocultures of each two amino acid auxotrophic *E. coli* strains were analysed in regard to their ability to cooperatively grow in minimal medium. Monocultures of these strains were tested for their performance in minimal medium during several days of incubation. Finally the initial performance of selected consortia on different surfaces, representing spatial structures, was determined. In addition, the required design of a long-term coevolution experiment in a spatially structured environment, capable to test the hypothesis presented in the introduction, is investigated.

In the following chapter 3.2 the outcome of the long-term experiments is presented. In detail this is one *E. coli* wild type monoculture experiment and one *E. coli ΔtrpB* / E. *coli ΔtyrA* coculture experiment. Both were performed in different spatially structured environments. Relevant outcomes for the evolved coculture, such as optical density and the ratio of strains over time, are presented and supplemented with results of further analysis. Those comprise amino acid analysis data and the fitness comparison of evolved phenotypes with the ancestor. Lastly phenotypic appearance in evolved cocultures of auxotrophs and evolved wild-type monocultures is presented.

3.1 PRELIMINARY EXPERIMENTS

3.1.1 TEST FOR AUXOTROPHY

As a preliminary experiment it was recommended to ensure that the constructed auxotrophic *Escherichia coli* strains are specific for each defined amino acid. This is essential for a well defined consortium, consisting of two amino acid auxotrophs, in the conducted long-term experiment. The genetically modified strains were grown for 24 hours in minimal medium, minimal medium with 19 amino acids, except the amino acid they are auxotroph for, and minimal medium with the particular focal amino acid. In both former media strains were not able to grow. Growth was exclusively observed in minimal medium supplemented with the amino acid which they are auxotroph for. On average, optical densities between 0.1 and 0.3 were reached, as shown in Figure 3-1. In detail, *E. coli ΔleuB* reached th lowest OD, whereas *E. coli ΔargH* exhibited three-fold growth. Differences in the OD_{600} measured after 24 hours indicate either altering growth rates or maximum ODs of the auxotrophic strains, indicating individual demands, uptake rates, or metabolic utilisation of the focal amino acid. These characteristics were further tested in another experiment (3.1.2 Kinetic Growth). However, the outcomes of both experiments are contradictory and will be discussed in chapter 4.1.

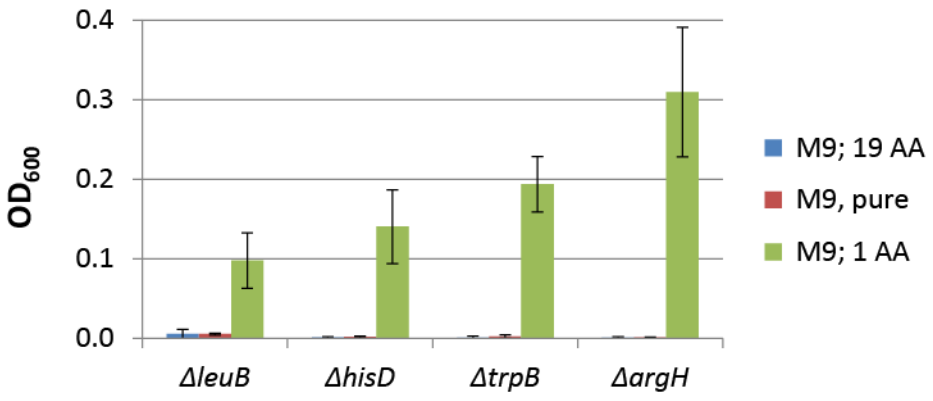


Figure 3-1: Dependency of selected amino acid auxotrophic *E. coli* strains on a specific amino acid. Strains were grown in M9-minimal medium 0.5% Glucose, either supplemented with all proteinogenic amino acids (each 100 µM) except the critical one, or the particular amino acid (100 µM), or without amino acid supplementation. Optical density (600nm) was determined after 24h growth at 30°C and 225rpm. (Error bars given as 95% CI; n=8)

3.1.2 KINETIC GROWTH

Amino acid auxotrophs should exhibit growth, dependent on the amino acid concentration in the medium. Therefore selected genetically modified strains were tested in regard to their growth in minimal medium supplemented with amino acid concentrations ranging from 1 µM to 500 µM. Figure 3-2 shows the outcome of the conducted experiment. Each strain was exclusively supplemented with its specific amino acid. The maximum OD_{600} and maximum growth rate were calculated from recorded growth curves. Strains either did not grow, or grew marginalyl in minimal medium with 0 µM, 1 µM and 5 µM amino acid, whereas the histidine auxotroph reached OD 0.05 already at 5 µM. The maximum OD increased with increasing amino acid concentration. This is not the case for the leucine and the histidine auxotroph, which reached the highest OD with 100 µM leucine and histidine, respectively. The highest desities were reached by *E. coli ΔleuB* with approximately OD_{600} 0.3, which is in contrast to experiment 3.1.1. As already mentioned, this will be discussed later. The other auxotrophs exhibited comparable maximum ODs of about 0.2. In one case, namely *E. coli leuB*, the OD decreased at 500 µM amino acid supplementation.

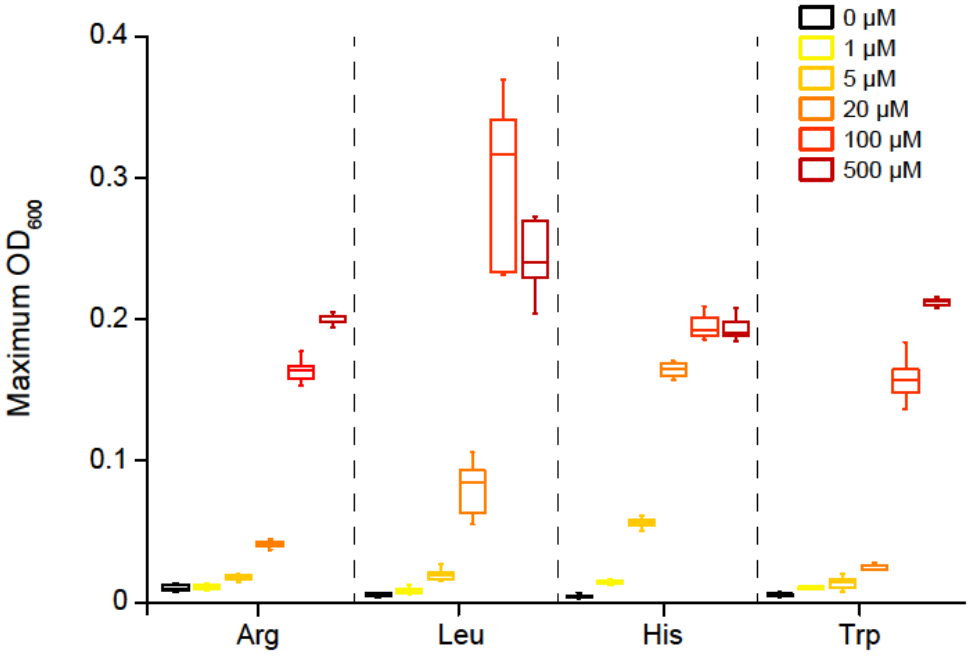


Figure 3-2: Maximum OD_{600} of auxotrophs supplemented with different amino acid concentrations. Cultures within a 96 well plate were grown in a plate reader for 24 hours. M9-minimal medium contained 0.5 % Glucose. OD_{600} was determined every 5 minutes and maximum optical density was calculated with R using 8 time points. (Errors given as 95% CI; n=8)

Strains increased in maximum growth rate with increasing amino acid concentration, as observed for maximum OD. Strains did grow at lowest rates with 0 µM, 1 µM and 5 µM amino acid, whereas the Histidine auxotroph started to increase in growth rate at 5 µM. Also for $\mu_{Max,}$ *E. coli ΔleuB* showed the highest values. The other auxotrophs exhibited comparable maximum growth rates, with values between 0.003 and 0.0035 OD_{600}/hour. The observed growth rates are consistent with the observed maximum ODs.

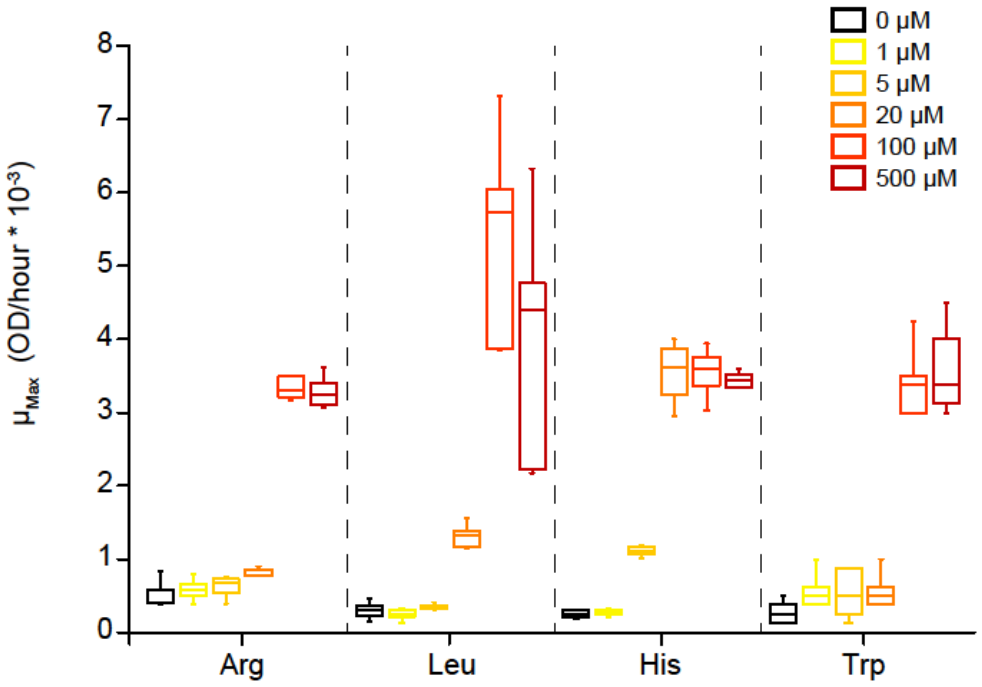
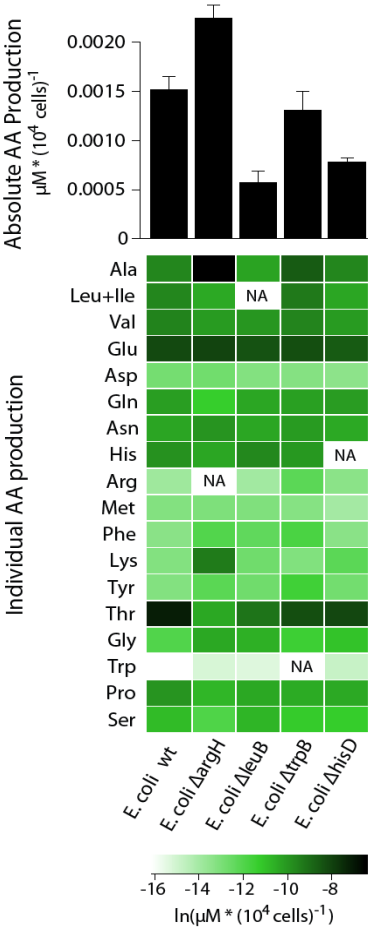


Figure 3-3: Maximum growth rates of cultures supplemented with different amino acid concentrations. Cultures within a 96 well plate were grown in a plate reader for 24 hours. OD_{600} was determined every 5 minutes and maximum growth rates were calculated with R using 8 time points. (Errors given as 95% CI; n=8)

3.1.3 Release of Amino Acids

One requirement for the synthetically established by-product interaction is that amino acids, especially the focal ones, are released by both partners of a consortium. This is a prerequisite for sufficient cross-feeding. Therefore LC-MS/MS was performed to quantify amounts of amino acids in the supernatant of monocultures grown in minimal medium. In Figure 3-4, absolute and individual amino acid concentrations are summarized for the *E. coli* wild type as well as auxotrophs. The four auxotrophic strains analysed, were already available at this time point, whereas more auxotrophs were constructed in the following month. Concentrations between $1*10^{-7}$ µM to $>9*10^{-3}$ µM amino acid in 10^4 cells were detected. The total amounts released by the analysed strains varied. The observed profiles did not change globally for all amino acids (see also Figure A-7-2 for an overview). Hence, only a minority of individual concentrations, predominantly alanine and threonine, and in one case lysine (*E. coli ΔargH*), were effected. Whereas *E. coli ΔleuB* and *ΔhisD* released less than the wt, the total concentration increased in the case of *E. coli ΔargH* and remained at the level of the wild type for *E. coli ΔtrpB*.



The *E. coli* auxotrophs had to be supplemented with one amino acid in the preculture. Those measured concentrations, which might only reflect remaining supplemented amino acid in the supernatant, were excluded from further analysis.

Figure 3-4: Absolute and individual amino acid release normalized to 10^4 cells. Individual concentrations of amino acids in culture supernatants of the *E. coli* wild type and four different auxotrophs were determined via LC-MS/MS after 18 hours incubation at 30°C and 225 rpm in M9 minimal medium. Total concentrations represent the sum of individual concentrations. NA: Not analysed. (Error bars given as 95% CI; n=5)

3.1.4 COCULTURE

For a long-term experiment using an obligate consortium of two amino acid auxotrophs, all combinations of nine different knockout mutants were analysed in regard to their ability to cross feed amino acids. For the proposed evolution experiment, finally one combination with positive growth effects was selected.

Each coculture consisted of two amino acid auxotrophic *E. coli* strains. Given growth effects in Figure 3-5 were determined by comparison of the growth in coculture and the growth of the respective single mutants in monocultures. Hence, the relative fold growth to monocultures is presented in this figure. The extent of cooperation within cocultures alterred with the mutants used, as already shown in Wintermute and Silver [62]. Several auxotrophic strains were capable to grow cooperatively in a consortium. *E.coli ΔthrC*, *E.coli ΔilvE*, *E.coli ΔtrpB* and *E. coli ΔtyrA* showed positive or neutral interactions in most combinations with other auxotrophs. Among these strains *E.coli ΔthrC* was the most compatible strain, showing positive interactions with every auxotroph tested. In contrast, *E. coli ΔhisD*, *E. coli ΔargH*, *E. coli ΔleuB* and *E. coli ΔpheA* appeared to grow in less cases synergistic, with *E. coli ΔhisD* interacting positive in only one combination. The most competitive interaction, 0.75-fold, was observed in the coculture of *E. coli ΔargH* and *ΔmetB*, whereas the most positive interaction, 5.75-fold, was detected in *E. coli ΔthrC-ΔilvA*. Overall only a few consortia grew at least 2-fold better than in monoculture. Those combinations, *E. coli ΔthrC-ΔilvA*, *ΔthrC-ΔtyrA*, *thrC-ΔpheA* and *ΔtyrA-ΔtrpB*, were selected for further preparative characterizations. Finally, the consortium *ΔtyrA-ΔtrpB* was chosen for the long-term experiment, because of a couple of reasons, which are described in chapters 3.1.7 and 3.1.5, and discussed in chapter 4.2.

The biggest issue in the coculture experiment were auxotrophic strains in monoculture, exhibiting growth, in some cases even to the level of the wild type. To further explore this phenomenon, a kinetic growth experiment was performed with monocultures of all amino acid auxotrophs and the wild type.

