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**The effect of diversity on productivity
within cooperative amino acid cross-
feeding consortia of auxotrophic
*Escherichia coli***

Bachelorarbeit

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Kurzfassung

Betrachtet man Mikroorganismen in ihrer natürlichen Umgebung, stellt sich heraus, dass sie fast nie isoliert vorkommen. Bakterielle Gemeinschaften sind omnipräsent und meist sehr divers in ihrer Struktur. Diese Vielfalt lässt ungemein viel Raum für inter- und intraspezifische Interaktionen. Für das Phänomen der mutualistischen, mikrobiologischen Abhängigkeiten sind bereits viele Beispiele beschrieben und untersucht. Um diese Gemeinschaften in ihrem ökologischen Kontext zu begreifen, ist es wichtig diese Netzwerke in Abhängigkeit von ihrer Diversität zu untersuchen. Die Keio Collection auxotropher *Escherichia coli* Stämme ermöglicht die Erstellung von synthetischen bakteriellen Konsortien, welche auf symbiotischen Abhängigkeiten - wie "cross-feeding" basieren. Auf dieser Grundlage wurden in dieser Arbeit verschiedene Konsortien auxotropher *Escherichia coli* Stämme hinsichtlich ihrer Produktivität in Abhängigkeit von der zu Grunde liegenden Diversität untersucht. Ferner wurden diese Konsortien noch in ihrer Zusammensetzung charakterisiert. Herausgefunden wurde, dass die Produktivität der Konsortien mit zunehmender Diversität steigt, wobei der Anteil der Einzelstämme am Konsortium mitunter sehr unterschiedlich sein kann. Unter Bedingungen eines Vollmediums bleiben die Anteile ungefähr gleich groß. Über diese Untersuchung hinaus werden in dieser Arbeit verschiedene Ursachen für die Resultate diskutiert, wobei herausgearbeitet wird, dass die "Sampling-, Selection-" und "Niche Complementary-" Effekte eine bedeutsame Rolle spielen. Auch wurde die Black Queen Hypothese (BQH) in diesem Zusammenhang diskutiert. Als Grundlage dafür wird auch auf die aktuelle Forschung von Kollegen der Arbeitsgruppe Experimentelle Ökologie und Evolution des Max Planck Instituts für chemische Ökologie Bezug genommen. Außerdem wurde die Bedeutung der einzelnen Aminosäuren für das Bakterium als proximate Ursache für die Konsortienzusammensetzung erörtert. Diese Arbeit bildet einen weiteren wichtigen Baustein für das Verständnis biologischer Gemeinschaften und veranschaulicht die Verwendung synthetischer Konsortien für die Beantwortung biologischer Fragestellungen.

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1. Introduction: The Emergence of Bacterial Auxotrophy

When microorganisms are observed in nature, it is found that they almost never occur in isolation – bacterial communities are omnipresent and often very diverse. The variety of different microorganisms leaves much room for interaction. For the phenomenon of mutual microbiological dependencies, such as nitrogen fixation or other syntrophies, many examples are described [1, 2]. To understand the ecology of these communities, it is important to investigate the mechanisms of microbial networks regarding their underlying diversity. The creation of a collection of amino acid auxotrophic biosensors from the Keio Collection [3] offered to create synthetic bacterial consortia in which dependencies by cross-feeding play a key role. Based on this the relationship of the productivity from the diversity of consortia of auxotrophic *Escherichia coli* will be examined and characterized here.

1.1 Black Queen- and Public Goods Hypotheses

A problem that microbiologists have in the study of bacteria from the environment is the "great plate count anomaly": Only a tiny fraction of the bacteria can be cultured under laboratory conditions [11-13]. Several reasons may be responsible for this phenomenon. Under laboratory conditions, the abiotic properties cannot be created, for example, pH, temperature, pressure, providing necessary and avoiding harmful substances, which are needed by bacteria to thrive.

On the other hand uncultivable organisms obligatory need their interaction partners. The mutualistic relationships of bacteria may have already reached a complexity that they are obligatorily tied to their partners [2]. This tie may itself be caused by different mechanisms. In the following one way of the emergence will be described. Many microorganisms produce so-called public goods - they intendedly or unintendedly leak substances (e.g. amino acids or vitamins), which are used by their neighboring cell's metabolism [4, 14-16] (Figure 1 A). Division of labor can reduce the over-all production costs for such goods in a community and subsequently it is not selected for prototrophy because it is not beneficial in comparison [17, 18].

Due to genetic drift such auxotrophic bacteria are common as endosymbiotic bacteria [18, 19]. For free-living bacteria another explanation for the genome erosion is more plausible: the Black Queen Hypothesis. After Morris *et al.* microorganisms discard extra genes, similar to the card-game "Hearts" where the players try to ditch the queen of spades. According to this, a loss of traits for the production of public goods brings a selective advantage because this often reduces costs. Consequently, this causes also dependencies between those organisms [18]. Depending

on which public goods are produced, specific niches arise and those who occupy these create in turn other niches through the provision of public goods – division of labor emerges [20-22] (Figure 1A). The effectiveness of the consortium depends among other factors on how expensive, how leaky and how required the public goods are. On the other hand, it is also - according to the Black Queen Hypothesis - possible that so-called "beneficiaries" and "helpers" emerge. The "beneficiary" would benefit from the products of the "helper" without contributing net public goods to the community [18]. So it would have a selective advantage over the helpers. In function of the density ratios it may also reduce the overall productivity (Figure 1 B, C).

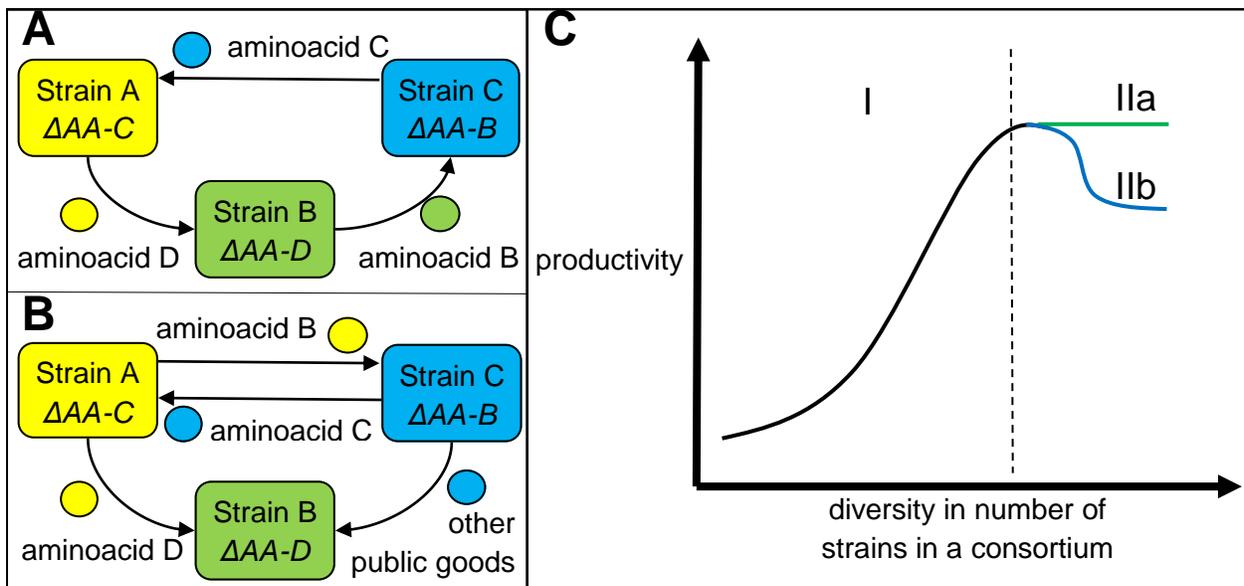


Figure 1. Expected relationships between the mutualistic effects.

A shows the case that all strains are interdependent. **B** shows a consortium where a "beneficiary" (strain B) and „helper“ (strain A, C) are present. **C** shows the expectation that with increasing diversity of a consortium productivity increases (part I), because synergistic effects increase. Part IIa shows the case that from a certain diversity a maximum productivity is achieved and potential negative effects do not reduce productivity. IIb shows that after reaching a maximum productivity the synergistic effects are diminished again due to negative effects.

On the one hand the productivity of the consortium should increase with a greater initial variability of the traits because of the effects described in 1.2. On the other hand, the productivity may also decrease when the species compete for the same spatial niche, occupy the same functional role and differ only in the amount of resource demand [5] which is related to the Black Queen Hypothesis.

The effectiveness of the consortium can be measured by productivity which accords to the cell growth over time [23]. Biological diversity on the one hand is considered as a diversity of species, but on the other hand ecologists consider diversity increasingly also as the level of functionality - so called functional diversity. Since the exact relationship between species

diversity and functional diversity (although they usually correlate) is not clearly understood and discussed controversial [6, 24, 25], reference will be made here on the functional diversity.

1.2 Sampling-, Selection-, Niche Complementary Effect

The complex relationships between microorganisms with their environment have a great impact on humans [4]. Although knowledge about the phylogeny and character of the bacteria has grown almost exponentially in recent decades, much remains hidden. When the individuals in a community obligatory depend on each other, it is obvious that there probably exist mutualistic relationships. The more diverse and the more different these partners, the more possibilities for interactions exist. But what has this diversity for an effect on productivity?

There are different effects which influence the relationship between productivity and diversity. The “sampling effect” says there is competition for a limiting resource between all involved species. An initial trait variability causes competing species with different productivities. Subsequently it is selected for the more productive ones [5]. The “selection effect” [6, 7] goes in the same direction. This effect indicates that higher species diversity increases the probability of the presence of species with a particularly important trait which can dominate the ecosystem. Loreau also describes a “niche complementary effect”: With a greater diversity a greater range of functional characteristics will be represented. This allows a more efficient exploitation of resources in a spatially or temporally variable environment [5, 7]. Loreau, Tilman and Diaz refer to plants [5-8] but is it possible to monitor these effects in bacterial communities too?

One opportunity to examine this is due to auxotrophic bacteria of the Keio Collection [3, 9]. Wintermute *et al.* already showed with the help of the Keio Collection that synthetically generated auxotrophic *E.coli* strains develop a synergistic effect in the consortium, which is reflected in improved growth [10]. With a similar system the question will be addressed here: how does the growth of such a community behave with increasing diversity?

1.3 Expectation of this Study: Productivity will increase with increasing Diversity

In the case described above, the bacteria are forced to form a community here. The more interaction partners, the more likely it is that cross-feeding arises. Due to the described effects (see 1.1, 1.2), it is likely that with increasing diversity better interactions are formed and thus productivity increases. The emergence of “beneficiaries” can also cause decrease in productivity with increasing diversity at a certain level of productivity (Figure 1 C).

The hypothesis of this study is that productivity increases with diversity in our synthetic consortia. In this context, the character of the communities will be described and discussed – more precisely – the composition of the consortia and potential fitness advantage over the wild type.

2 Materials and Methods

2.1 Media and Solutions

LB-Agar (Carl-Roth, Karlsruhe, Germany)

Yeast extract	5 g/l
Tryptone	10 g/l
NaCl	5 g/l
Kobe Agar	16 g/l

Minimal medium for *Azospirillum basiliense* (MMAB)

(according to Vanstockem *et al.* [26])

K ₂ HPO ₄ dibasic	3 g/l
NaH ₂ PO ₄ monobasic	1 g/l
KCl	0.15 g/l
Na ₂ MoO ₄	0.1g
Fructose (autoclaved separately)	20 g

Salt:

MgSO ₄ * 7 H ₂ O	0.3 g/l
CaCl ₂	0.01 g/l
FeSO ₄ * 7 H ₂ O (in EDTA)	0.0025 g/l

TA-Agar¹ (developed by Levin *et al.* [27] and modified by Lenski *et al.* [28])

Tryptone	10 g/l
Yeast extract	1 g/l
NaCl	5 g/l
Kobe Agar	16 g/l
L(+) Arabinose (autoclaved separately)	10 g/l
Tetrazolium indicator dye ²	1 ml/l

Amino Acid Stock Solutions:

The corresponding mass of each amino acid (arginine, lysine, histidine, threonine, tryptophan, proline, leucine, isoleucine, methionine, phenylalanine, tyrosine) was dissolved in either bidistilled water to receive a stock solution of 10 µmol/ml. From this stock solution the needed volume was added to the final MMAB-medium to achieve a final amino acid concentration of 100 µmol/l.

¹ TA = Tetrazolium and Arabinose

² (Sigma T8877), added separately by sterile filtering

2.2 Strains

In the analysis of the cross-feeding 11 auxotrophic strains were used (see S1 – Strain List). The used strains came from the Keio Mutant Library [3]. Gene deletions of the terminal gene of the corresponding amino acid to receive auxotrophy were established by Bertels *et al.* [9]. Each single amino acid synthesis gene was replaced with a Kan^R containing cassette due transduction and homologous recombination.

From these strains further substrains were created:

In this study the ara⁻, Kan^R – Strains were also chromosomally labeled with a cassette containing a fluorescent marker, either eGFP or mCherry (see 2.3.2). The used plasmids were constructed by Bertels *et al.* [9] and Scholz, S. [29].

2.3 Methods

2.3.1 Measurement of the Optical Density and fluorescence Intensity

Two devices were used for the measurement of the optical density (OD₆₀₀). The first spectrometer was the Spectramax 190 (Molecular Devices, Sunnyvale, USA) which is only capable of measuring the optical density. The second spectrometer was the Tecan F200 luminescence reader (Tecan, Männedorf, Switzerland) which was also used to monitor the fluorescent strains. The used software for single measurements was the iControl software (Tecan, Männedorf, Switzerland) and for kinetic measurements Magellan software (Tecan, Männedorf, Switzerland).

For measuring in the Spectramax transparent 96 chimney microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were used. For the Tecan F200 black 96 chimney microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were used.

2.3.2 Creating fluorescently labeled Strains

From the strains *E.coli* Top Ten PGRG+mCherry and *E.coli* Top Ten PGRG36+EGFP [29] the plasmids were extracted and transformed into the competent auxotrophic *ara*⁻, Kan^R strains by heat shock method. The used protocol for the chromosomal insertion was developed by McKenzie *et al.* [30].

Creating competent *E.coli* strains

The rubidium chloride method (NEB) was used to obtain chemo-competent *E.coli* cells. An overnight culture (30 °C) was streaked on LB agar directly from the glycerol stock. Of these, a colony was picked and grown in 5 ml of LB medium overnight (30 °C, 220 rpm). This was then diluted 1:20 with LB medium to get a final volume of 5 ml and let grow again until the culture reached an OD₆₀₀ of approximately 0.6. Subsequently, the cells were placed on ice and centrifuged (15 min, 4000 x g, 4 °C). The pellet was resuspended in 1.5 ml TFB1. The cell suspension was then centrifuged again and the supernatant discarded. After this 160 µl TFB2 was added, and the pellet was resuspended and divided into 6 tubes, which were then stored at -80 °C.

Extraction of plasmids

The *E.coli* strains harboring the respective plasmids were grown over night (30 °C, 220 rpm) in LB medium (supplemented with 100µmol/l ampicillin, which was the selective marker). The plasmids were then extracted using the GeneJET™ Plasmid Miniprep Kit (Fermentas) and stored at 4 °C.

Transformation

The competent cells were taken from the glycerol stock directly on ice and treated with 50 ng of plasmid and incubated for 20 minutes. This was followed by an incubation at 42 °C for 50s (heat shock). Then immediately 1 ml of LB medium was added and incubated for 30 min (30 °C, 220 rpm). The culture was then plated on LB agar (supplemented with 100 µmol/l ampicillin) and incubated at 30°C. At this step the selection for the transformed cells took place. After colonies were visible with at least three colonies a dilution plating was done on LB medium (supplemented with ampicillin). Of these, several colonies were then picked and re-cultured in LB medium (without ampicillin) incubated (30 °C, 220 rpm). At this point, the transposase should act. From this culture, then streaks were made on LB-agar (without ampicillin) and incubated overnight at 42 °C (loss of the plasmids). Cultures thereof were prepared in LB medium

overnight (30 °C, 220 rpm) and analyzed for fluorescence. From fluorescent cultures a cryoculture was prepared.