	Thr	Phe	Ile	His	Arg	Tyr	Trp	Leu
Phe	2.41							
Ile	5.75	1.47						
His	1.65	0.87	0.99					
Arg	1.46	0.97	1.44	0.86				
Tyr	4.25	1.21	2.14	0.94	1.89			
Trp	1.64	1.30	1.83	0.95	1.27	2.38		
Leu	1.36	0.79	1.03	0.80	0.79	1.11	1.14	
Met	1.98	0.81	0.95	0.75	0.80	1.13	1.11	0.90

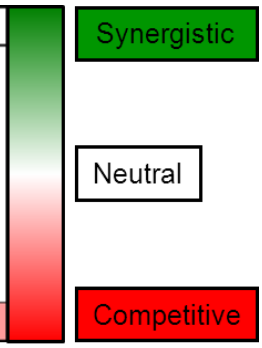


Figure 3-5: Growth of consortia relative to monocultures. Abbreviations of amino acids represent the respective auxotrophic strain. Mono, - and cocultures were grown for 72 hours at 30°C in minimal medium and OD_{600} was determined. Given values are quotients of the final OD of consortia with the sum of each 50% final OD of both respective monocultures. Colour code indicates antagonistic interactions (red), neutral or no interactions (white), and cooperative interactions (green).

3.1.5 GAIN OF FUNCTION

The genetically modified strains were already used for a variety of experiments in the working group, which share one common parameter: maximum incubation time of 24 h. In this time slot, the auxotrophs merely were capable of a few cell divisions, indicated by very less growth above the detection limit in minimal medium. For experiments, lasting from a few days to several weeks this clear dependency to amino acid supplementation had to be verified. Auxotrophic strains were incubated in minimal medium without amino acids for 72 hours in a plate reader, detecting the optical density every 30 minutes. The outcome was very diverse for different auxotrophs. The maximum OD of two strains stayed below an acceptable level, which can be tolerated to term those strains still auxotroph. This is true for example for *E. coli ΔtrpB* and *E. coli* Δ*proC*, whereas *E. coli ΔtyrA*, *ΔmetA*, *ΔilvA*, and *ΔpheA* grew to the level of the wild type or even higher. The highest optical density was reached by *E. coli ΔtyrA.* Clear consistent differences however were observed for maximum growth rates. All tested auxotrophs showed significant lower μ_{Max}-values compared to the wild type. Growth rates positively correlate with increasing maximum OD, but are not even half as high compared to the wild type. The capability of amino acid auxotrophs, especially *E. coli ΔtyrA* playing a major role in the long-term experiment, will be discussed further.

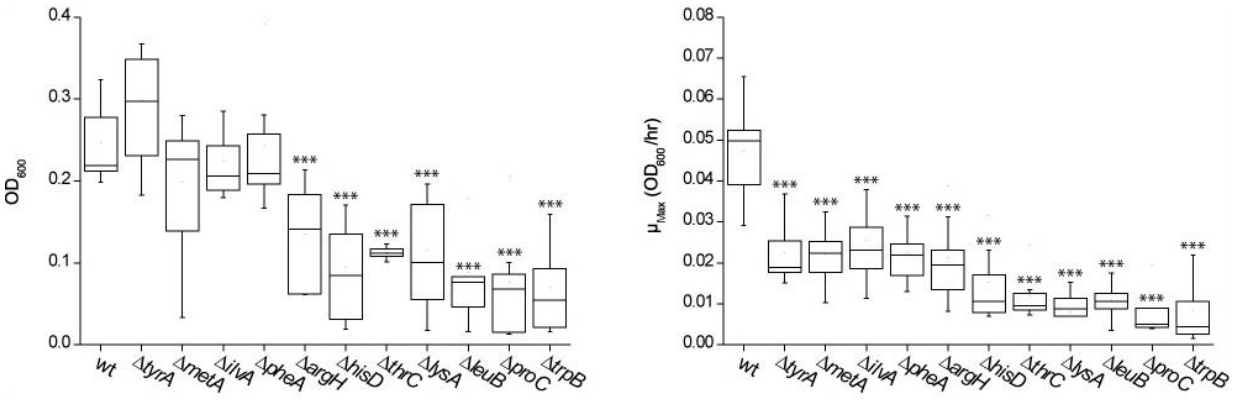


Figure 3-6: Growth of auxotrophs in minimal medium. Shown are maximum optical density (600 nm) and maximum growth rate of eleven amino acid auxotrophs and the wild type in minimal medium. Growth curves of bacterial cultures were measured in a plate reader for 72 hours at 30°C. OD_{max} and μ_{max} were automatically calculated by the Magellan software, using eight time points for every parameter to process. Significant difference to wild type is indicated by stars (#Posthoc; $p<0.001$)

3.1.6 PARTNER EQUILIBRIUM IN COCULTURE

In preparation of the long-term experiment, a coculture of amino acid auxotrophs was tested in different environments. The experimental design was similar to the proposed evolution experiment. Microcosms contained either agar (spatial structure), or liquid medium (spatially non-structured environment). A coculture of *E. coli ΔtyrA-ΔtrpB* was monitored during four days incubation (Figure 3-7). The aim of this experiment was to get insights into the initial performance of the selected consortium, and to define the optical density for inoculation and incubation time during the long-term experiment. Therefore, colony forming units and ratio of partners were characterized in the different treatments.

Independent of environment and initial density of cells, the tryptophan auxotrophs increased in frequency after two days, while the tyrosine auxotrophs decreased in frequency. For the liquid microcosms this trend lasted till the end of the experiment. After four days incubation, tyrosine auxotrophs exhibited the lowest numbers of cfu in the liquid environment. The consortia in this treatment showed $6*10^5$ cfu/ml of *E. coli ΔtyrA*, when inoculated with an initial OD_{600} of 0.005 and 0.01. The cultures with a higher initial OD_{600} of 0.02 showed less decrease for *E. coli ΔtyrA* to only $2*10^6$ cfu/ml. The tryptophane auxotrophs increased in cfu and finally reached $1*10^7$, $3*10^7$, and $6*10^7$ cfu/ml, when inoculated with 0.005, 0.01, and 0.02 OD_{600}, respectively. Consortia on the agar-surface seemed to aspire similar ratios, independent of inoculated density of cells. The tyrosine auxotrophs exhibited after four days on agar approximately $2.5*10^6$ cfu/ml for all treatments. The tryptophane auxotrophs showed between $2*10^7$ and $5*10^7$ cfu/ml, which is comparable to the values in liquid medium, when inoculated with OD_{600} of 0.01 and 0.02.

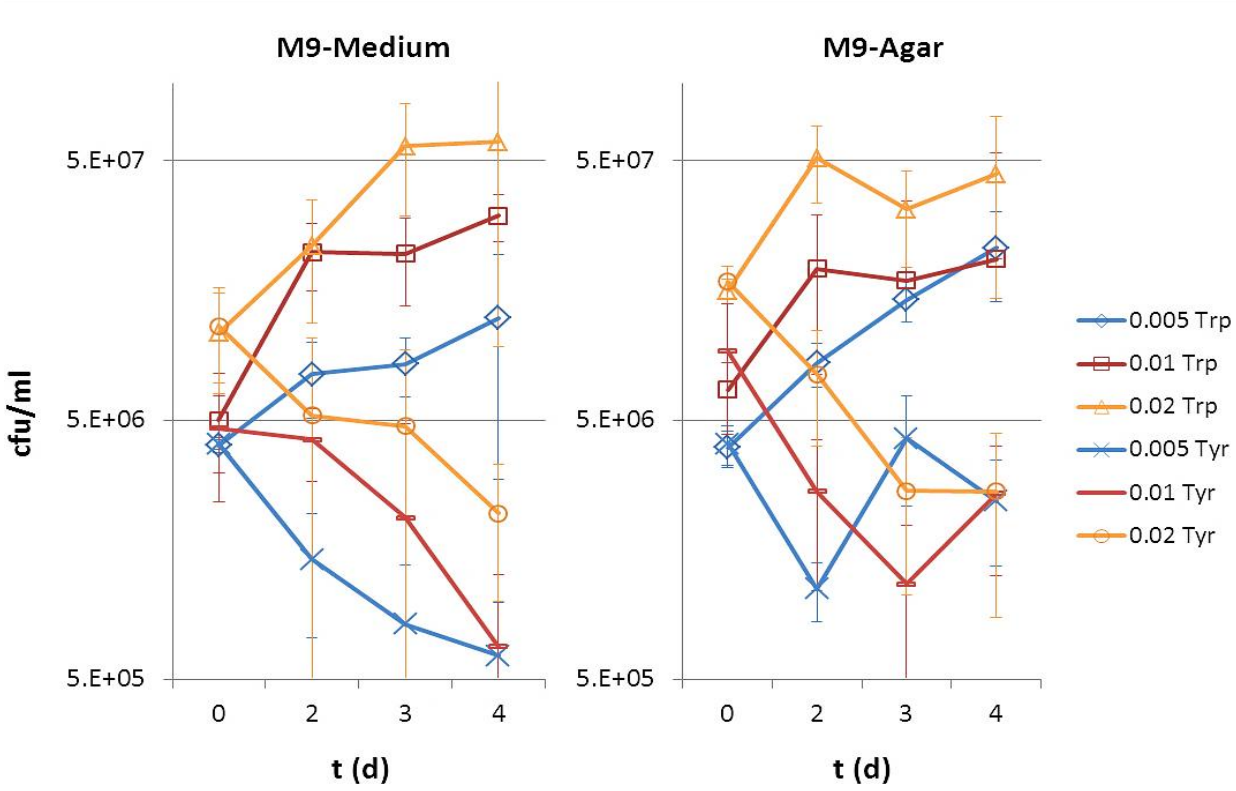


Figure 3-7: Partner equilibrium of cross-feeders. Microcosms were inoculated with different initial optical densities of *E. coli ΔtyrA* and *E. coli ΔtrpB*. Microcosms contained either 4 ml M9-broth or 4 ml M9-agar. Colour code in the legend indicates strains from the same treatment. Cultures were either shaken at 225 rpm (broth) or kept static (agar) at 30°C. Dilutions of cocultures were plated on TA-Agar after 2, 3, and 4 days and colony forming units and thereby ratio of both partners were determined. (Errors given as 95% CI; n=6)

3.1.7 Spatial Structure

For the propagation and cfu determination of consortia, growing on agar, these have to be removed from the surface and separated in liquid medium. This may be accomplished by scratching off the biomass, which is time-consuming for dozens of samples. As an alternative, filter discs may enable highly reproductable outcomes as well as simplified handling in serial transfers and increased numbers of biological replicates. They can be easily removed from an agar surface and vortexed in liquid medium, when carrying a colony of cross-feeding bacteria. In preparation of the long-term experiment several filter discs were tested in regard to the growth of two consortia and homogeneity of outcomes in biological replicates (Figure 3-8). In the case of Celluloseacetate,- and Polyethersulfonefilters consortia grew different. *E. coli ΔpheA* and *ΔthrC* performed better on PES than on CA. In contrast *E. coli ΔtrpB* and *ΔtyrA* showed completely opposite results. The highest outcomes were on average $8*10^7$ cfu/ml on CA and Nylon. On Nylon-membrane strains were capable to reach increased cfu/ml in both combinations, but exhibited greater variation than on the other filters. The growth was most consistent on agar surface without filter. Both consortia grew to the most similar level and showed the lowest variety in colony forming units, if compared to the other treatments. As a consequence of these outcomes experiments in spatially structured environments were performed without filter discs.

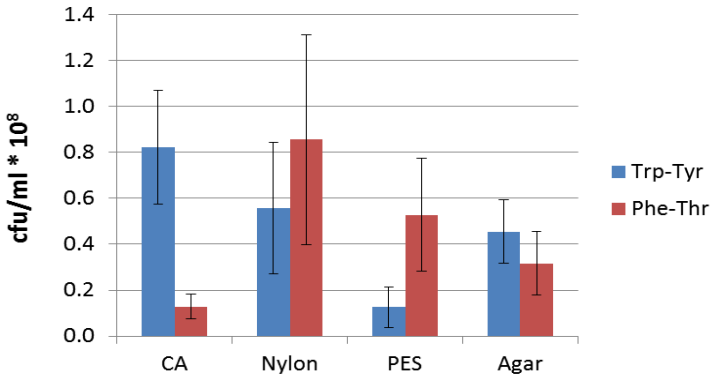


Figure 3-8: Screening of bacterial growth on filter discs as spatial structures. Different filter discs on M9-agar in comparison to normal agar surface were analysed for two consortia of amino acid auxotrophic *E. coli.* cultures were incubated for 4 days at 30°C and cfu was determined. CA: Cellulose acetate; Nylon: Nylon-membrane; PES: Polyethersulfone. (Errors given as 95% CI; n=6)

3.2 LONG-TERM EXPERIMENTS

For *E. coli* several long-term experiments were already performed using minimal medium and low concentrations of carbon sources [41]. In the present experiments, higher concentrations of carbon source (0.5 % glucose or fructose) were applied to provide resources for the release of amino acids, due to overflow-metabolism#. During the *E. coli* wild type experiment, evolved auxotrophs might therefore have a fitness benefit in the community. Hence, the *E. coli* wild type experiment was performed to test the hypothesis of emerging auxotrophic subpopulations in long time scales of incubation, which receive a benefit by using a public good, which is released by other genotypes.

In addition, as the main experiment of the presented work, the results of an *E. coli* coevolution experiment with amino acid cross-feeding auxotrophs are presented.

3.2.1 *E. COLI* WILD TYPE

This evolution experiment was performed for total 86 days in three environments, differing in the extent of spatial structure. Increasing productivity was observed during the first 25 days of incubation in the shaken cultures, with highest OD_{600} of 1.2 on average (Figure 3-9A). The shaken cultures represent spatially non-structured environments. Furthermore, the strain's performance was also of interest for spatially structured environments like agar-surface and non-shaken liquid medium (Figure 3-9C, and B, respectively). In those two treatments, the *E. coli* wt did not increase in fitness during the long-term experiment except a few time points of escalating increase followed by decrease in optical density. Results appear to be most homogeneous for liquid non-shaken microcosms. The optical densities measured of this treatment in the early experiment are nearly similar to the final values of approximately OD_{600} 0.2. In contrast, the other treatments, especially the agar-surface, showed diverse optical densities between biological replicates in the same environment. ODs in the agar treatment showed also quiet similar optical densities at the beginning and the end of the experiment with OD_{600} values of approximately 0.3. On average, the far highest optical densities were achieved by liquid shaken microcosms with OD_{600} of 1.6,

followed by agar microcosms and lately liquid non-shaken environments with minor differences to the latter.