Used Buffers for Labeling:

TFB1 Transformation buffer 1 (NEB)	Adjust pH to 5.8 with 0.2 Acetic Acid
RbCl	12 g/l
MnCl ₂ * 4 H ₂ O	10 g/l
1 M Potassium Acetate, pH 7.5	30 ml
CaCl ₂ * 2H ₂ O	1.6 g/l
Glycerol	150 ml

TFB2 Transformation buffer 2 (NEB)

0.5 M MOPS, pH 6.8	20 ml
RbCl	1.2 g/l
CaCl ₂ * 2H ₂ O	11 g/l
Glycerol	150 ml

2.3.3 Consortia Experiments

Eleven conditionally lethal auxotrophic strains were selected from the Keio Collection [3]. Each strain is auxotrophic for a particular amino acid and shows individually growth in LB medium and no considerable growth in MMAB medium [9]. These strains were also used to create fluorescent strains with a chromosomal marker gene. The strains were prepared in different compositions together in minimal medium and showed growth, so the existence of cross-feeding can be assumed. Via measurements of the optical density, fluorescence, colony forming units (cfu) on selective media the total productivity and the proportions of the individual strain at the consortium were measured (Figure 2).

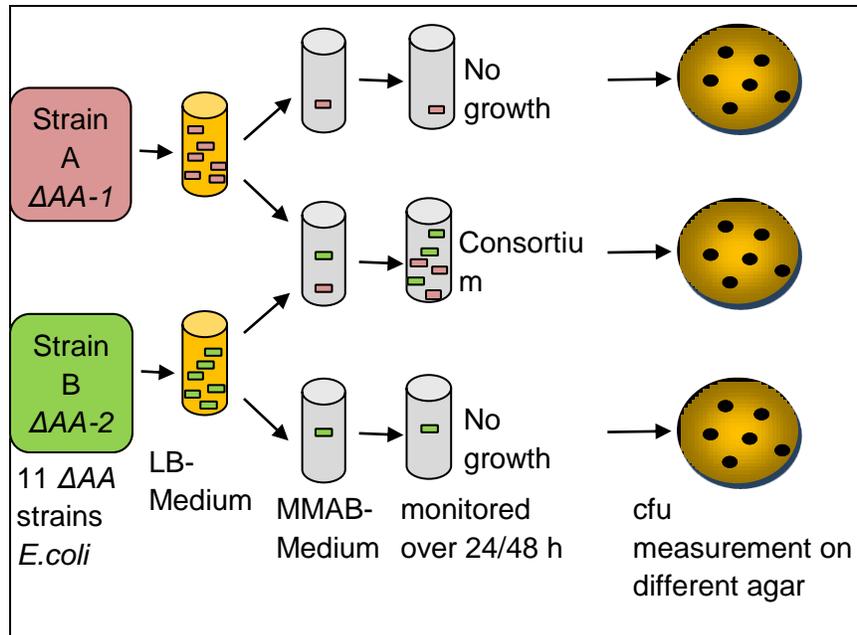


Figure 2. The experimental design in principle.

Eleven auxotrophic strains of the Keio collection were chosen and grown in LB medium. After that they were diluted and different compositions of consortia were created. In this figure an example for a consortium of two strains is pictured. They were grown in MMAB medium. In MMAB medium they showed no growth alone but in most consortia. The measurements were done by optical density, fluorescence and cfu. Picture made after Wintermute *et al.* [10].

2.3.3.1 Kinetic Measurements in general

The cultures were streaked from the glycerol stock onto LB agar and grown over night (30 °C). Subsequently, the colonies were picked from these plates. These were grown overnight in 5 ml LB-medium (30 °C, 220 rpm). Then they were washed three times with MMAB medium. In the MMAB medium the OD (600 nm) was measured using the Tecan or Spectramax and diluted to an OD₆₀₀ of 0.1. From these dilutions various compositions were created in which the strains had the same share.

In order to achieve the desired dilution, these compositions were themselves diluted with MMAB medium (or MMAB medium with supplemented amino acids). The volume of the microtiter plates in each well was 200 µl. For the programs of the devices in the experiments see S2 – Programms.

To obtain growth curves that have completed their growth within 48 hours, a pre-experiment was made. In this, consortia of all eleven strains were inoculated with a different dilution. Based on these results a dilution of 1:60 and 1:100 for the compositions was chosen in the following experiments.

2.3.3.2 Experiments to measure Productivity/Diversity

From strains 1 and 4 – 33 (see S1 – Strain List) with an OD₆₀₀ of 0.1, consortia of different diversity levels ranging from two, up to eleven strains, were prepared. Each diversity level was replicated seven times though composition of individual consortia per diversity level differed. The compositions were then diluted 1:60, and 200 µl of each composition was placed in a transparent microtiter plate, plus a corresponding uninoculated MMAB medium as blank. The wild-type was the positive control, the negative control were the single auxotrophic strains in MMAB medium. Results see 3.1.

2.3.3.3 Experiment Comparison Wild-type/Consortia

For this experiment, the auxotrophic strains 1 and 4 – 33 were used (see S1 – Strain List). 200 µl 1:60 diluted consortia consisting of 11 strains and the wild type separately were placed in a microtiter plate, and the growth kinetics were measured in Spectramax (program in the supplementary). To measure the proportions of the various strains in the consortium, each consortium consisted of 11 ara⁻ and one ara⁺ and vice versa. After measurement 200 µl of 10⁻⁶ and 10⁻⁷ dilutions were plated on TA agar and the cfu was determined. Additionally the samples were inoculated on MMAB agar as negative control. Results see in 3.2.

2.3.3.4 Experiment to analyze the Distribution of the Dstrains in the Consortia via cfu

For this experiment, the strains 1 and 4 – 33 were used (see S1 – Strain List). With these, a consortium of 11 auxotrophic strains was composed and diluted 1:60 with MMAB medium. 200 µl of these compositions were put into the microtiter plate and incubated in the Spectramax. The device measured the OD (600 nm) for the incubation time. Then these samples were diluted 10⁻⁴ and 10⁻⁵, and plated on amino acid supplemented MMAB agar. Supplementation always included the respective amino acid for which each strain was auxotrophic. Thus, each sample of the microtiter plate was plated on 11 different MMAB agar plates with three replicates. The cfu was determined by counting. As control for prototrophy the samples were streaked out on unsupplemented MMAB agar with a dilution of 10⁻³. The measurement in the Spectramax was terminated after 24 h, as the stationary phase has been reached because a dying of the bacteria should be prevented. Results see 3.3.

2.3.3.5 *Experiment to analyze Distribution of the Strains in the Consortia via fluorescently labeled Strains*

For this experiment, the strains 1 and 34 – 57 were used (see S1 – Strain List). With these consortia of 10 auxotrophic strains were created. These had the feature that 9 strains were unlabeled and the tenth with either mCherry or eGFP was labeled. Thus, there were 11 different consortia for each color, each with 2 replicates. These were put in a black microtiter plate with a dilution of 1:100. Each strain was also put into the microtiter plate with MMAB medium supplemented with all 11 amino acids and also unsupplemented with the same dilution. As a positive control the prototrophic strains were inoculated the same way. The reason for just 10 instead of 11 strains was due to the fact that the chromosomal labeling for the isoleucine auxotroph could not be accomplished at this time. The measurement was performed by the Tecan M200. Results see 3.3.

2.3.4 Statistical Analysis

The data was collected in Excel 2010 (Microsoft Corporation). The calculation of the maximum growth rate (MGR) and the regression lines were done also in Excel 2010 (Microsoft Corporation). For the calculation of the regression line see S3 – Calculations. Statistical tests were done with SPSS (IBM Corporation) and ORIGIN8G (Originlab Corporation).

3 Results

3.1 More diverse Consortia have higher Fitness

Wintermute *et al.* has been shown that amino acid auxotrophic *E.coli* from the Keio-Collection establish mutualistic relationships [3, 10]. To test whether the productivity increases with increasing diversity following approaches were used based on these mutualistic relationships. The productivity and maximum growth rate (MGR) of different compositions of consortia was determined and compared. First compositions of consortia were mixed, consisting of 2 until 11 strains. The members of the consortia were chosen randomly with no duplicate. These compositions were inoculated in MMAB-Medium. Then the OD₆₀₀ was measured over time. The composition of 10 and 11 strains will not be portrayed below due to errors (see S4.2 – Errors of Consortia of 10 and 11 Strains).

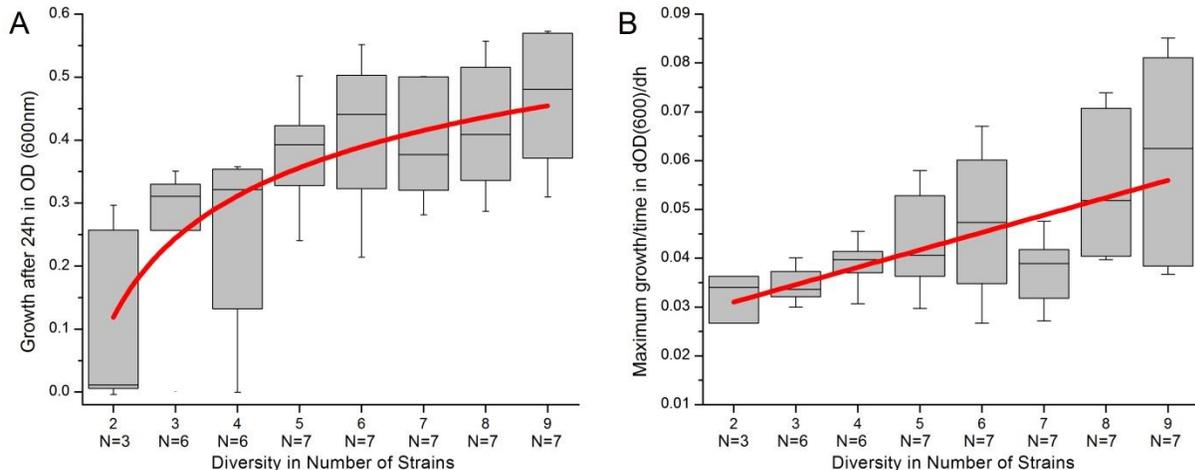


Figure 3. Productivity after 24 h and maximum growth rate (MGR) of the consortia with increasing diversity in number of auxotrophic strains.

A: Attained growth after 24h of different consortia. Red - logistical adaptation of the average values (ANOVA: $F = 169.6$, $p < 0.001$). The average productivity increases with diversity. The biggest increase can be discerned in the consortia 2-6. It seems that the maximum productivity is achieved at a consortium of six different strains. If the non-functioning consortia would still be counted, then the result would be even more precipitously in the first area. B: Maximal growth rate (log-phase). Consortia, which showed no growth, were excluded. Red - linear fit of the average values (ANOVA: $F = 21.6$, $p < 0.001$). The average maximum growth rate increases across all consortia considered almost linearly, wherein the standard deviation is greater, too.

In compositions with low diversity (compositions of 2, 3 and 4 strains) it happened that some compositions showed no growth above an $OD_{600} \geq 0.02$ even after more than 48h so it can be assumed that no consortia were formed. Probably not each composition can develop a syntrophic relationship. Even if only the growing compositions (=consortia) were compared, an

increase in productivity is visible with increasing diversity³ and an increasing maximum growth rate⁴ (MGR) (Figure 3). This shows that the increase of productivity is not just caused by the effect that some compositions simply do not establish cross-feeding but the probable existence of other effects. The productivity remains constant for the diversity of 6 and more strains.⁵ The assumption of Figure 1C was met while a decrease in productivity with a high diversity due to competition effects (see Figure 1C IIb) could not be shown. Figure 3A shows that with a diversity of 6 strains in a composition the maximum productivity is reached and does not increase much further.

3.2 The prototroph Wild-Type grows faster than the Consortia

The auxotrophic bacteria in the consortium differ in their needs and their output of different amino acids relative to the wild type [31]. It might be that the wild-type *E.coli* of this study does also absorb non-essential amino acids the same as the large number of developed biosensors based on *E.coli* [32]. When species occupy the same niche and fulfill the same function, only their resource consumption is different, then increasing diversity may lower the productivity [5]. This leads to the assumption that in this study the wild type should show a higher performance than the consortia which was indeed the case (Figure 4). The wild-type performs much better than the consortia of 11 strains in terms of productivity and MGR.

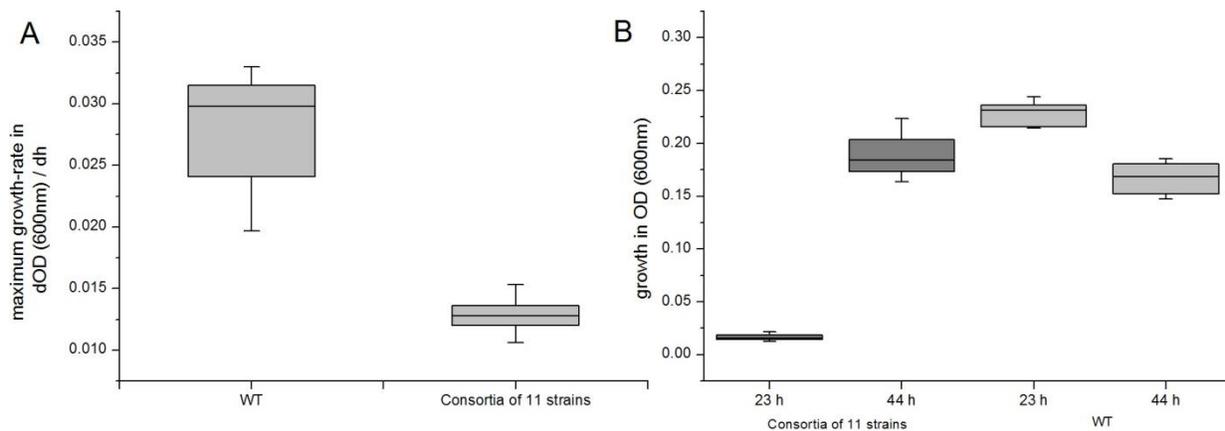


Figure 4. Fitness comparison of consortia with 11 AA⁻ strains with the wild type.

Productivity after 24 h and maximum growth rate (MGR) of consortia with 11 auxotrophic strains in MMAB medium. A: The wild type (N = 12) grew three times faster than the consortia (N = 12) (two sample t-test, $\mu = 0$, $p < 0,001$). B: The wild type (N = 12) after 23 h has a much larger growth reached (maximum OD in the experiment) than the consortium (N = 12) (two sample t-test, $\mu = 0$, $p < 0,001$). After 44 h, the OD (600 nm) value decreases (two sample t-test, $\mu = 0$, $p < 0,001$).

³ Kruskal-Wallis Test for consortia with a diversity of 2 – 6: $p = 0.002$, Figure 3A

⁴ Kruskal-Wallis Test for consortia with a diversity of 2 – 9: $p = 0.009$, Figure 3B

⁵ Kruskal-Wallis Test for consortia with a diversity of 6 – 9: $p = 0.509$, Figure 3A

3.3 Distribution of the strains in the consortia

3.3.1 Development of Strain Distribution in the cross-feeding Consortia

Due to the different physiological properties of the amino acid auxotrophic strains, they should have different growth in the consortium. It was observed that after 48 h the consortium of 10 strains consists of more than 90% of only of 5 different strains (Figure 5). These results were achieved due to fluorescent strains. Problematically is that fluorescence can only be detected when the strain is growing at all. The linear calibration range of the OD₆₀₀/fluorescence correlation is not sensitive for an OD₆₀₀ below 0.05. It can be assumed that the initial inoculated strains such as the leucine auxotrophic simply could not be measured, but are not extinct in the sample.