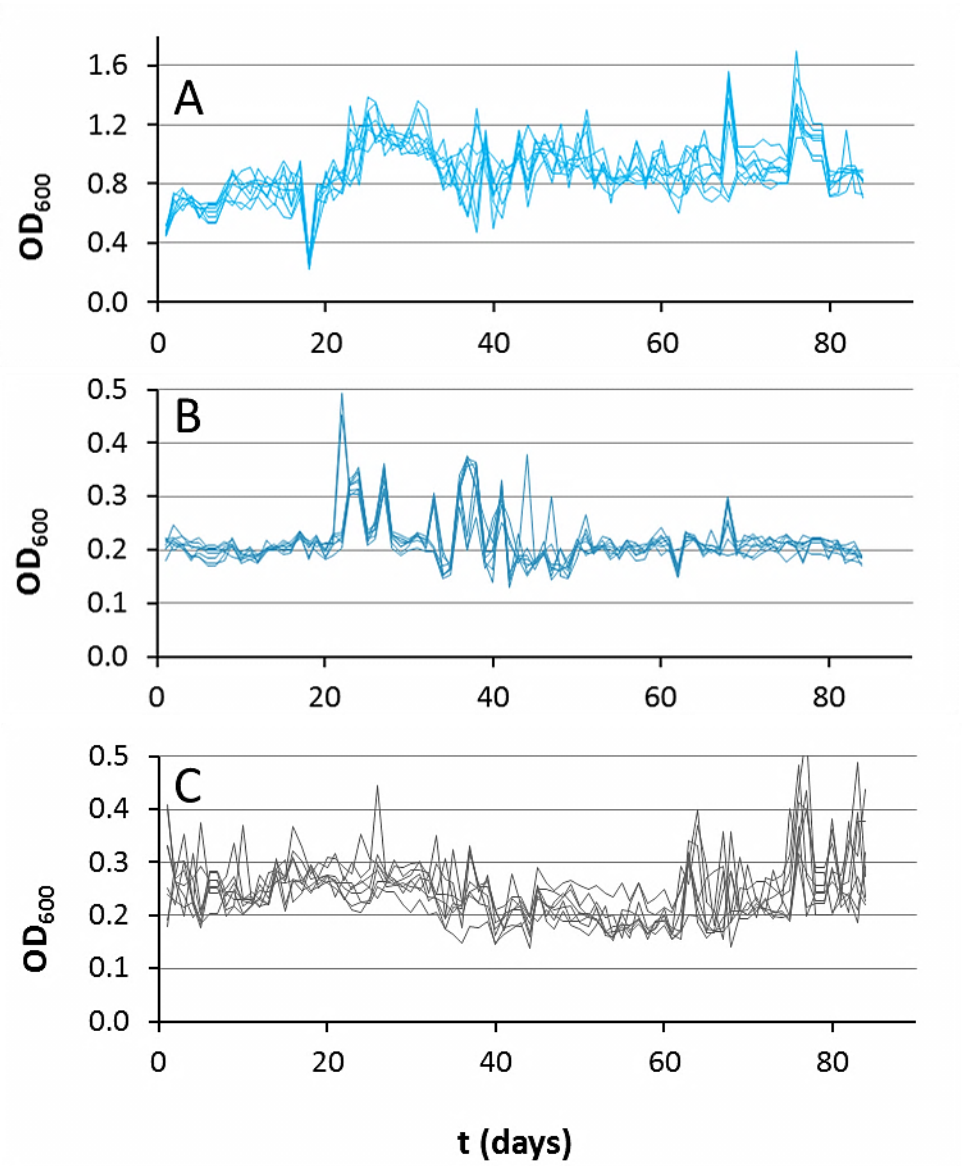


Figure 3-9: Growth of the *E. coli* wt in different environments. Each eight replicates (four arabinose +ve and four arabinose -ve) for each environment were incubated at 30°C and transferred every 22-24 hours for a total duration of 86 days. OD_{600} was measured at every transfer. A) Liquid shaken microcosms (225 rpm); B) Liquid unshaken microcosms; C) Agar microcosms; MMAB-medium or agar was used (0.5 % Fructose).

The screening for evolved *E. coli* auxotrophs after 400 generations did show that there were apparently no auxotrophs with high enough frequency to be detected. From selected biological replicates, each 500 colonies were screened for auxotrophies. If the cultures did harbour auxotrophs, the frequency must have been below 0.5% of total population.

3.2.2 *E. coli* Cross-Feeding Consortium

A long-term incubation with the consortium *E. coli ΔtrpB* and *E. coli ΔtyrA* was performed in a spatially structured environment and spatially non-structured reference environment. The coevolution experiment lasted in total seven weeks. At every transfer, glycerol stocks were prepared, and the optical density as well as the number of colony forming units and the ratio of partners was monitored. The coevolved populations were analysed for amino acid release and fitness in both treatments. This was performed to determine evolved cooperative phenotypes and the adaptation of populations to the environment, where strains coevolved. In these analyses, the ancestor strains of *E. coli ΔtrpB* and *E. coli ΔtyrA* were included, to quantify the improvement in growth and release of amino acids in coevolved consortia.
After the first week of incubation, the cocultures were propagated to microcosms with fresh medium. Especially in the case of liquid microcosms, growth after was not sufficient in every case to reach the initially set density of OD 0.005 after 1:20 propagation. This furthermore would theoretically imply the untimely end of the experiment. During inoculation, cultures were therefore diluted 1:20, as well as 1:4 to ensure that the bacterial strains would not be washed out over time. This strategy was necessary, because the initial evolutionary dynamics and the development of the consortia were completely unknown and unpredictable at this time point.

3.2.2.1 OD and Ratio of Consortium

To compare all microcosms, concerning their total productivity, optical density was measured at every transfer. Over time, all cocultures increased in productivity, when compared to the initial values, as shown in Figure 3-10. However, the liquid shaken cocultures increased in optical density in significant lower quantity, compared to the cocultures in the spatially structured environment (Linear mixed effects model in R; comparison of treatment over time; $p < 0.001$). The consortia on agar already reached higher densities after one week of incubation with an OD_{600} of 0.3. In general, densities constantly increased during the long-term experiment, with an OD_{600} of 1.1 after seven weeks of coevolution. In the contrary, liquid cultures did not exhibit improved growth during the first

two transfers. In 1:20 propagation, those cultures reached their maximum OD_{600} of approximately 0.55 already after five transfers, whereas cultures in 1:4 dilution did exhibit the highest OD_{600} with 0.05 on average after seven transfers.

Results are not shown for agar 1:4 since these microcosms reached a maximum OD already after a few days of incubation after every transfer (data not shown). These cultures then had to wait until the next transfer in starvation. In the agar microcosms, it was more appropriate to dilute the coculture to a defined OD of 0.005 (see Figure 3-10). Therefore, after 4 weeks incubation a new series of microcosms was found from the agar 1:20 cocultures. This was to test, whether the 1:20 treatment caused accumulation of cells and thereby the increase of optical density over time. Agar microcosms with 0.005 initial OD cannot show such an accumulation, but exhibited comparable productivity than the same environments with 1:20 propagation.

As an outcome of an evolution experiment, it always appropriate to calculate the number of generations. It can be calculated via the logarithm of the dilution, which is 20 in this case, to the base of two. This makes 4.3 generations each week and approximately 30 generations after seven weeks of coevolution.

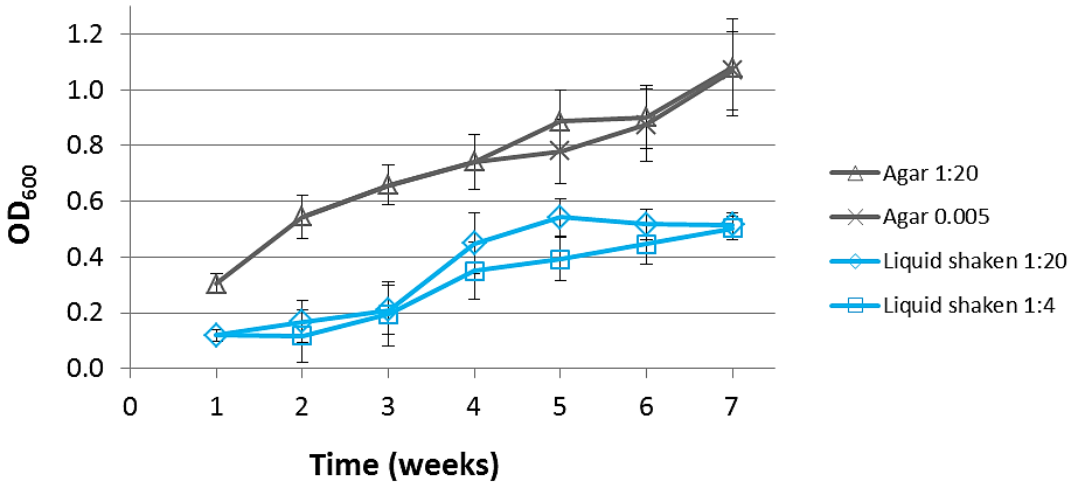


Figure 3-10: Total productivity of microcosms. Consortia of *E. coli ΔtrpB* and Δ*tyrA* were incubated at 30°C in minimal medium or on agar surface (0.5 % Glucose). Liquid microcosms were shaken at 225 rpm. Every seven days cultures were propagated with dilutions indicated in the figure legend and optical density (600nm) was determined. (Errors given as 95% CI; n=12, consisting of 6 *ΔtrpB* ara+/*ΔtyrA* ara- and 6 *ΔtrpB* ara-/*ΔtyrA* ara+ combinations)

Total numbers of living cells were also determined at every transfer on TA-agar (see Figure A-7-4). Cfu increased in time in most of the cocultures, but differences between agar and liquid microcosms in 1:20 treatment were not as distinct as in the case of optical densities. These results will be discussed in chapter 4.

The numbers of colony forming units were divided to calculate the ratio of these strains during the seven weeks of incubation. Ratios are given for 1:20 propagated microcosms. Like already observed in the partner equilibrium experiment (Chapter 3.1.5), the initial applied ratio of circa 1:1 of both strains is not stable and shifted towards the Tryptophan-auxotroph during the first week. Only one consortium was performing different (Fig. 3-11 D). The common initial trend started to become diverse in the second week. The liquid cultures and also five of six Trp ara+/Tyr ara- agar-cultures remained at a higher proportion of Tryptophan-auxotrophs, but five of six Trp ara-/Tyr ara+ agar-cultures shifted to either similar proportions of partners or in 3 cases to Tyrosine-auxotrophs representing the majority of the consortium. During the following transfers, consortia independent of treatment and environment exhibited mostly unique ratios. The agar-evolved consortia Trp ara+/Tyr ara- continuously had a ratio below one, except one consortium. The reverse combinations Trp ara-/Tyr ara+ did show increased quantities of Tyrosine-auxotrophs for several cocultures. The same is valid for the same combinations, which are liquid-evolved. In those cases ratios shifted during the last three weeks towards *E. coli ΔtyrA*. In Figure 3-11B most of the consortia exhibited a ratio below one throughout the experiment.

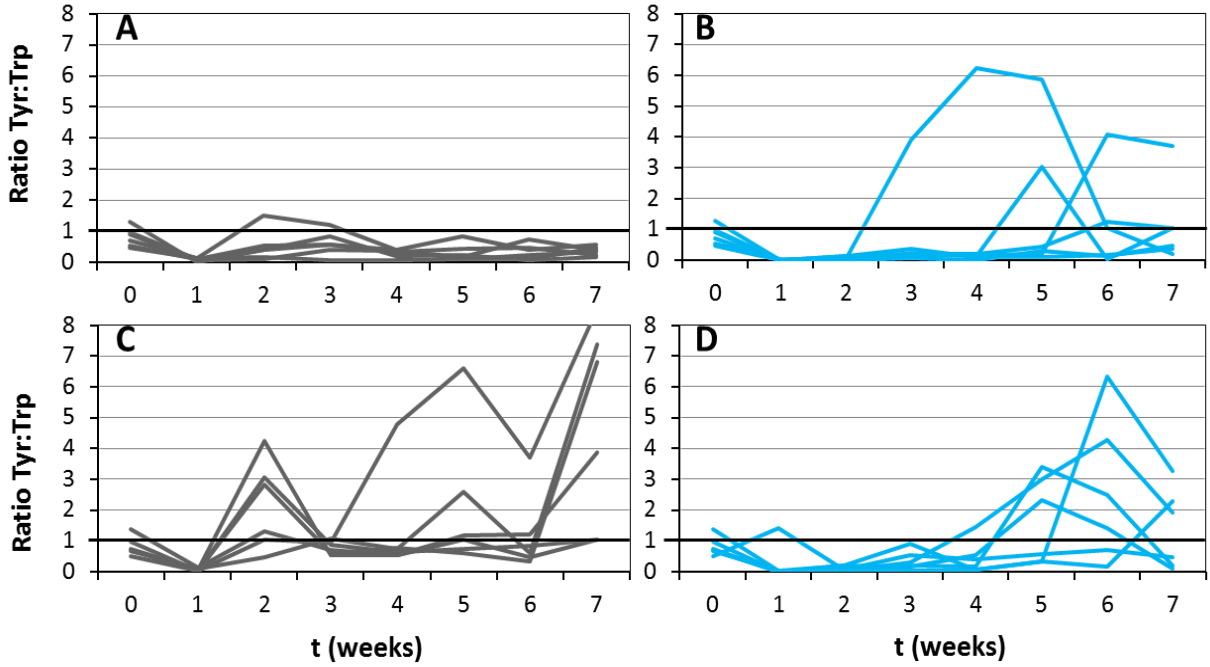


Figure 3-11: Ratio of partners during the long-term experiment. Agar-microcosms *E. coli ΔtrpB* ara+, *E coli ΔtyrA* ara- B) Liquid-microcosms *E. coli ΔtrpB* ara+, *E. coli ΔtyrA* ara- C) Agar-microcosms *E. coli ΔtrpB* ara-, *E. coli ΔtyrA* ara+ D) Liquid-microcosms *E. coli Δ trpB* ara-, *E. coli ΔtyrA* ara+; Results are shown for individual 1:20 propagated cocultures.

At every transfer, dilutions of cocultures were plated on minimal agar to check for revertant phenotypes. These phenotypes recovered the ability to form the focal amino acid they were auxotroph for. Indeed, phenotypes, forming single colonies on M9-agar, were detected (Figure A-7-5). These phenotypes could not be quantified, since satellite colonies started to surround these colonies. This was also caused by the long incubation time of three to four days, until colonies were formed. Revertant phenotypes were therefore neither able to grow like the wild type, nor able to dominate the population of a microcosm.

3.2.2.2 Fitness Comparison

To test the adaptation to the abiotic environment and differences in fitness of evolved consortia and the ancestors, strains were incubated similar to the procedure of the long-term experiment in microcosms. In addition, strains of former liquid-evolved consortia were grown on agar and agar-evolved consortia were grown in liquid medium to detect specific adaptation to the environment during the evolution experiment. Eight evolved populations were selected from total 48 microcosms.

Consortia grown on agar-surface did show higher cell numbers compared to the liquid cultures. This was most dominant for the agar-evolved consortia. In the liquid-evolved consortia growth was only in two cases, -+ L1 and +-L5 at the level of agar-evolved consortia. The ancestors had higher productivity on the agar-surface compared to the liquid cultures and showed less growth than the evolved consortia, except the -+ L5 consortium grown on agar, which performed comparable to the ancestor.