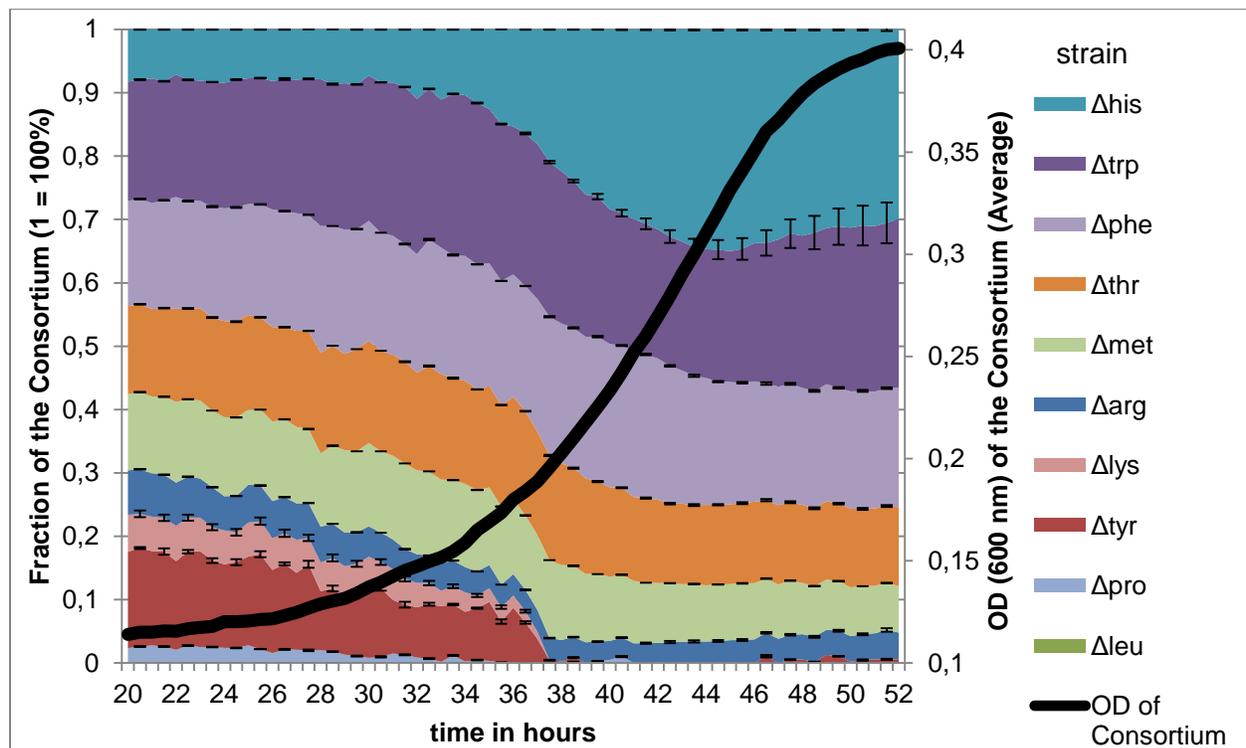


Figure 5. Evolution of the composition of the consortium over time.

The results come from 10 consortia (N = 2), which had the same composition of auxotrophs but one strain was labeled with the fluorescent protein mCherry. Subsequently, the percentage of the consortium of the strain was based on the fluorescence (see S3 – Calculations). Colored areas: left axis, errors given as 95%. The growth of the total consortium is shown by the black line (N = 20, right axis, without error bars).

The standard curve was based on single strains growing in supplemented MMAB medium. It has to be mentioned that the fluorescence can be different when the strain is growing in the consortium instead of growing alone.

3.3.2 Comparison of the Distribution of Strains in the Consortia with different Methods and Media

Two different experiments with similar diversity (10 and 11 strains), measuring methods, devices, and dilutions have been applied. Despite the differences between these experiments the consortia consisted of 80 % of the same 5 strains after their log phase (Figure 6). The negative control was made of the same initial composition of 10 strains and grew in supplemented MMAB medium. Here, a substantially more equal distribution can be detected (Figure 7).

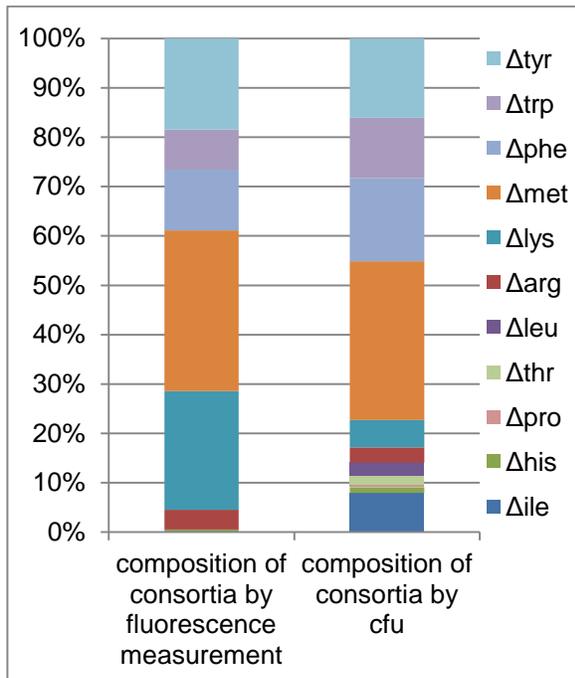


Figure 6. Comparison of the method of fluorescence measurement and cfu to obtain the composition of the consortia over time.

These are the results of two independent different experiments. The left bar shows the ratios of the AA⁻ *E.coli* in the consortium after 48 h at an initial inoculation of 1:100 in MMAB medium. The proportions were calculated from the measured fluorescence (S3 – Calculations). The consortium consisted of 10 AA⁻ *E.coli* (all strains were in the initial inoculation except the isoleucine auxotrophic, N = 2). The right bar shows the conditions at an initial inoculation of 1:60 in MMAB-Medium. The proportions were calculated by counting the cfu. The consortium consisted of 11 AA⁻ *E.coli* (isoleucine auxotroph included, N = 3).

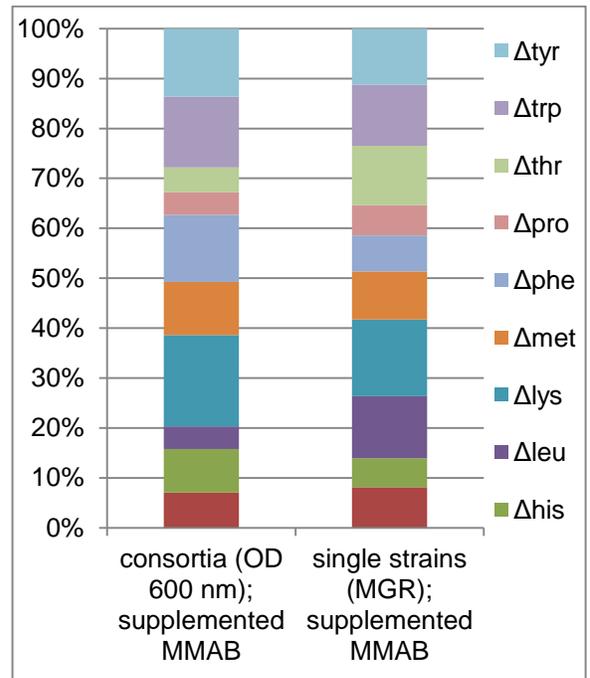


Figure 7. Analysis of strain distribution in compositions of 10 strains by OD (600 nm) measurement and single strain MGR in supplemented MMAB medium.

The left bar shows the ratios of AA⁻ *E.coli* in the consortium after 24 h. The proportions were calculated from the measured fluorescence. The right bar shows the maximum growth rate of the separately growing single strains in supplemented MMAB Medium ($\Delta OD_{600} / \Delta t$ in %)

Auxotrophic *E.coli* of a certain composition should start to grow in a minimal medium supplemented with amino acids as they would grow alone supplemented with amino acids because they do not depend on the amino acid excretion and absorption of the individual consortium members.

Figure 7 shows growth in OD₆₀₀ of the consortia and the MGR of the individual strains, both in supplemented MMAB medium. Since the strains which had grown alone reached very different optical densities the MGR was used for comparison. The growth behavior of the consortium in supplemented MMAB-Medium is very similar to the single strain MGR. Therefore it can be assumed that no cross-feeding has been established.

4 Discussion

4.1 Productivity and Diversity

4.1.1 The Influence of the Sampling-, Selection-, and Niche Complementary Effect

Each strain in the consortia favors different amino acids based on its auxotrophy which causes the various traits and therefore the diversity. It was found that with increasing initial diversity consortia actually have a higher productivity (see 3.1). The relationship appeared to be a logistic or linear relationship (Figure 3). With a diversity of five strains, the maximum productivity seems to have already reached more than 90% of its maximum capacity. This relationship could be reproduced by Merker, H. (unpublished data). The observed increase in productivity in the context of the evolving diversity suggests the presence of the effects described initially: sampling-, selection-, niche complementary effect (see 1.2). The larger the initial diversity, the greater the possibilities of interaction and more effective strain combinations prevail. Ineffective combinations are probably outcompeted.

It was found that in the consortia with a diversity of 10 and 11 strains more than 80 % of the cells consist of only five strains over time. This was confirmed by two different methods (see 3.3). Thus, the relationship between productivity and biodiversity – productivity increases with biodiversity – see in grasslands by Tilman *et al.* [25] holds true in bacteria and therefore probably other microorganisms. As the result of growth in supplemented MMAB medium shows (Figure 7), the availability of nutrients already changes at the considered community very much – there is no selection pressure for cross-feeding anymore and the distribution of strains remains almost the same as in the beginning. Such as Hooper *et al.* implies [33], this relationship productivity versus diversity strongly depends on multiple external factors. Using the system developed in this study to investigate bacterial communities can contribute in the future to investigate these ambiguities.

4.1.2 Comparison of auxotrophic Strains with prototrophic Strains

As described before in 1, auxotrophic bacteria are found in nature frequently and auxotrophy is often beneficial. However, in this study the prototrophic wild-type showed a better performance according to MGR than the consortia consisting of auxotrophic strains. Probably the cross-feeding is more costly but the question comes up, when do the auxotrophic strains show the same or better performance than the wild-type. Maybe here the “niche complementary effect” [5] can explain the observation: the more different the niches, the greater the possibility of better performance. As de Bello *et al.* show, the more different the characteristic properties of organisms are, the more likely is niche differentiation [34]. The properties of the organisms of this study do differentiate only in one trait and therefore the niche differentiation is low. Consequently, if the niche differentiation is low the positive complementary effects (e.g. division of labor) are low too and cannot compensate the costlier cross-feeding.

According to this the more auxotrophies one organism has, the more different the niches and subsequently the better the performance of consortia. Too many auxotrophies instead would be disadvantageous because the organism would become too sensible to changes in the environment. Interestingly, this statement is substantiated with the findings of D'Souza, *et al.* (unpublished data): among auxotrophic Eubacteria including *E. coli* most had two or more auxotrophies while bacteria with only one auxotrophy occurred rarely. Furthermore D'Souza *et al.* showed that the auxotrophies of *E.coli* which are also monitored in this study may have specific fitness advantages and disadvantages compared with prototrophic strains, depending on which genes were deleted when the strains grew supplemented with amino acid. When these strains were grown in co-culture with a prototrophic wild type in amino acid supplemented medium, most auxotrophic strains needed less amino acid to maintain the same growth as if they were grown alone in supplemented medium. This indicates that the auxotroph benefits due to the “helping” prototrophic wild type which is in line with the Black Queen Hypothesis. Therefore auxotrophy may be beneficial only when grown with with prototrophic strains.

A comparison of consortia consisting of bacteria with multiple- and bacteria with fewer auxotrophies or the prototrophic wild-type would be interesting. In another long-term experiment similar to Lenski *et al.* [28] in a chemostat it could be looked for the emergence of multiple auxotrophies out of strains with only one auxotrophy which may be caused due to loss of function mutations as Hottes *et al.* predicts [17].

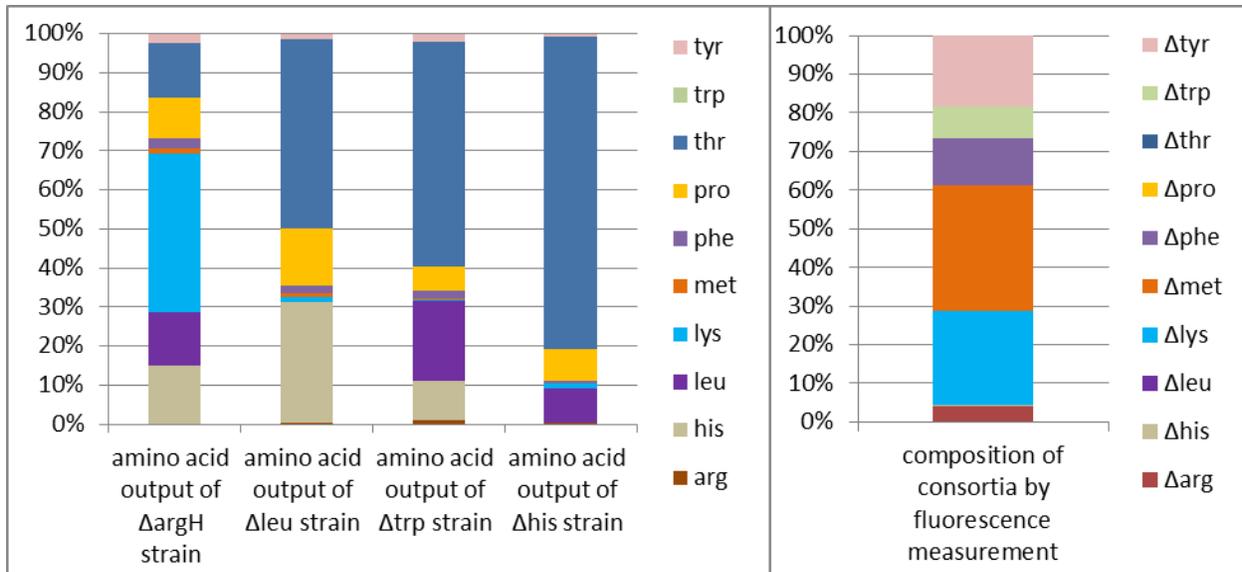
4.2 Causes for the Distribution of the Strains in the Consortia

After discussing general the productivity versus diversity relationship the characterization of the consortia is discussed here. There are various cooperation mechanisms which may be responsible for the strain behavior: Hamilton's rule, kin selection, direct reciprocity, indirect reciprocity, network reciprocity and group selection [36]. Kin selection and Hamilton's rule is not discussed here because of the limited size of this study and the lack of data which would be needed. Since the cooperation is based on cross-feeding there is probably only a tit-for-tat mechanism instead of an indirect cross-feeding as Nowak describes [36].

4.2.1 Analyzing the Cost / Benefit Ratio of the Strains in the Consortia – Direct Reciprocity

The growth of individual strains depends on multiple factors. Relatively easy to be determined are the leaked amount of a required amino acid in the consortium and the growth per amount of amino acid.

For the first part there is missing data, however, it can be said that proline, threonine, leucine and histidine have a big share in the total leaked amino acids of the four considered strains. Unfortunately those do not play an important part in the consortia of 10 and 11 strains (Figure 8). Furthermore, only a very low output of methionine could be measured whereas the methionine auxotrophic constitutes a high share in the consortia of 10 and 11 strains. Due to lack of data no correlation between output and growth under cross-feeding conditions can be determined.



For the second part there is a complete data set of growth per amino acid concentration (Figure 9). If just taken the growth per amino acid as source for expectations the correlation with the share of the strain distribution of the consortium would fit for about 7 of 11 strains. Especially the three strains which are auxotrophic for methionine, phenylalanine and histidine do not fit into the correlation. The growth per amino acid plays certainly a big role, but since the leaking of the amino acids is not equal and other mechanisms are also of importance, it is not possible to define a certain conclusion out of these data.