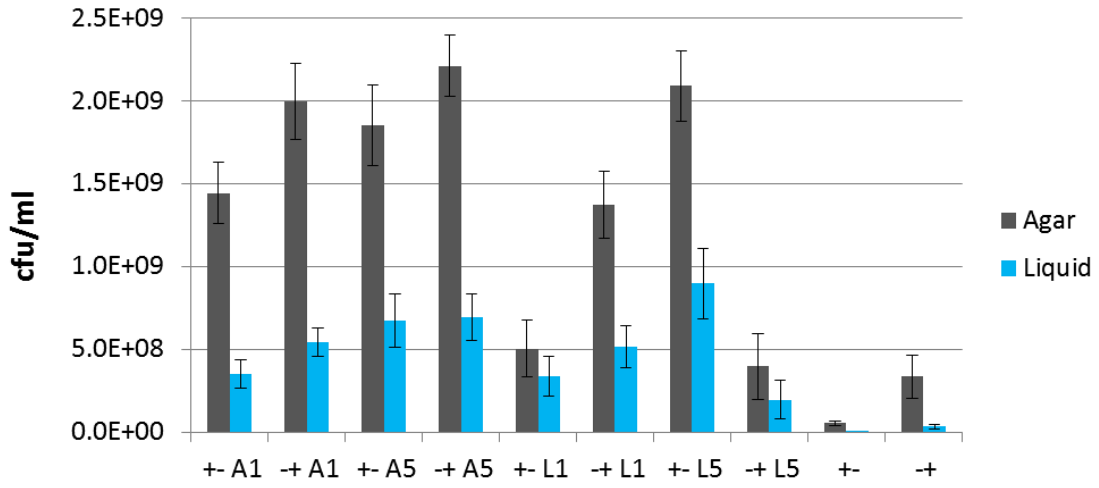


Figure 3-12: Growth of evolved consortia and ancestor in structured and unstructured environments. Microcosms were inoculated with evolved consortia and the ancestors and incubated at 30°C for 7 days, in analogy to the LTE in M9-minimal medium, and agar. Thereafter cultures were plated and cfu was determined. A: Agar; L: Liquid; 1 or 5: Number of replicate. (Errors given as 95% CI; n=8) For statistics, Mann-Whitney-U Test was performed: Agar-evolved and liquid evolved consortia on agar: $p<0.001$; and in liquid: no significant difference; Agar-evolved on agar and in liquid: $p<0.001$; Liquid-evolved on agar and in liquid: $p<0.05$; Agar-evolved on agar vs. Liquid-evolved in liquid: $p<0.001$.

The liquid-evolved consortia did not exhibit enhanced growth in liquid medium, when compared to the agar-evolved ones, whereas there are significant differences in three of four cases, when cell numbers on agar are compared between these groups.

As already mentioned growth relative to the ancestor is in every case increased in the evolved populations, except the consortium -+ A5. Consortia showed independent of the environment they adapted to higher relative growth in liquid culture. This is due to very low numbers of cells in the ancestral consortium in liquid medium. (Discussion: On this point has to be considered that the fitness experiment was running one week. During this incubation time, the reference consortia improved already differentially in fitness. This uneven fitness of ancestors in agar and liquid environment mainly caused the observed differences of relative fitness in Table 3-1.)

Table 3-1: Growth of evolved consortia relative to the ancestors. The normal quotient of averages of derived cell numbers in each treatment are given with true quotients of 95% CI. Averages of evolved consortia are compared to averages of ancestors in the same treatment.

	Environ-ment	Normal quotient ($cfu_{evolved}/cfu_{ancestor}$)	+ve 95% CI	-ve 95% CI
+- A1	Agar	26.5	-7.9	12.9
	Liquid	280.8	-198.7	-900.0
-+ A1	Agar	6.0	-2.2	4.9
	Liquid	16.5	-6.3	13.7
+- A5	Agar	34.0	-10.2	16.6
	Liquid	538.8	-379.7	-1719.4
-+ A5	Agar	6.6	-2.2	5.1
	Liquid	21.1	-8.8	19.1
+- L1	Agar	9.2	-4.3	7.1
	Liquid	268.5	-201.6	-912.7
-+ L1	Agar	4.1	-1.6	3.6
	Liquid	15.8	-7.1	15.3
+- L5	Agar	38.3	-10.6	17.3
	Liquid	717.1	-504.0	-2282.3
-+ L5	Agar	1.2	-0.8	1.7
	Liquid	6.0	-4.2	9.2

3.2.2.3 Amino Acid Release of Evolved Phenotypes

After the long-term experiment, two lines of microcosms were selected for further analysis. The same agar and liquid-microcosms, as in the fitness experiment, number 1 and 5 of the identical treatment with 1:20 propagation were chosen (3.2.2.2). These cocultures did not show phenotypes capable to form small colonies, when plated on M9-agar to check for revertant subpopulations (see Figure A-7-5). The evolved consortia consisted of different phenotypes, which could be distinguished on TA-agar. The most predominant phenotypes which either had different size or intensity/shape of red stain were selected for amino acid analysis. Since the auxotrophs had to be supplemented with tyrosine or tryptophan, the detected concentrations were excluded from further analysis and indicated with “NA” in Figure 3-13. In total production, many of the agar-evolved phenotypes release more amino acids then the ancestor. Three phenotypes released more than 20-fold of amino acids and one out of them even approximately 100 times more than the ancestor. Five phenotypes did exhibit either lower or approximately the same amount of released amino acids. In liquid-culture evolved phenotypes did release significant lower amounts of amino acids than phenotypes evolved on agar (Linear mixed effects model in R, $p<0.01$). Four phenotypes released more amino acids as the ancestor, while the remaining ones released the same or in most cases less amounts.

Decreased concentrations of amino acids relative to the ancestor were observed in many cases for alanine and glycine. Proline was released in every case in elevated quantity and in half of the phenotypes at least more than 20-fold. Glutamine and glutamate were detected in increased concentrations in most of the agar-evolved phenotypes. Moreover, glutamate dominated the amino acid profile in culture supernatants of the agar microcosms number 5. Tyrosine and tryptophan are of special interest, since the utilized consortium is cross-feeding these amino acids. Increased release of tryptophan was observed for all phenotypes analysed (Figure 3-13). Clearly elevated amounts were thereby observed for three phenotypes, which evolved on agar surface. Phenotypes, evolved in liquid environment, also released higher amounts of tryptophan, but less pronounced than in agar populations. Increased tyrosine concentrations relative to the ancestor were only released by three out of

six phenotypes, which were isolated from one agar-population. All samples from liquid microcosms released either less tyrosine than the ancestor did, or approximately the same amount in one case.

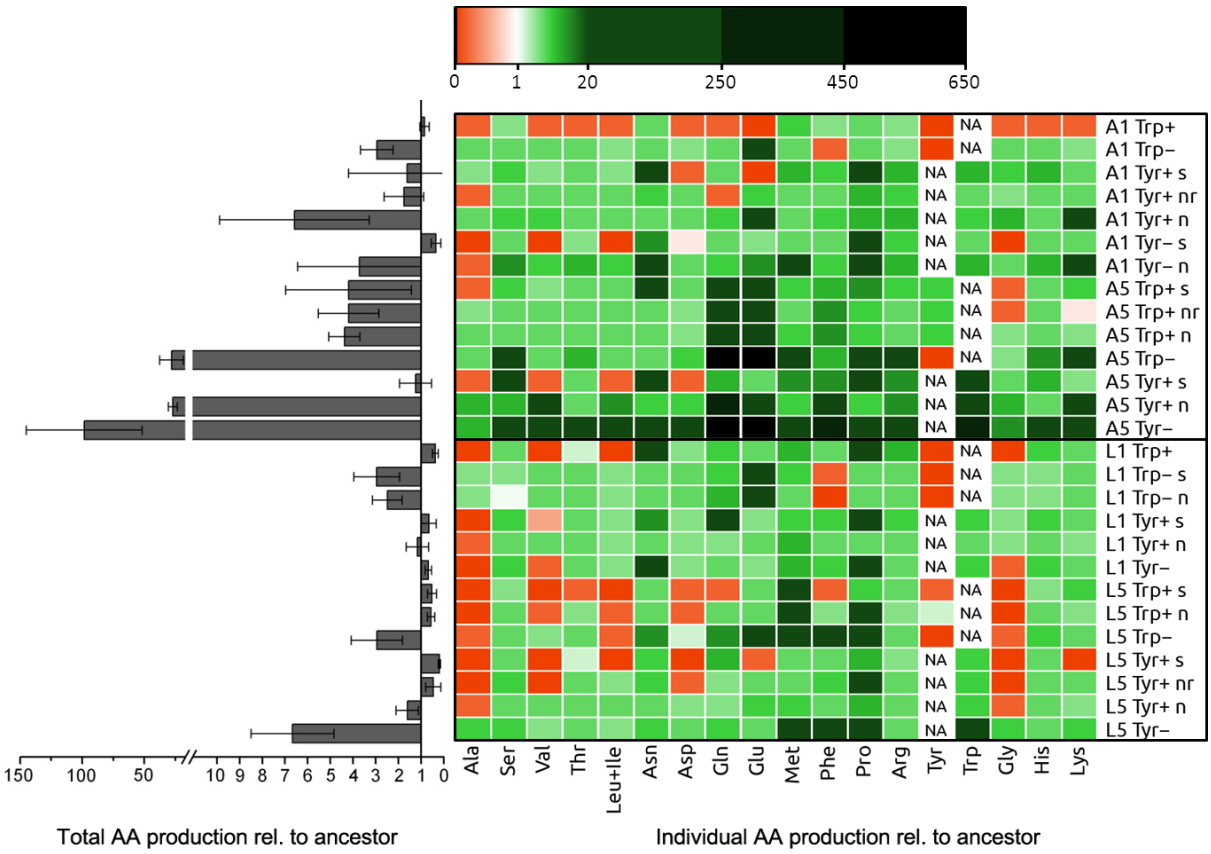


Figure 3-13: Total and individual amino acid release relative to the ancestor. Individual concentrations of *E. coli* auxotrophs determined in supernatants of *E. coli* cultures via LC-MS/MS after 24 or 48 hours incubation at 30°C in M9 minimal medium. Total concentrations represent the sum of individual concentrations divided through the median of the respective ancestor. Abbreviations consist of A for agar or L for liquid, 1 or 5 for the number of microcosm, + or – for arabinose-activity, and s (small), n (normal), nr (normal red) for phenotype. If not other mentioned, phenotype is normal. NA: data 'not available'.

3.2.3 EVOLVED PHENOTYPES

After the evolution experiments, the consortia, as well as the wild type were plated on TA-agar. This was on the one hand done for contamination control. No contaminations were found. On the other hand, phenotypic diversity was analysed. Phenotypes of randomly picked biological replicates evolved on agar surface are shown, which indeed exhibit diversity. In Figure 3-14 colonies, shown in picture A2 and A3, were for example categorized as small phenotypes of *E. coli ΔtyrA* ara+, whereas A6 shows the normal phenotype of *E. coli ΔtyrA* ara+. The colony in picture A1 is a slimy phenotype of *E. coli ΔtyrA* ara+, which also was observed in other populations. Pictures A4 and A5 show normal phenotypes of *E. coli ΔtrpB* ara-. These categorized phenotypes were analysed for amino acid release in chapter 3.2.2.3. The wild type long-term experiment also resulted in different evolved phenotypes. The size of colonies is not as differentiated as in the coevolved consortia. However, when plated after 50 days incubation, no phenotypic diversity was observed for the wild type.

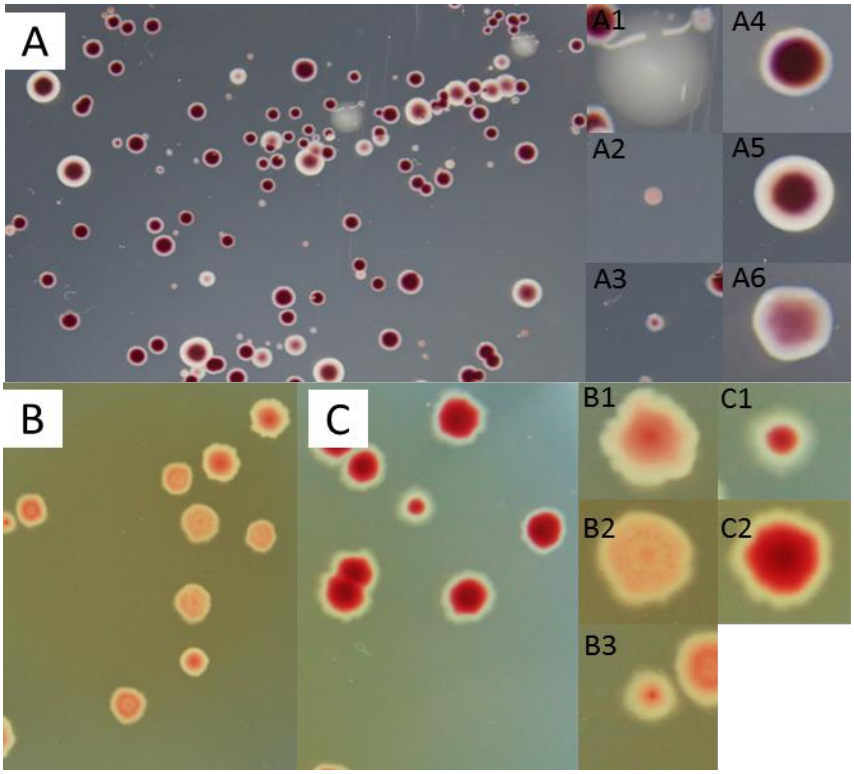


Figure 3-14: Phenotypic diversity after the evolution experiments. Appearance of diluted cultures on TA-agar is shown. A) Coevolved auxotrophs *E. coli ΔtrpB* and *E. coli ΔtyrA* after 28 days incubation on agar. A1 – A6, B1 – B3, C1 – C2) Magnification of phenotypes; B and C*) E. coli* wild type after 86 days incubation on agar. Red colonies represent arabinose negative phenotype, whereas white/reddish colonies are arabinose positive phenotypes. (Pictures were taken with a digital camera. Therefore, magnification cannot be given.)

4 DISCUSSION

The main aim of this thesis was to perform a long-term coevolution experiment, to determine effects of a spatially structured environment on the development of a synthetically designed obligate by-product interaction. Spatially non-structured reference environments were incubated in parallel to quantify hypothesized positive effects of the spatial structure. Consortia on agar surface were suggested to perform better than in liquid culture. Cooperative phenotypes were proposed to evolve in both *E. coli* strains on agar and presumably not in the liquid shaken cocultures. Furthermore, these cooperative phenotypes are expected to release significantly higher amounts of amino acids as populations in liquid, as an indication of true cooperation.

The interacting bacteria were monitored during coevolution, with regard to productivity and ratio of partners. The release of amino acids was finally analysed in derived phenotypes of both treatments. These parameters will be further discussed between both environments in chapter 4.6 to test the proposed hypothesis of spatial structure promoting the evolution of cooperative interactions. Furthermore, the fitness of derived phenotypes and the ancestors will be compared. Finally, in chapter 4.8 phenotypic diversity will be discussed between these derived coevolved strains and a performed *E. coli* wild type long-term experiment.

In preliminary experiments, genetically modified *E. coli* strains were used to establish a cross-feeding interaction based on proteinogenic amino acids. To enable a more focused discussion, the preliminary experiments, predented in chapter 3.1, were categorized. First of all, growth-characteristics of monocultures comprising the test for auxotrophy (3.1.1) and the kinetic growth experiments (3.1.2) will be examined, followed by observations made in the coculture (3.1.4) as well as the partner equlibrium in coculture experiment (3.1.6). The phenomenon of revertant phenotypes within auxotrophic populations, investigated during a 72 hours kinetic-growth experiment, is discussed in chapter 4.3. This chapter includes all experiments being effected by growth of revertant phenotypes in monoculture or in coculture (3.1.2, 3.1.4, 3.1.5, 3.1.7, and 3.2.2). The release of amino acids is critically reviewed in chapter 4.4, including the outcome from preliminary experiments (3.1.2) and the

long-term experiment (3.2.2.3). The design of the long-term experiment will be discussed in chapter 4.5, with the relevant experiments 3.1.6 and 3.1.7.

4.1 GROWTH CHARACTERISTICS OF AMINO ACID AUXOTROPHS

Genetically modified *E. coli*, exhibiting the lack of a terminal amino acid biosynthesis gene, showed a clear auxotrophy for one focal amino acid. Figure 3-1 demonstrates the incompetence of auxotrophic strains first to synthesize one proteinogenic amino acid and second to convert another supplemented amino acids into the required one. Thus, prototrophy was synthetically restricted to generate specific demands for a single amino acid in 11 strains, respectively.

The demand for a certain metabolite is a prerequisite for an obligate cross-feeding interaction. Amongst others, this parameter was investigated in the experiments in chapter 3.1.1 and 3.1.2, which were performed with the 11 auxotrophic *E. coli* strains. The individual maximum optical densities in Figure 3-1 indicate differences in demands, uptake rates, or metabolic utilisation of the focal amino acid among strains. It can be concluded from the kinetic growth curves in Figure A-7-1 that the maximum density is reached after 24 hours incubation. Thus, the uptake rate is unlikely to cause the differences in maximum optical densities. If the import of an amino acid would be the limiting step during the exponential growth phase, the maximum OD would be reached at a later time point. Hence, either the demand of amino acids for the synthesis of the proteome differs, or the focal amino acid is in parallel degraded, while it is utilized in the ribosome. The modified cell is eventually not evolutionary prepared for the scenario that one supplemented amino acid has become focal and should therefore not be degraded. Regulatory elements for such an event are unlikely to exist in the former prototrophic strain. Unfortunately, the distribution and content of total intracellular amino acid amounts in *Escherichia coli* has not been investigated, but is currently investigated in our lab (Shraddha Shitut, personal communication). Therefore specific demands are not yet known. If the maximum ODs are compared between the abovementioned experiments 3.1.1 and 3.1.2, they are not consistent for *E. coli ΔargH* and

E. coli ΔleuB. There is no definite explanation for this observation, but different growth conditions might be a reason. Cultures were incubated in 96 deep-well plates, containing 1 ml medium, with continuous access to oxygen for the test for auxotrophy (3.1.1). The kinetic growth experiments in contrast took place in a 96-well plate, containing 0.2 ml of medium, and being sealed with a transparent sticker to prevent evaporation. This may have caused oxygen limitation and hence altered optical densities.

Growth rates and maximum ODs however correlate within the kinetic growth measurements (3.1.2). This concentration dependent growth, already shown in Bertels *et al.*, was used there in the development of auxotrophy-based biosensors [63]. The increase of OD is reported to be linear within increasing amino acid concentrations between 0.5 µM to 10 µM for histidine and tryptophan and 1 µM to 50 µM for arginine and leucine [63]. This is consistent with the observations for increasing concentrations in Figure 3-2, showing a distribution related to a saturation curve. The benefit of the supplied particular amino acid at low concentrations is most pronounced for *E. coli ΔhisD*, followed by *E. coli ΔleuB* and finally *E. coli ΔargH* and *E. coli ΔtrpB*. This is in accordance to the abovementioned publication. Maximum growth rates are at the highest level with 100 µM amino acid supplementation, which reflects on average the best result in growth for all auxotrophs tested. Thus, this concentration was standardized for preculture supplementation of auxotrophs. A negative effect of increased amino acid concentration in *E. coli ΔleuB* was observed for 500 µM leucine, as maximum optical density and growth rate decreased in comparison to 100 µM supplementation. In *E. coli* L-leucine binds to the global regulator leucine responsive protein (Lrp), which subsequently inhibits the L-valine biosynthesis [64]. This could cause deregulation and therefore decreased maximum growth rate, as well as OD. It is also been reported that high concentrations of certain amino acids, in this case valine, inhibit growth of *E. coli* cultures [65].

4.2 COCULTURE AND PARTNER EQUILIBRIUM

Before a coculture experiment was performed, the initial density after inoculation had to be defined. Theoretical and empirical precedents have established that metabolic cooperation is enhanced at intermediate cell densities when the concentration of cross-fed metabolites is sufficiently high to improve cooperative fitness [45, 49]. Consortia of amino acid auxotrophic *E. coli* strains were already cultured in two other approaches. Initial densities, which allow sufficient cross-feeding are reported there with ~$1*10^7$ cells per ml [62, 66]. Therefore, cocultures were inoculated with an initial density of 0.005, which represents approximately $5*10^6$ cells per ml (see Figure 3-7).

The coculture experiment was performed to select consortia for the long term coevolution experiment. The capability of nine amino acid auxotrophs to grow cooperatively in a total number of pairwise 36 combinations was investigated. The interactions ranged from competitive to strongly positive interactions. These effects appeared to be consistent for some strains. The ability to cross-feed was most prominent for *E. coli ΔthrC* and competitive interactions were not observed for this strain. Contrasting examples in terms of competitive growth are *E. coli ΔhisD* and *E. coli ΔmetB*, which show synergistic growth only in combination *with E. coli ΔthrC*. In order to explain the extent of synergistic growth, profiles of released amino acids could be compared with the performance of two auxotrophs in coculture. The profiles of four strains were analysed: *E. coli ΔhisD*, *E. coli ΔleuB*, *E. coli ΔargH*, and *E. coli ΔtrpB*. The remaining auxotrophic strains were not analysed on this time point, because they were not yet constructed. There are two szenarios, which could explain the compatibility of *E. coli ΔthrC* to exhibit positive interactions in coculture. Either the amino acid release of this strain was favourable for every other amino acid auxotroph, or the other strains released higher amounts of threonine. The profile of released amino acids actually reveals that the latter explanation is more likely. Figure A-7-2: Amino acid release normalized to OD_{600} of 1.Figure A-7-2 shows threonine to be one of the predominant amino acids, which are released in considerable amounts by the *E. coli* wild type, *E. coli ΔtrpB* and *E. coli ΔhisD*. However, *E. coli ΔleuB* and *E. coli ΔargH* release lower amounts of threonine, which is consistent with the lower synergistic growth effects in coculture with *E. coli ΔthrC*.

Hence, the *E. coli* threonine-auxotroph probably does not exhibit universal potential in cross-feeding itself. The strain rather benefits from released amino acids of a partner. However, the profile of released amino acids was not determined for *E. coli ΔthrC*.

The before mentioned contrasting examples in terms of competitive growth are *E. coli ΔhisD* and *E. coli ΔmetB*. These strains show synergistic growth only in combination with *E. coli ΔthrC*, which was already explained. The amino acid profile of *E. coli ΔhisD* does not explain the competitive interactions, which is also the case for other competitive, but also for some synergistic interactions. Interestingly, other synergistcally growing consortia would theoretically not be favoured by the released amounts of amino acids. *E. coli ΔtrpB* and *E. coli ΔtyrA* grow in coculture more than 2-fold better than in monoculture, but in amino acid analysis only traces of tyrosine were detected in *E. coli ΔtrpB* culture-supernatants (Figure 3-4, Figure A-7-2). Furthermore, the cocultures containing the strains *E. coli ΔleuB* and *E. coli ΔilvE* cannot be evaluated with regard to released amino acids, because the analytical method is not able to discriminate between leucine and isoleucine. Therefore, these auxotrophs were excluded from potential consortia for the long-term experiment. In summary, three consortia came into consideration for the coevolution experiment: *E. coli ΔthrC-ΔtyrA*, *thrC-ΔpheA*, and *ΔtyrA-ΔtrpB*. These combinations exhibited appropriate cooperative growth, when compared to monoculture.

A comparable approach was recently published, in which *E. coli* strains, lacking the ability to synthesize a focal metabolite, were cocultured [62]. This study also comprises cocultures of *E. coli* amino acid auxotrophs. They report that a proportion of consortia grew synergistically, especially when the partners had a knockout mutation in distinct biosynthetic pathways, but no strain was found to be universally cooperative. By contrast, consortia with the best capability to show synergistic growth observed in the present study, consisted not exclusively of auxotrophs from distinct pathways. *E. coli ΔthrC-ΔilvA* and *E. coli ΔtyrA-ΔtrpB*, showed amongst four others the most pronounced cooperative interactions. Moreover, *E. coli ΔthrC* exhibited synergistic interactions in combination with every other strain, representing a universally cooperative phenotype. Possibly, this is due to the comparably low number of combinations with other auxotrophs, tested here. Unfortunately, there is no comparison possible for *E. coli ΔthrC*, and also *E. coli ΔilvA*, *E. coli ΔpheA*, and *E. coli ΔmetA*, because these strains were not used in the discussed publication. Since they are not the

focus of this study, competitive interactions will not be compared in detail with the results in Wintermute and Silver [62]. In general, less positive interactions of the strains *E. coli ΔhisD* and *E. coli ΔleuB* with other auxotrophs agree with the results of Wintermute and Silver [62]. Most relevant for this study is the outcome for the coculture of *E. coli ΔtyrA-ΔtrpB*. Synergistic growth was reported for both strains in coculture, with *E. coli ΔtrpB*, dominating the population [62]. This was also shown in chapter 3.1.6, where these strains were grown on agar and in liquid medium.

In the publication of Wintermute and Silver [62], growth of monocultures was maximum eight-fold during four days of incubation. This is not consistent to observations made here. Emerging revertant phenotypes most likely influenced the outcome of the presented coculture experiment. Those phenotypes started to grow in monocultures predominantly after 24 hours incubation and altered thereby the extent of synergistic growth in cocultures. This phenomenon will be discussed in detail in the following chapter.

4.3 THE PHENOMENON OF REVERTING AUXOTROPHS

In the literature it was so far not reported that amino acid auxotrophs, which lack a terminal amino acid synthesis gene, can regain the ability to grow prototrophic in minimal medium. In contrast, revertant phenotypes were observed in several experiments, presented in chapter 2.3.6.2. These phenotypes grew more slowly than the *E. coli* wild type, but occationally reached its maximum optical density (chapter 3.1.5). The auxotrophic strains used in this study were tested for confirmation of auxotrophy in the following protocol: Within the procedure of genetical modification, the derived knockout-mutants were tested for kanamycine-resistance and incubated in unsupplemented minimal medium for several days, to confirm the targeted gene being replaced by the kanamycine-cassette. This is why these strains were considered as auxotroph, also during increased incubation time. In contrast, amino acid auxotrophs started to grow in monoculture in the performed experiments. Even in coculture, for example during the long-term incubation of *E. coli ΔtrpB* and *E. coli ΔtyrA*, revertant phenotypes were observed.

Two possible szenarios may have led to the erroneous assumption of auxotrophy and could explain the unexpected growth of the putative auxotrophs. First of all, the initial density, as well as preculture conditions were different: For the confirmation of auxotrophy, single colonies were picked from an over-night LB-agar plate and aliquotes of 1 ml minimal medium were inoculated. On the other hand precultures were grown for approximately 16 hours in minimal medium, supplemented with the focal amino acid of the particular strain. Subsequently, the preculture was centrifuged and the pellet was resuspended in pure minimal medium, followed by the inoculation of the desired experimental setup with an initial optical density of 0.005. The initial load of cells was therefore considerably lower in the former protocol during the confirmation of auxotrophy. Cellular density and therewith associated proteins, peptides, and amino acids, released by starved cells, might be a critical factor for the potential to revert to prototrophy. A sufficient load of focal amino acid could enable a subpopulation to a few cell divisions, providing one critical mutation, which leads to a revertant cell. The same effects may have occurred in cocultures. The second possible scenario is the storage length of genetically modified *E. coli* on agar plates in the fridge. Usually, a bacterial strain is considered to be nearly inactive at 4°C, especially *E. coli*, with optimum growth at 37°C. Therefore, strains were stored for a maximum of two weeks at 4°C, until they were streaked from a glycerol stock. During repeated experiments the observation of reverting auxotrophs was made (personal communication, Holger Merker). The assumption regarding adequate inactivity during storage in the fridge is most likely not true for amino acid auxotrophs. The resulting hypothesis is that the longer the bacterial strain is stored at 4°C, the higher its capability to generate revertants and the faster those phenotypes emerge. Since the knockout mutants were tested for auxotrophy directly after their generation, storage conditions could never have an effect. In consequence, *E. coli* auxotrophs should not be stored longer than one week at 4°C before they are incubated longer than 24 hours. Additionally, double knockout mutants are currently constructed, which lack two amino acid synthesis genes in one pathway, to increase the stability of auxotrophy during upcoming long-term experiments.

Indeed, amino acid auxotrophic *E. coli* were already observed to grow in unsupplemented minimal medium (Felix Bertels, diploma thesis). However, those strains had knockouts in

different pathways than the strains used in this thesis. Moreover, these knockouts were not located at terminal positions in the biosynthetic pathway.

The described phenomenon of revertant auxotrophs affected almost every experiment in this thesis incubated longer than 24 hours. First indications for growth of monocultures are shown in Figure A-7-1. The bacterial strains do not clearly enter the stationary growth phase, after consuming the comparatively low amounts of 5 µM histidine for *E. coli ΔhisD*, or 20 µM focal amino acid for the remaining three strains. At this time point, the possibility for growth without amino acid was not taken into account for these strains and moreover, the magnitude was nearly at the detection limit of the analytical method. Hence, this was not further investigated. It cannot be stated therefore that these observations exclusively were caused by revertants. In the coculture experiment (chapter 3.1.4) however, revertant phenotypes were clearly observed. Amino acid auxotrophic strains in monoculture exhibited optical densities, comparable to the wild type. Possible consequences for the outcome of this experiment were already discussed. The results, presented in chapter 3.1.5, also demonstrate the capability of amino acid auxotrophs to gain prototrophic growth. However, the revertant auxotrophs in monoculture do not exhibit comparable growth in the experiments, presented in chapter 3.1.4 and 3.1.5. This might be an effect of different storage times of the strains in the fridge, since both experiments were performed at different time points. Another reason might be again culture conditions, as explained in the previous chapter. Unlimited oxygen supply therefore may influenced the emergence of revertant phenotypes. However, a definite explanation of the observed differences is not possible based on the available data. Relevant for the performed long-term experiment is that the strain *E. coli ΔtyrA* exhibited one of the most different optical densities in both abovementioned experiments. However, the auxotrophy of this strain, to remain stable during long incubation times, was confirmed in the experiments 3.1.6 and 3.1.7 (data not shown). This is why the strain was used in the long-term experiment, regardless to the outstanding high OD, reached in the gain of function experiment. The last example for observed revertants within the preliminary experiments is the evaluation of spatial structures (chapter 3.1.7). Dilutions of the cocultures were also plated on M9-agar after the experiment to test for the ability of a single strain to form a colony. The consortium *E. coli ΔthrC-ΔpheA* exhibited growing colonies, thus revertants, which were even able to form

single colonies after being substreaked on M9-agar. This combination of auxotrophs was therefore not useful for a coevolution experiment. The consortium *E. coli ΔtrpB-ΔtyrA* did not form single colonies, when plated on M9-agar and was used in consequence for the long-term experiment. However, emerging revertants were later observed during the long-term experiment (see Figure A-7-5). This is problematic for a long-term coevolution experiment, which requires bacterial strains to be constantly dependent on a partner. Interestingly, revertant phenotypes never dominated a population, as further discussed in chapter 4.6.

4.4 AMINO ACID ANALYSIS

The profile of released amino acids was analysed for two purposes: First, to get insights into the profile in culture supernatants, because there is no information available so far for *E. coli*. This was recommended to eventually allow predictions about potential combinations of amino acid auxotrophs for cross-feeding consortia. Second, to identify overproducers and eventually cheaters, evolved during the long-term experiment. Results of both measurements will be compared in this chapter, whereas the measured profiles of the evolved phenotypes will be discussed in detail in chapter 4.6.