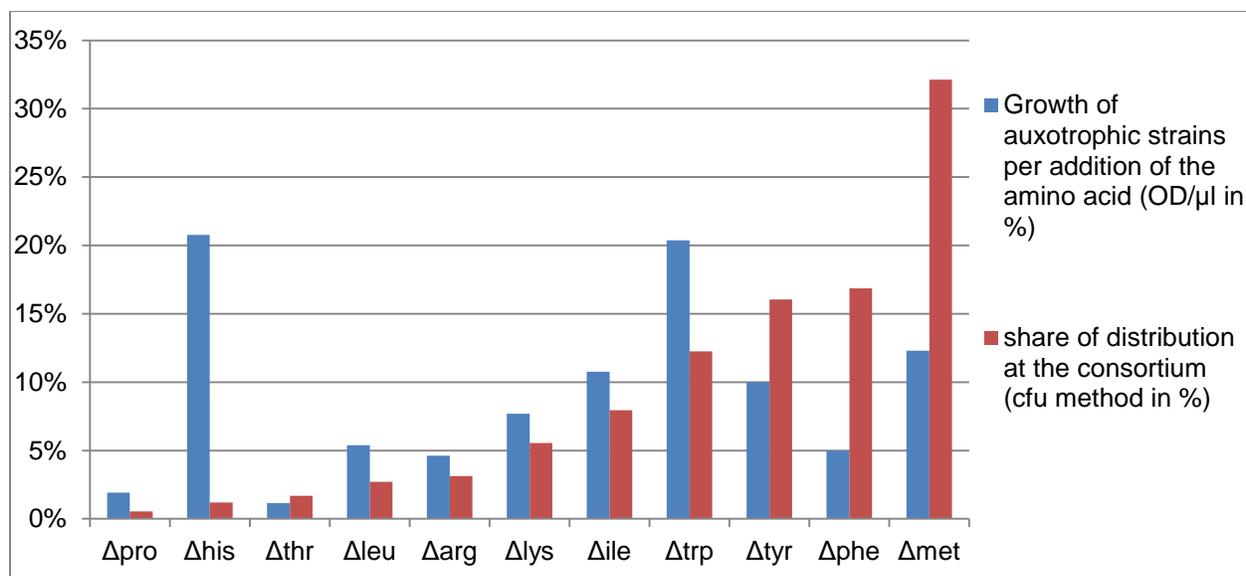


Figure 9. Growth of auxotrophic strains per addition of the amino acid.

MMAB medium as described in this study was used. The values of the blue bars include represent the maximum linear growth gain per μl amino acid. The red bars represent the percentage share of the strain distribution as also shown in Figure 6 left bar ([37] and unpublished data, modified).

Other proximate causes of the strain distribution in the consortium can be for example in the intake costs. Also, the mobility of the amino acids must be taken into consideration [38, 39]. If the amino acids are the only metabolites which are important for consideration for the cross-feeding the mobility of those probably played no role because they are all zwitterions and very mobile in solution. Other metabolites were not reviewed for this study. But their presence cannot be completely ruled out.

Figure 10. Aminoacid uptake mechanisms of *E.coli* K12 MG1655.

These data were collected using the EcoCyc database [40]. On the left there is the amino acid and on the right its uptake mechanism of *E.coli* K12 MG1655.

L-leucine	ABC transporter Sodium pump
L-histidine	ABC transporter
L-arginine	ABC transporter
L-isoleucine	ABC transporter Sodium pump
L-methionine	ABC transporter
L-proline	Proton symport Sodium pump ABC transporter
L-phenylalanine	Proton symport
L-tyrosine	Proton symport
L-threonine	Proton symport
L-tryptophane	Proton symport
L-lysine	Proton symport ABC transporter

Using the database EcoCyc (<http://EcoCyc.org>) different amino acid intake mechanisms could be listed [40] (Figure 10). For example, a proton symport is used for the lysine intake and for the leucine intake is either an ATP-binding cassette transporter (ABC transporter) or a sodium pump responsible. Beside the methionine-auxotrophic the five major strains of the consortia of 10 and 11 strains in unsupplemented MMAB medium have all a proton symport in common. As shown with the previous points there is no mono-causal relationship apparent. This database is based on the *E.coli K12 MG1655* genome which is closely related to the strains used in this study. But there remains a high uncertainty whether the data can be applied for the *E.coli K12 BW25113* strains.

4.2.2 Influence of spatial Structures to exclude Beneficiaries – Group Selection

Another mechanism responsible for the distribution of the strains could be spatial structures, which could be observed in cross-feeding *E.coli BW25113* and *Acinetobacter baylyi* (Pande, S. and Freund, L. unpublished data). The experimental conditions were very similar to these in this study.

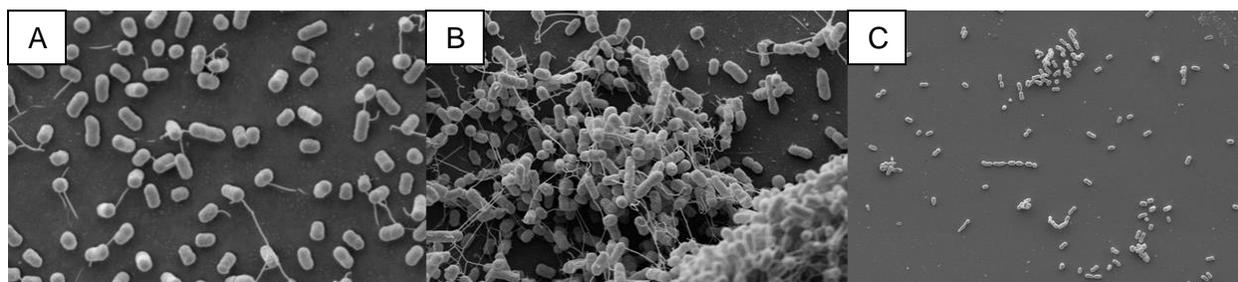


Figure 11. Electron microscopy images of auxotrophic *E.coli*, *Acinetobacter* and cross-feeding co-culture. A: *E.coli* pure culture; 1 cm = 5 μ m, WD = 5 mm, EHT = 10.00 kV. B: *E.coli* and *A. baylyi* co-culture; 1 cm = 7.5 μ m, WD = 4 mm, C: *A. baylyi* pure culture; 0,5 cm = 7,5 μ m, WD = 3 mm. Microtubes are visible in the A and B. Due to these tubes a nutrient exchange is expected to be found. For all pictures EHT = 10 kV; Source: Pande, S. and Freund, L. unpublished data.

These spatial structures were visible after only 12 h of incubation time in shaken minimal media which look similar to biofilm formations (Figure 11). It seems that *E.coli* and *A. baylyi* form small tubes and may exchange nutrients this way. Interestingly, the *E.coli* BW25113 strain is known for very poor biofilm formation [41] and so is *Acinetobacter baylyi*. Due to these spatial structures cheating bacteria or “beneficiaries” might be excluded. It is plausible to find spatial structures made by cross-feeders since it is well known that the evolution of cooperation is highly supported by spatially constructed environments [42-45]. The clusters with fewer beneficiaries will have a selection advantage and therefore cheating will be suppressed.

5 Summary and Outlook

“Is the increase of ecosystem productivity through multiplication of mutualisms a general trend?” With this question, Egbert Giles Leigh closed his review “The evolution of mutualism” which is dealing with general mutualistic interactions [46]. This study tries to address this question. Here, the ecological impact of diversity on productivity was analyzed with synthetic consortia of auxotrophic *E.coli*. The influence of diversity showed a positive correlation towards productivity. This relatively simple setting can now be brought to other and also more complex questions. For example there are also many effects of diversity on productivity described [47-49].

It was found that with increasing initial diversity consortia actually have higher productivity, which seems to be a logistic relationship. This relationship could be verified by repetition of similar experiments by Merker, H. (unpublished data). The distribution of strains in the consortium of 10 and 11 strains changed considerably over time whereas in both diversities in the consortia more than 80 % of the cells consist of only five strains. The accuracy of this result can be confirmed even more because two completely different methods were used.

The distribution of individual strains within the consortium is probably not only due to one proximate reason. None of the discussed possible correlations can explain the distribution in total. Unfortunately there is data missing which could have the potential to be the major cause for the distribution – the leakage of amino acids. However a global-scale metabolic network analysis also would be able to disclose the proximate causes for the observation of strain the distribution in the consortium. McClosekey *et al.* say that exactly this approach - the study of metabolic networks at the level of interspecific interactions and evolutionary processes - has a huge potential in the future [50]. Additionally new effects as the monitored spatial structures by Pande, S. and Freund L. can be analyzed.