Interestingly, compared to the wild type the absolut amounts of released amino acids were alterred by the knockout of a terminal amino acid synthesis gene in auxotrophic strains (Figure 3-4 and Figure A-7-2). The minority of amino acid concentrations is thereby highly altered. Solely alanine and threonine concentrations were predominantly changed, indicating that the global regulation was not strongly effected. One supporting example would be the stringent response, being generally activated by unloaded t-RNAs, which bind to a ribosome, indicating amino acid starvation. In cause of the the ppGpp concentration increases and the stringent response is activated. However, measured ppGpp-levels in amino acid auxotrophic strains revealed low concentrations of this signal molecule (Glen D'Souza, unpublished data) and thereby no stringent response, which would have explained globally changed amino acid metabolism.

Enormous differences between the measured amino acid profiles in the preliminary experiments (chapter 3.1.3) and after coevolution (chapter 3.2.2.3) were detected. The values within each analysis are consistent, but not between the experiments. Only one strain, *E. coli ΔtrpB* ara+, was analysed in both measurements. Although the total amount of released amino acids was the same, individual concentrations differed between analyses (Figure A-7-2 and Figure A-7-6). The protocol for amino acid analysis was identical between measurements. Since there are no profiles of amino acids in culture supernatants published for *E. coli* yet, it cannot be decided, whether one analysis was inaccurate. Differences between preculture conditions or sample preparation are not documented.

Furthermore, differences in released amino acid concentrations between ara+ and ara- phenotypes of the same strain are presented in Figure A-7-6. The arabinose negative strains release less amino acid than the respective arabinose positive strains. Reasons for this observation are not clear. During the construction of these strains, clones of ara- strains were used to generate the ara+ phenotype. Possibly, slightly different phenotypes were selected by coincidence, which differed in amino acid release.

4.5 Design of the Long-Term Experiment

In preparation of the evolution experiment, several parameters had to be characterized in advance. To quantify positive effects of the spatial structure, a reference environment was necessary for comparison. Therefore, the spatially structured environment was complemented with a spatially non-structured environment, which was otherwise treated identically. Both treatments were intended to be kept as comparable as possible, to exclude any difference in the abiotic and biotic environment, except the spatial structure. In the basic idea, small permeable filter-discs, placed on the surface of agar, should harbour a cross-feeding consortium. At every transfer during coevolution, the bacterial strains should be washed from the discs and an aliquote of the population should found the new generation. As a consequence of the inhomogenous outcome in the experiment 3.1.7, it was

decided against these discs. In the meanwhile small screw-bottles (Figure 2-1) were found to be advantageous.

To select a cross-feeding consortium, a screening was performed, to detect synergistic effects in cocultures. From total 36 cocultures, containing different combinations of *E. coli* auxotrophs, one combination was finally selected for the coevolution experiment. This cosortium was *E. coli ΔtrpB-ΔtyrA*, as already explained in chapter 4.3. The incubation time for the finally selected consortium was another parameter to be defined. In the experiment "partner equilibrium in coculture", the consortium *E. coli ΔtrB* and *E. coli ΔtyrA* was analysed for growth and ratio of partners during four days incubation in the abovementioned screw-glasses. Since after four days no growth was observed for *E. coli ΔtyrA*, the incubation time of the long-term experiment was necessarily increased to one week. The propagation regime was another parameter to be set. The consortia in both treatments were suggested to exhibit different maximum ODs and therefore would require different dilutions at every transfer. However, one common standard dilution (in this case 1:20) had to be set after the first week of coevolution to maintain direct comparability between treatments. Alternatively, other test series were found from the standard treatments. One dilution stragedy (1:4) was found directly after the first week of incubation for both treatments from the 1:20 treatment, because of very low optical densities at this time point. This was to prevent cross-feeding consortia to be washed out over time, since their performance during the first transfers was unpredictable. Another parallel test series was found from cultures in the agar 1:20 treatment after four weeks of coevolution, which was constantly propagated with an optical density of 0.005 after inoculation, thus a constant number of cells. This was done, because there was the possibility that these consortia increased in optical density over time, only because of the 1:20 dilution. The number of inoculated cells might have increased over time, due to increasing final ODs, but constant inoculation volume. This might have caused an artefact, since increased optical density was used as a measure of increased productivity. However, the growth in OD_{600} 0.005 microcosms was at the same level like the 1:20 treatment (Figure 3-10).

4.6 LONG-TERM COEVOLUTION EXPERIMENT

The investigation of mechanisms, generating the evolution and stability of mutualistic interactions, is a major challenge for evolutionary biology [16, 24]. Indeed, there exists a large body of theory and an increasing amount of computational approaches [38]. However, there are only a few empirical tests, which address especially the evolution of cooperation in a spatially structured environment. Therefore, a long-term coevolution experiment was designed, to investigate the importance of spatial structures for the evolution of cooperation from a by-product interaction.

The discussion will be structured in the order, given by the hypothesis in chapter 1.5:

1) The growth of the consortium improves over time. Total productivity represented by optical density as well as total counts of colony forming units of both partners (cfu) increase.

During coevolution, the optical density was measured at every transfer to quantify productivity. The consortia in both treatments, on agar and in liquid medium, were able to elevate their optical density over time (Figure 3-10), but on agar throughout the experiment significantly higher than in liquid medium ($p<0.001$). Finally, detected optical densities were 2-fold higher for agar. Thereby, the spatially structured consortia exhibited the capability to increase in productivity continuously, starting with the first treatment. Spatial structures might therefore provide a surrounding to inefficient by-product interactions, which allows sufficient cross feeding and adaptation to defined partners during early cooperation. In contrast to the agar surface, the reference cultures even decreased in optical density until the second propagation. This initial instability was also observed in an evolving syntrophic interaction in liquid medium [39]. Overall, the growth was significantly better in the agar treatment, than in the spatially non-structured environment. This is in perfect accordance to hypothesis 1. Interestingly, the performance of cocultures was marginal affected by the propagation regime. On agar, cultures improved similar in growth, independent of being diluted 1:20 or being set to OD_{600} of 0.005. A related observation was made for the liquid cultures in a less pronounced extent, resulting in identical optical densities after seven weeks coevolution. There is a critical concentration of cells reported for an amino acid cross-

feeding consortium to gain sufficient cross-feeding with 10^7 cells per ml [66]. However, initial densities of partners seem to become less important over time for sufficient cross feeding. The theoretically calculated number of ~30 generations is not assumed to fit the actual one. As observed in experiment 3.1.6, the initial interaction is characterized by the decline of one partner and growth of the other. There might have been oscillating cell numbers in both partners during one week of incubation, which caused much more generations due to one strain feeding on a starving partner and vice versa. Shou *et al.* reported similar dynamics in cross-feeding yeast populations [45]. Hence, the consortia exhibited most likely generations, which are some orders higher than calculated.

2) The ratio of partners is not stable but exhibits dynamics and fluctuation.

Coevolving auxotrophs theoretically exhibit differing fitness, caused by uneven amounts, uptake rates, and demands of cross-fed amino acids. The initial ratio of partners a by-product interaction is thereby theoretically most likely unequally distributed. Figure 3-7 demonstrates this assumed uneven distribution in the consortium, and thus an initial fixed ratio and Figure 3-11 shows the dynamics in the cross-feeding populations during coevolution. In accordance, Hosoda *et al.* demonstrated stable distributions of growing amino acid auxotrophic consortia independent of initial density of cells [59]. Shou *et al.* reported population dynamics in the ratio of partners in a synthetic obligatory cooperative system of yeasts [45], which is in accordance to the outcome in this work. Overall, emerging cooperative phenotypes and evolved increased efficiency in amino acid uptake might have contributed to rapid shifts in the ratio of partners. Thus, *E. coli ΔtyrA* possibly was capable to dominate the population in several microcosms, because of increased release of tyrosine by the partner or evolved elevated uptake rate of tyrosine. Treatment-specific observations for the ratio of partners were not made.

3) Cooperating phenotypes, which release increased amounts of amino acid, evolve in both strains. Concentrations of released amino acids are higher in the evolved strains as compared to the ancestors.

If two strains actively invest into an interaction to the mutual benefit of both partners, this can be termed cooperation. In this study, a by-product interaction was synthetically established between amino acid auxotrophs. The strains *E. coli ΔtrpB* and *E. coli ΔtyrA* were initially capable of growing on released traces of the amino acids tyrosine and tryptophan, thereby entering a positive feedback-loop. After coevolution, phenotypes of both strains released elevated amounts of tyrosine or tryptophan relative to the ancestor in one out of four agar-evolved populations. Thus, cooperation apparently evolved in this population. Generally, phenotypes that had evolved on agar surface released significantly higher amounts of amino acids, than phenotypes that had evolved in liquid medium ($p<0.01$). In the latter populations, no cooperator-phenotypes were detected for *E. coli ΔtrpB*, whereas three out of six phenotypes of this strain did cooperate on agar surface. These findings provide some evidence for the spatially structured environment promoting the evolution of cooperative interactions.

However, important for the outcome of amino acid analysis are the preculture conditions. Bacterial strains were grown in monoculture, which was supplemented with 100 µM amino acid, before the supernatants were analysed. However, there is evidence that the release of amino acids changes in the presence of another strain (Personal communication, Samay Pande). This observation is supported by a study, where changes in gene expression were detected in cocultures of amino acid auxotrophs *E. coli* strains, in comparison to monocultures [59]. Hence, altered gene expression is a possible explanation for different profiles of released amino acids in cross-feeding consortia. To test this assumption, biosensors might be used to monitor tyrosine or tryptophan concentrations in a growing coculture [63].

Another interesting observation is the presence of cheating phenotypes in coevolved populations. Cheaters are expected to obtain benefits cooperative phenotypes offer, but do not return benefits [12]. In the context of the performed coevolution experiment, cheating subpopulations were detected. Characterized by decreased relative amounts of released

amino acids, these phenotypes were observed predominantly in spatially unstructured environments.

4) Cooperators pay a cost for releasing increased amounts of amino acid.

This hypothesis was not experimentally tested, yet. The evolved phenotypes, which were selected for amino acid analysis, did not exhibit similar growth rates. Especially the small colony-forming phenotypes, which also released very small amounts of amino acids, did grow very slowly. This is why these strains cannot be used for a direct competition experiment[46], capable to detect fitness and therefore fitness decreasing costs.

5) The fitness of evolved consortia is higher than in the ancestor and at the highest level in the environment, where the long-term experiment took place.

As demonstrated in Figure 3-12, evolved consortia exhibit higher fitness compared to the corresponding ancestors. The agar-evolved consortia exhibit higher fitness on agar surface than in liquid medium, which is also in accordance to the hypothesis. However, cocultures, which evolved in liquid medium, showed higher fitness on agar, which was in two cases comparable to the agar-evolved consortia in the same environment. Moreover, there is on average no difference between consortia evolved in different treatments, when grown in liquid environment. These observations indicate that the adaptation to the abiotic environment is not crucial for the cocultures performance. Rather the effect of the environment, to be spatially structured or not, seems to be important for the cross-feeding interaction in consortia. In general, agar surface, and therefore spatial structure is suggested to have an immediate positive influence on cross-feeding. This conclusion is further discussed in section 4.9 describing a possible explanation for the immediate advantage on agar.

4.7 *E. COLI* WILD TYPE EVOLUTION EXPERIMENT

From previous studies, it is already known that *E. coli* rapidly increases in fitness during the first 100 generations when grown in liquid medium [26]. This was observed for cultures in the liquid shaken environment, which predominantly increased in maximum OD during the first 400 generations (~52 days incubation). Another evolution experiment confirms this observation [35]. In contrast, there was no such elevation in fitness observed, when *E. coli* was incubated in non-shaken liquid culture. The only parameters, which were obviously different between these treatments, are access to oxygen and the shaken or fixed incubation. Whereas all oxygen available is diffusing into the shaken culture, oxygen diffusion is more passive in non-shaken cultures. The latter growing population might therefore enter anaerobic growth much earlier than the former one. The result is acidification at an earlier time point in the non-shaken culture, which decreases maximum OD, but does not explain fitness to remain at the same level during several weeks of evolution.

4.8 PHENOTYPIC DIVERSITY

Phenotypic diversity was observed in both evolution experiments. This is in accordance to other long-term experiments, which were performed in an environment with single carbon source [42]. This outcome is remarkable, since the cocultures were transferred only seven times, during seven weeks of coevolution. The wild type, on the contrary, evolved for more than 12 weeks in the same environment, but did not exhibit more phenotypic diversity than coevolved consortia. Additionally, the number of phenotypes was approximately the same in the spatially structured and non-structured environment of the coevolution experiment (Figure 3-13). In contrast, Saxer *et al*. 2009 suggested that a spatially structured environment restricts public availability of by-product resources, which limits ecological opportunity and thereby diversity [35]. In the same study it was also reported that the marker genotype, which was like in this work either arabinose positive or negative, had an influence on the

detection of phenotypes. Arabinose positive strains did not exhibit large colony morphs, while arabinose negative genotypes lacked small colony morphs. Some related observation was made in experiment 3.2.2.3, where fewer phenotypes were detected in arabinose positive strains (see also Figure 3-13).

4.9 SCIENTIFIC SIGNIFICANCE

In this section, the presented observations will be linked to the literature to point out the relevance of the outcome of the coevolution experiment and for comparison with corresponding studies. This section will mainly focus on observed diversity, productivity, and detected cooperative phenotypes.

Phenotypic diversity, which was investigated in the coevolved consortia, had the same extent in both treatments, approximately. Currently, two studies examined the development of *Escherichia coli* populations on the surface of agar and in liquid medium. Habets *et al.* demonstrated the presence of diversification in spatially structured environments on condition that the population structure was kept intact at each daily transfer [34]. In another study, Saxer *et al.* addressed the maintenance of diversity in liquid-evolved strains on agar surface [35]. Their conclusion was that diversity as well as fitness declines, when liquid-evolved strains are incubated for several transfers on agar. However, they are also arguing that this was an effect of mixed population structure at every transfer, as investigated in Habets *et al*. to be negative for diversity [34]. Moreover, diversification was also observed for *Pseudomonas fluorescens* in a liquid heterogeneous environment, termed “adaptive radiation” [46]. Concluding, in analogy to this study, phenotypic diversity was detected in different environments in other approaches, which investigated prototrophic monocultures.
Cheating phenotypes did also evolve during the long-term experiment in both treatments. The presence of cheaters was already reported in publications for example in *Pseudomonas aeruginosa* strains [36, 67] and empirical studies with cheating phenotypes were performed with social bacteria [68]. Based on a theoretical model, Pfeiffer and Bonhoeffer predicted that spatial structure should increase the resistance of cooperative associations against non-cooperative cheating types [33]. In addition, Koschwanez *et al.* proposed from studies with

clustering yeasts that close proximity enhances the efficiency of the use of growth-promoting secretions and promotes the exclusion of cheaters [69]. In analogy, the cooperative production of siderophores by *Pseudomonas aeruginosa* was promoted in viscous medium and the fitness of mutants lacking the ability to form siderophores, thereby saving their production costs, decreased with increasing viscosity of the medium [36]. These publications describe that a population of cooperators is able to coexist with cheaters in a spatially structured environment. Although cheaters were present in both treatments after coevolution in this study, in particular the agar microcosms increased in productivity over time, which is in line with the listed conclusions above.

The next issue, which will be discussed, is productivity. As presented in section 3.2.2.1, the coevolved consortia, which interacted on agar surface, exhibited in comparison to liquid-evolved cocultures significantly higher productivity. This advantage, facilitated by the spatial structure, was already noticeable after one week of coevolution. After the coevolution, the growth of consortia was determined on solid as well as in liquid medium, to test for fitness and adaptation to a specific treatment during the long-term experiment. Surprisingly, two liquid-evolved consortia, which were incubated on agar during the fitness experiment (Figure 3-12), were capable to grow to the level of agar-evolved consortia. This indicates the following: The spatially structured environment has an immediate positive effect on a cross-feeding interaction and the adaptation to this abiotic factor is not necessarily important. Partners might profit from improved conditions for exchanging metabolites, due to close proximity and limited diffusion of shared goods. Indeed, there is evidence that the distribution of metabolites in structured populations enables the total community to be maintained [37, 70]. In Biofilms, furthermore, functional distribution of syntrophic partners occurred to maximize the efficiency of fluctuating metabolites and environmental conditions [71]. Another side-effect of spatial structure might be that benefits such as products, which are secreted into the environment (i.e. the public goods) are preferentially directed towards individuals that are genealogically more closely related to the cooperating individual (i.e. its kin) [33, 72]. In addition, Shapiro suggested that close proximity in biofilms might allow for metabolic interactions and enhance communication, potentially leading to coordinated behaviours [73]. Lastly, it is also known that yeasts benefit by elevated enzyme

concentrations, when cells form aggregates [69], and *P. aeruginosa* benefits by decreased siderophore diffusion in viscous medium [36]. Taken together, close proximity of cross-feeding partners and additionally limited diffusion of shared metabolites are simple factors, which enhance the exchange of goods like amino acids. The feedback loop, which is thereby generated more rapidly than in liquid shaken medium, might have been sufficient to facilitate the immediate fitness benefit in consortia on agar surface. Furthermore, in fact, there was each one cooperative phenotype detected in the liquid-evolved consortia, which exhibited higher fitness than assumed on agar. This phenotype might have generated a positive feedback-loop with the partner, who was in terms of amino acid release still on the level of the ancestor, but this is only one possible assumption. Since the fitness experiment was incubated one week, former liquid-evolved consortia might have evolved a cooperative trait in both partners on the agar surface and performed therefore better.

Cooperative phenotypes, which released more amino acid than the corresponding ancestor, were detected in this study and presented in most cases only one partner in a consortium. In one case on agar, both partners had started to invest into the interaction by the elevated release of amino acids during coevolution. Moreover, phenotypes evolved in a spatially structured environment did release significantly higher amounts of amino acids, when compared to the liquid, spatially unstructured environment. In another study, Hansen *et al*. utilized a two-species community consisting of a host (*Acinetobacter sp*.) and a commensal (*Pseudomonas putida*) for an evolution experiment [37]. Because of coevolution in a spatially structured environment, the coculture was more stable as well as productive, but the interaction developed into a kind of exploitation. The evolution of an exploitative interaction could have arisen because of the initial dependency only based on benzyl alcohol and benzoate, possibly ruling out any cooperative solution for the populations. Hence, this publication did not focus on the evolution of cooperation. There is only one approach so far where an obligate syntrophic mutualism was designed to perform evolution experiments in spatially structured environments: Hillesland & Stahl showed initial erratic growth of *Desulfovibrio vulgaris* and *Methanococcus maripaludis*, which stabilized over time [39]. Interestingly, in this case cocultures that evolved in a heterogeneous environment did not differ from those evolved in a homogeneous liquid non-shaken environment, which probably

was due to the special type of ecological interaction investigated. The uptake of hydrogen as a syntrophic interaction might not be suitable to evolve towards a mutualism, since there is no other alternative for *M. maripaludis* to enhance its cooperation than feeding on hydrogen. In addition, the study does not describe how evolved mutualism could be detected in this syntrophic interaction. Lastly Harcombe *et al.* established an obligate by-product interaction between *Salmonella*, feeding on the partners waste products and the methionine auxotroph *E. coli* [38]. There it is demonstrated that cooperation requires reciprocation and spatial structure. In contrast to the presented work, *Salmonella* was genetically modified to exhibit a precondition for cooperation, which was overproduction of methionine. As the next step, the coculture of the *Salmonella* overproducer and *E. coli* was incubated on agar for several days until *Salmonella* started to release the overproduced methionine. It might be not correct to term this phenotype an evolved cooperator, because most of the steps towards cooperation were already synthetically established. There was also no evolved cooperation shown being directed from *E. coli*. This finally implies that within the presented thesis the positive effect of spatial structure on the evolution of a former by-product interaction to bidirectional cooperation was demonstrated. Auxotrophic *E. coli* strains started to produce enhanced amounts of amino acids and released them to the mutual benefit of the respective partner, without any additional genetic modification needed. Cooperative phenotypes were isolated form one coculture, which evolved on the surface of agar, representing a spatial structure. Moreover, cooperative consortia were significantly more efficient on agar surface than in liquid culture.

4.10 FUTURE PERSPECTIVE

The long-term coevolution experiment presented in this work appeared to be capable to test the hypothesis regarding spatial structures. The positive effect of a spatially structured environment on the evolution of cooperation from an obligate synthetic by-product interaction was demonstrated. Since this represents a novel approach there were many relevant observations made. For further long-term experiments with amino acid auxotrophs, several improvements can be suggested:

The incubation time of seven days was chosen, to provide enough time for coevolution to occur. Since cocultures on agar started to improve in productivity already during the first seven days, the incubation time could be reduced to three days. Moreover, cocultures were observed to sufficiently cross-feed at lower densities after a few weeks of coevolution. The density after inoculation can be therefore set to OD_{600} of 0.001. This might even increase the benefit of evolved cooperative phenotypes. When the incubation time is increased and the dilution is increased at propagation, the *E. coli* wild type could also be included into the experiment for direct comparison with coevolved consortia. In the presented experiment, cryo-stocks of precultures were not prepared. The ancestors, used for further analysis of the coevolved strains, are therefore taken from different cryo-stocks of the original strain collection. In upcoming evolution experiments, samples of precultures should be stored at -80°C. The bacterial strains carried a kanamycin-resistance, integrated into the genome. Combined in a consortium, it was not possible to separate both populations of auxotrophs. Therefore, additional antibiotic resistances can be introduced into the genome. After coevolution, the partners can then be separated, when each partner carries an individual resistance. This would enable the possibility to combine auxotrophs of different evolved consortia and the specificity of adaptation to a partner can be studied.

The emergence of revertant phenotypes was a big issue in the performed experiments. This is why double knockout mutants are currently constructed. Those genetically modified strains will exhibit two missing genes in one biosynthetic pathway of an amino acid. This potentially will reduce the capability of mutant strains to grow in monoculture and coculture in long time-scale incubations.

As another future perspective, interspecies interactions could be studied. The insights gained in this work, if in reality true, are suggested to be reproducible for the evolution of interspecies interactions. Therefore, amino acid based by-product interactions could be established between species of *Escherichia coli*, *Pseudomonas fluorescens*, or *Acinetobacter baylyi*.

Full genome sequencing of coevolved populations could be performed to detect responsible alterations, which caused phenotypes to become cooperative. Single nucleotide polymorphisms, deletions, insertions, and insertion sequence movements are commonly observed sources of mutation [74]. Possible mutations might be located in regulatory elements, which control amino acid anabolism and catabolism, or the transcription of membrane-located amino acid transporters.

5 CONCLUSION

A synthetically designed cooperative by-product interaction was utilized to study the effect of a spatially structured environment on the evolution of cooperation. This interaction was established between the auxotrophs *E. coli ΔtrpB*, and *E. coli ΔtyrA*, each lacking the ability to synthesize one amino acid from the chorismate pathway, tryptophan, and tyrosine, respectively. Dynamics and productivity of a cross-feeding consortium were monitored during a long-term evolution experiment. The experimental design included agar and liquid shaken medium, representing a spatially structured and non-structured environment, respectively. To screen for cooperators and cheaters, phenotypic diversity was observed in evolved consortia of both treatments and amino acids were analysed in culture supernatants of evolved phenotypes from two biological replicates. Statistical comparison of the two treatments revealed significant differences for three parameters, as hypothesized. First, throughout the evolution experiment, positive effects on the productivity of cocultures were significantly higher in spatially structured consortia ($p<0.001$). Second, cross-feeding *E. coli* strains evolved on agar performed on agar surface in terms of fitness significantly superior than liquid-evolved strains on agar ($p<0.001$) or in liquid ($p<0.001$). However, there was no difference for both consortia, when fitness in liquid medium was compared. Third, the release of amino acids was partial remarkably elevated in agar-evolved consortia with a significant difference to liquid-evolved cultures of $p<0.01$. In one population derived on agar, actually both partners started to cooperate and exchanged elevated amounts of tryptophan and tyrosine. In contrast, clearly increased amounts of amino acids were only released by one phenotype in the spatially non-structured environment. Furthermore, cheating phenotypes were detected in both treatments. They are characterized by decreased release of amino acids relative to the ancestor. As another confirmed expectation, consortia, independent of treatment exhibited dynamics in the ratio of partners as well as phenotypic diversity.

Finally, results presented in this thesis support the predictions about positive effects of a spatially structured environment on the evolution of cooperation.

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7 APPENDIX

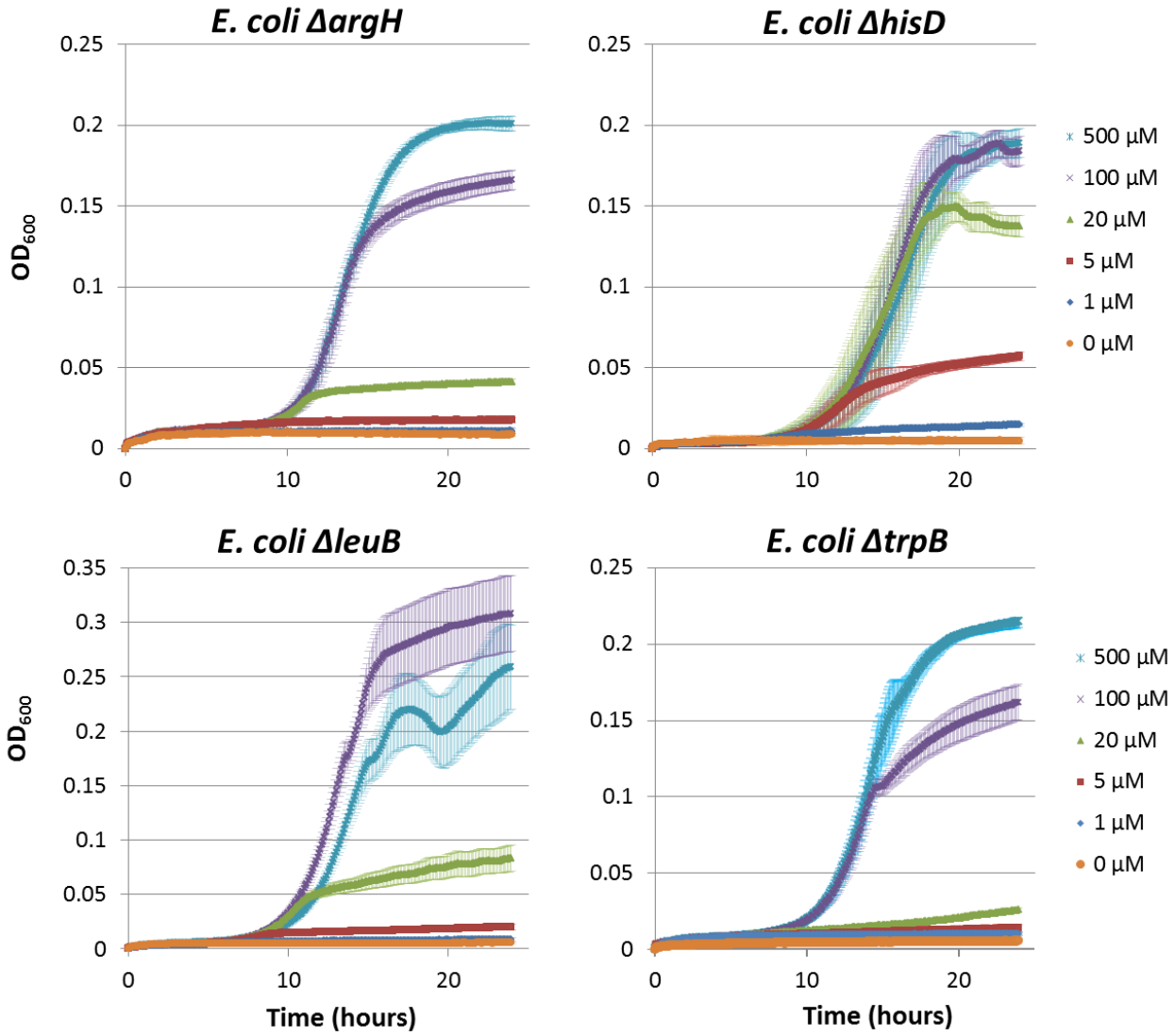

Figure A-7-1: Growth curves of cultures supplemented with different amino acid concentrations. Cultures within a 96 well plate were grown in a plate reader for 24 hours. M9-minimal medium containing 0.5 % Glucose was supplemented with increasing concentrations of the focal amino acid. OD_{600} was determined every 5 minutes. (Errors given as 95% CI; n=8)

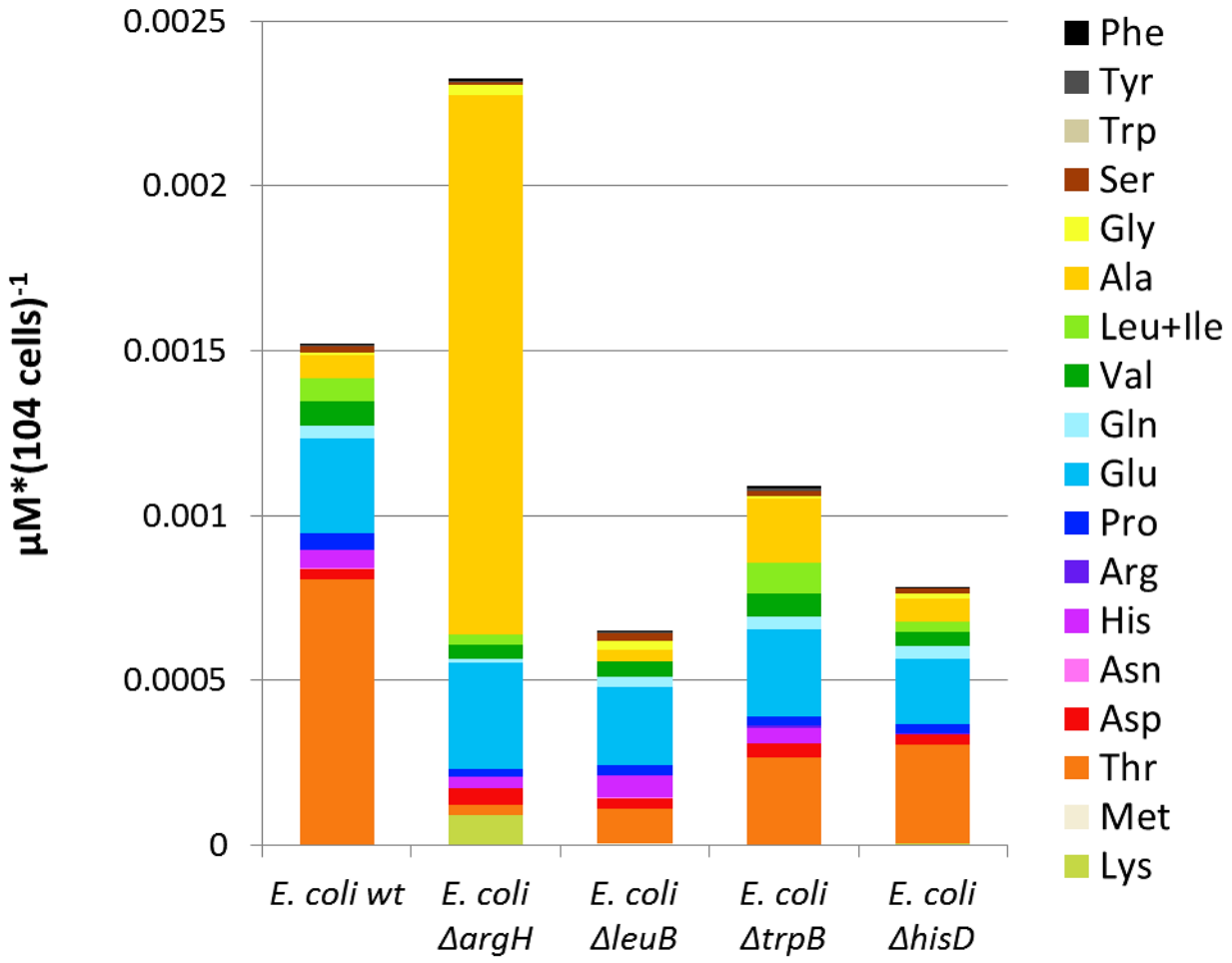

Figure A-7-2: Amino acid release normalized to OD_{600} of 1. Individual concentrations of the *E. coli* wild type and auxotrophs were determined in culture supernatants via LC-MS/MS. *E. coli* cultures were incubated for 18 hours incubation at 30°C in supplemented M9 minimal medium. Medium was not supplemented in case of the wild type. OD_{600} of the precultures was determined before analysis to normalize amounts of amino acids to optical density of precultures. 10^9 cells were suggested to exhibit an OD_{600} of 1. Concentrations, normalized to OD_{600} of 1, were therefore divided through 10^5 to receive µM/10^4 cells.

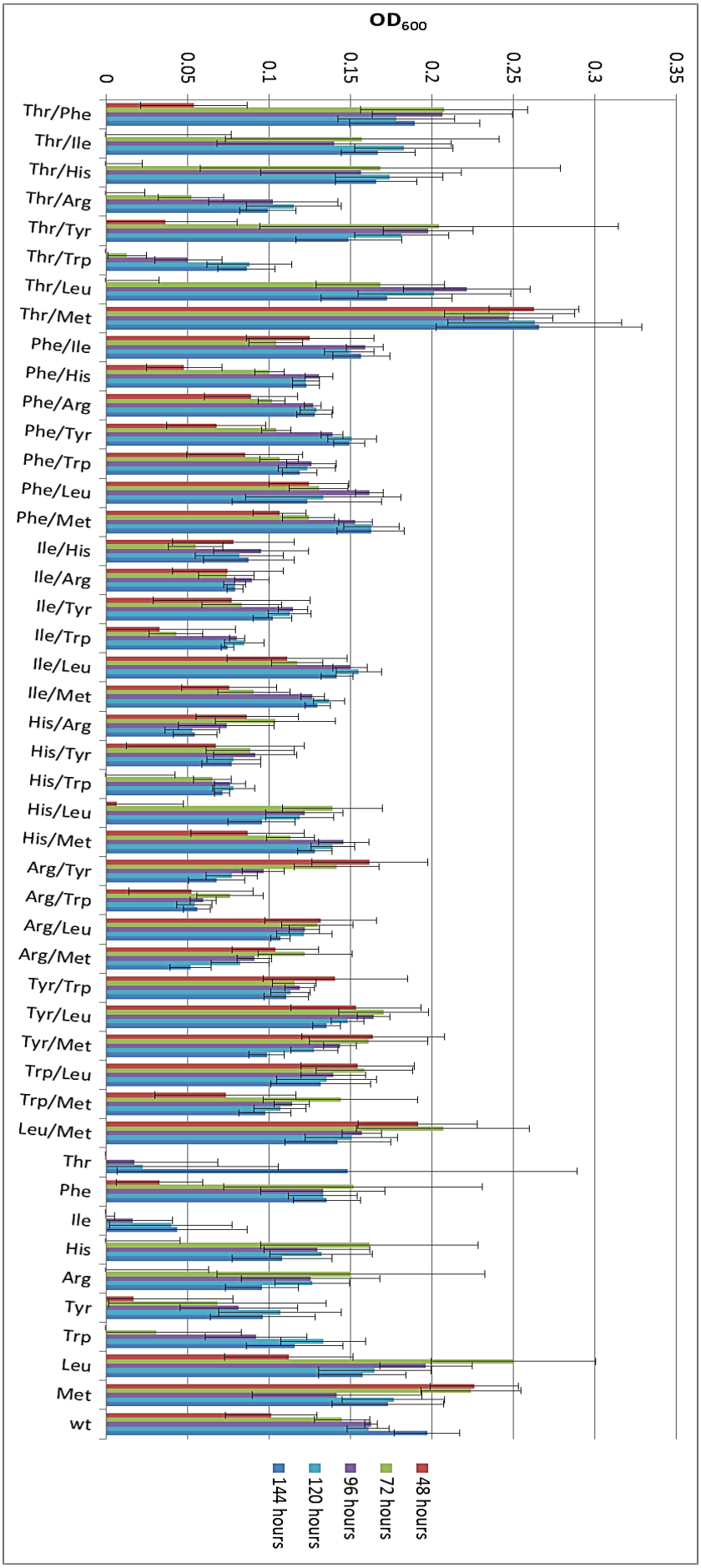

Figure A-7-3: Coculture experiment. Mono,- and cocultures were grown for 144 hours at 30°C in minimal medium and OD_{600} was determined every 24 hours, starting at 48 hours. Given values are quotients of the final OD of consortia with the sum of each 50% final OD of both respective monocultures.

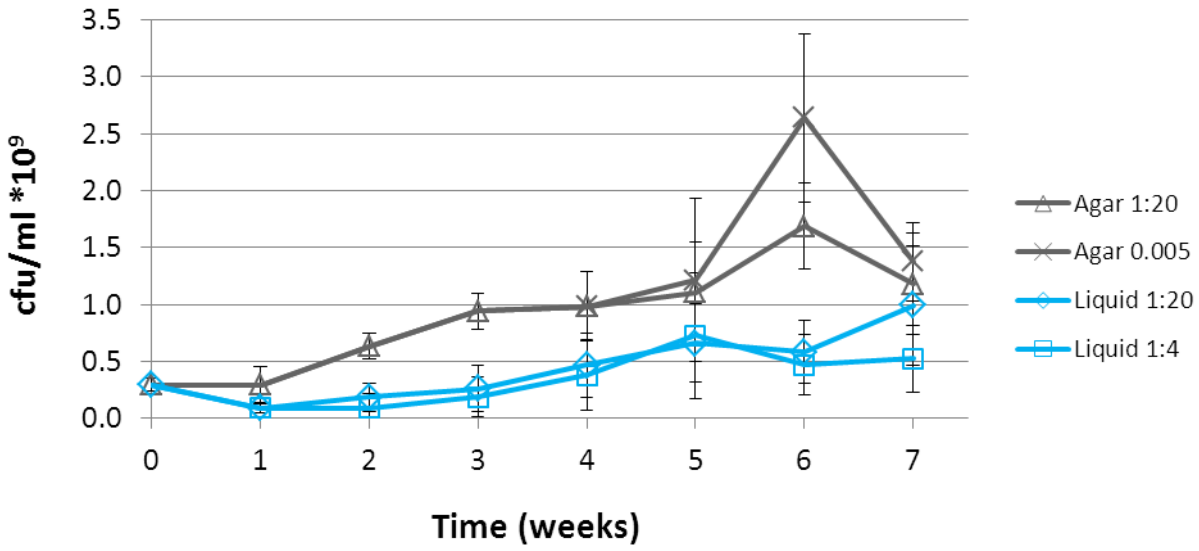

Figure A-7-4: Colony forming units in cocultures during the long-term experiment. At every transfer, cocultures were plated on TA-agar to determine numbers of living cells for both strains in each microcosm. (Errors given as 95% CI; n=12)

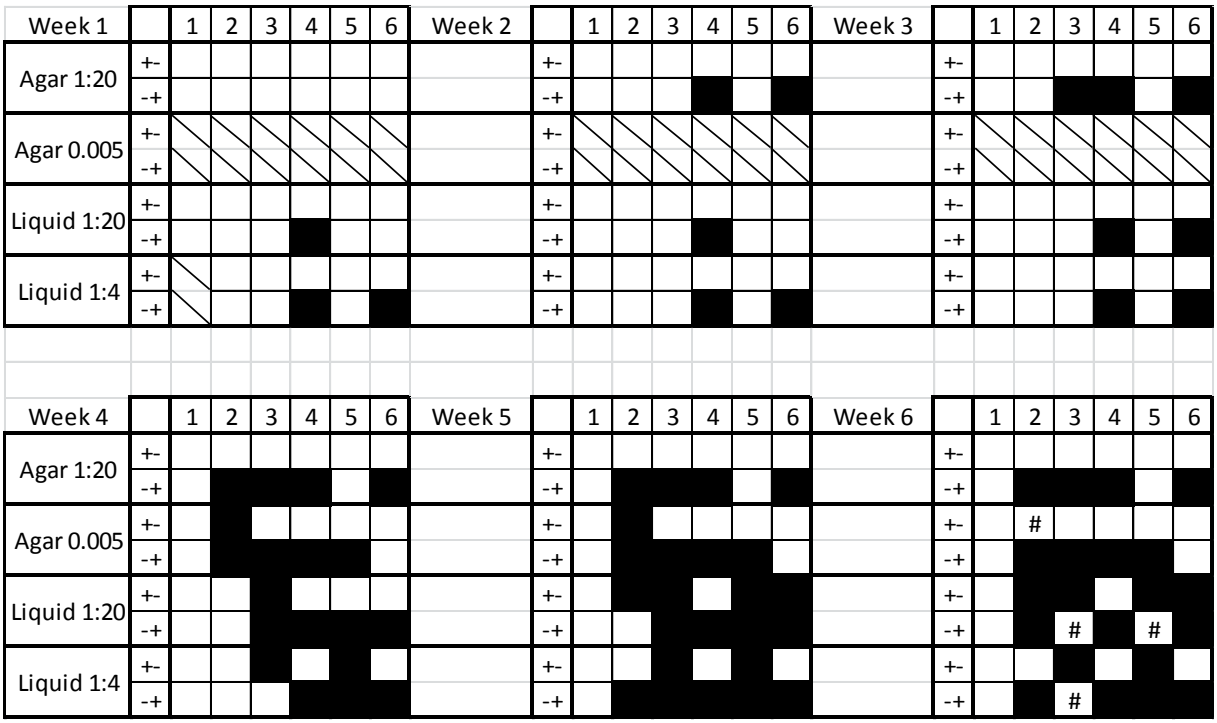

Figure A-7-5: Emergence of revertant phenotypes during the long-term experiment. Dilutions of cocultures were plated weekly on M9-agar to test for revertants. Plates were incubated for 3-4 days at 30°C. Formation of single colonies was evaluated as prototrophic growth, hence as revertant phenotypes. Observed revertants are indicated by a black field. ”#” indicates disappearance of these phenotypes.

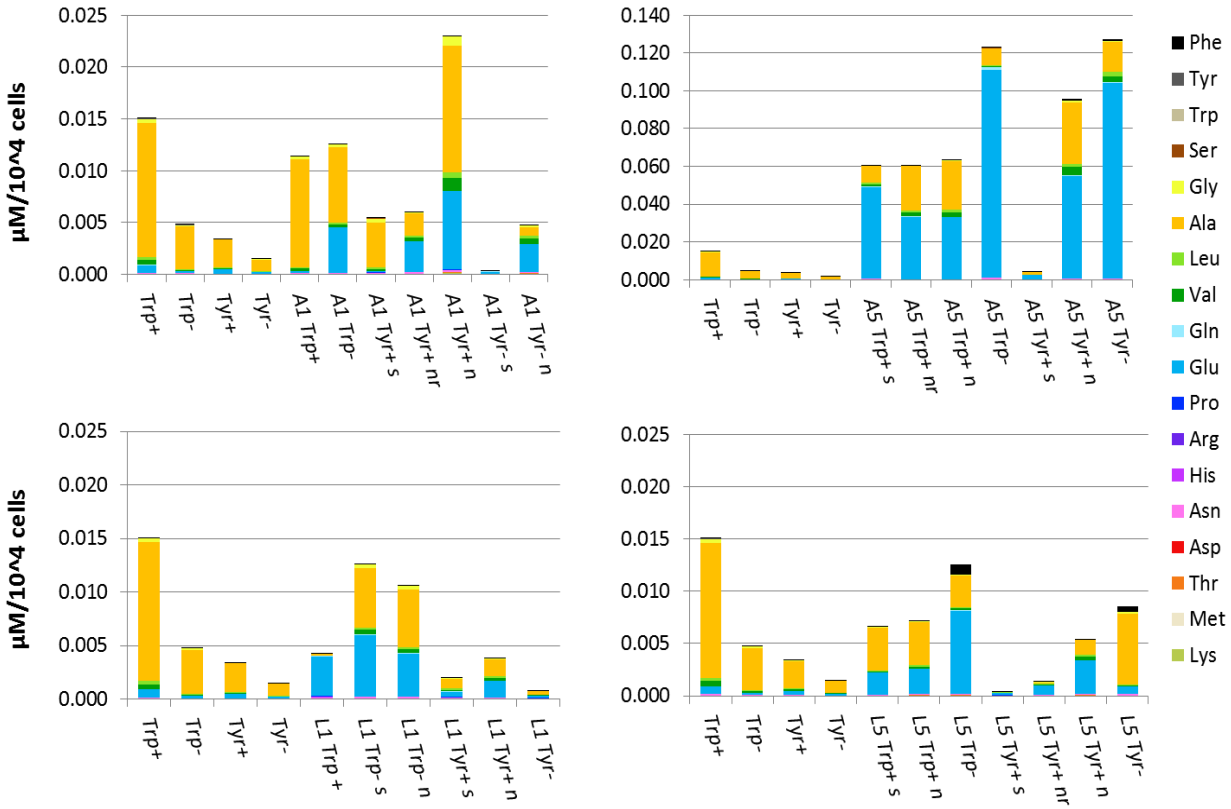

Figure A-7-6: Amino acid release normalized to 10^4 cells. Individual concentrations of the *E. coli* wild type and auxotrophs determined in supernatants of *E. coli* cultures via LC-MS/MS after 18 hours incubation at 30°C in M9 minimal medium. Total concentrations represent the sum of individual concentrations.

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Thesis selbständig und ohne unzulässige fremde Hilfe angefertigt habe. Die verwendeten Quellen sind vollständig zitiert.

Datum: 28.02.2013 **Unterschrift**_____

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