The study *in vitro* makes it easier to go from just a correlation towards a causality between ecological drivers, since this is a major problem in analyzing complex systems such as ecosystems [48]. For instance an expansion of the genome scale metabolic network reconstruction of *E.coli* [50] is expected due to the analysis of mutualisms. In combination with these networks the causality of the monitored correlations in bacterial ecosystems can be found. Moreover the evolution of bacterial spatial structures as biofilms can be studied more in detail. The question can be addressed how mutualists avoid cheaters. It may be also possible to get more understanding of the uncultivable bacteria which are found in nature and maybe even new culturing methods for them.

6 References

- [1] Kazamia E, Czesnick H, Thi TVN, Croft MT, Sherwood E, Sasso S, et al. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environmental microbiology* 2012;14:1466-76.
- [2] Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. *FEMS microbiology reviews* 2013;37:384-406.
- [3] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology* 2006;2:2006 0008.
- [4] West SA, Diggle SP, Buckling A, Gardner A, Griffins AS. The social lives of microbes. *Annu Rev Ecol Evol S* 2007;38:53-77.
- [5] Loreau M. Biodiversity and ecosystem functioning: recent theoretical advances. *Oikos* 2000;91:3-17.
- [6] Diaz S, Cabido M. Vive la difference: plant functional diversity matters to ecosystem processes. *Trends in ecology & evolution* 2001;16:646-55.
- [7] Loreau M. Biodiversity and ecosystem functioning: A mechanistic model. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:5632-6.
- [8] Loreau M. Separating sampling and other effects in biodiversity experiments. *Oikos* 1998;82:600-2.
- [9] Bertels F, Merker H, Kost C. Design and characterization of auxotrophy-based amino acid biosensors. *PLoS one* 2012;7:e41349.
- [10] Wintermute EH, Silver PA. Emergent cooperation in microbial metabolism. *Molecular systems biology* 2010;6.
- [11] Staley JT, Konopka A. Measurement of Insitu Activities of Nonphotosynthetic Microorganisms in Aquatic and Terrestrial Habitats. *Annual review of microbiology* 1985;39:321-46.
- [12] Pham VHT, Kim J. Cultivation of unculturable soil bacteria. *Trends in biotechnology* 2012;30:475-84.
- [13] Stewart EJ. Growing Unculturable Bacteria. *Journal of bacteriology* 2012;194:4151-60.
- [14] McInerney JO, Pisani D, Baptiste E, O'Connell MJ. The public goods hypothesis for the evolution of life on Earth. *Biol Direct* 2011;6.
- [15] Archetti M, Scheuring I. Game theory of public goods in one-shot social dilemmas without assortment. *J Theor Biol* 2012;299:9-20.
- [16] Kummerli R, Brown SP. Molecular and regulatory properties of a public good shape the evolution of cooperation. *Proceedings of the National Academy of Sciences of the United States of America* 2010;107:18921-6.
- [17] Hottes AK, Freddolino PL, Khare A, Donnell ZN, Liu JC, Tavazoie S. Bacterial Adaptation through Loss of Function. *PLoS genetics* 2013;9:e1003617.
- [18] Morris JJ, Lenski RE, Zinser ER. The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *mBio* 2012;3.
- [19] Kuo CH, Moran NA, Ochman H. The consequences of genetic drift for bacterial genome complexity. *Genome research* 2009;19:1450-4.
- [20] Odling-Smee FJ, Laland KN, Feldman MW. Niche construction. *American Naturalist* 1996;147:641-8.
- [21] Day RL, Laland KN, Odling-Smee J. Rethinking adaptation - the niche-construction perspective. *Perspect Biol Med* 2003;46:80-95.

- [22] Laland KN, OdlingSmee FJ, Feldman MW. The evolutionary consequences of niche construction: A theoretical investigation using two-locus theory. *Journal of Evolutionary Biology* 1996;9:293-316.
- [23] Stukus PE. *Investigating Microbiology - A Laboratory Manual for General Microbiology*: Harcourt Brace & Company; 1997.
- [24] Cadotte MW, Carscadden K, Mirotnick N. Beyond species: functional diversity and the maintenance of ecological processes and services. *J Appl Ecol* 2011;48:1079-87.
- [25] Tilman D, Knops J, Wedin D, Reich P, Ritchie M, Siemann E. The influence of functional diversity and composition on ecosystem processes. *Science* 1997;277:1300-2.
- [26] Vanstockem M, Michiels K, Vanderleyden J, Vangoel AP. Transposon Mutagenesis of *Azospirillum-Brasilense* and *Azospirillum-Lipoferum* - Physical Analysis of Tn5 and Tn5-Mob Insertion Mutants. *Applied and environmental microbiology* 1987;53:410-5.
- [27] Levin BR, Stewart FM, Chao L. Resource-Limited Growth, Competition, and Predation - a Model and Experimental Studies with Bacteria and Bacteriophage. *Am Nat* 1977;111:3-24.
- [28] Lenski RE, Rose MR, Simpson SC, Tadler SC. Long-Term Experimental Evolution in *Escherichia-Coli* .1. Adaptation and Divergence during 2,000 Generations. *Am Nat* 1991;138:1315-41.
- [29] Scholz S. Establishing of a flexible labeling strategy for soil bacteria with Fluorescent proteins [Diploma Thesis]: Friedrich-Schiller-Universität Jena; 2011.
- [30] McKenzie GJ, Craig NL. Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn7 without need for selection of the insertion event. *BMC microbiology* 2006;6.
- [31] Preussger D. The effect of spatial structures on the evolution of cooperative amino acid cross-feeding within bacterial consortia [Master Thesis]. Jena: Friedrich-Schiller-Universität Jena; 2013.
- [32] Chalova VI, Sirsat SA, O'Bryan CA, Crandall PG, Ricke SC. *Escherichia coli*, an Intestinal Microorganism, as a Biosensor for Quantification of Amino Acid Bioavailability. *Sensors-Basel* 2009;9:7038-57.
- [33] Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, et al. Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecol Monogr* 2005;75:3-35.
- [34] de Bello F, Carmona CP, Mason NWH, Sebastia MT, Leps J. Which trait dissimilarity for functional diversity: trait means or trait overlap? *J Veg Sci* 2013;24:807-19.
- [35] Mason NWH, de Bello F. Functional diversity: a tool for answering challenging ecological questions. *J Veg Sci* 2013;24:777-80.
- [36] Nowak MA. Five rules for the evolution of cooperation. *Science* 2006;314:1560-3.
- [37] Bertels F. Entwicklung eines synthetischen Modellsystems zur Untersuchung mutualistischer Interaktionen: Aminosäurequantifizierung und Markierungsstrategie [Diploma thesis]. Jena: Fachhochschule Jena; 2010.
- [38] Kummerli R, Griffin AS, West SA, Buckling A, Harrison F. Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *P Roy Soc B-Biol Sci* 2009;276:3531-8.
- [39] Le Gac M, Doebeli M. Environmental Viscosity Does Not Affect the Evolution of Cooperation during Experimental Evolution of Colicogenic Bacteria. *Evolution; international journal of organic evolution* 2010;64:522-33.
- [40] Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, Bonavides-Martinez C, et al. EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res* 2013;41:D605-12.
- [41] Wood TK, Barrios AFG, Herzberg M, Lee J. Motility influences biofilm architecture in *Escherichia coli*. *Applied microbiology and biotechnology* 2006;72:361-7.

- [42] Eastman JM, Harmon LJ, La HJ, Joyce P, Forney LJ. The onion model, a simple neutral model for the evolution of diversity in bacterial biofilms. *Journal of Evolutionary Biology* 2011;24:2496-504.
- [43] Koeppel AF, Wertheim JO, Barone L, Gentile N, Krizanc D, Cohan FM. Speedy speciation in a bacterial microcosm: new species can arise as frequently as adaptations within a species. *Isme Journal* 2013;7:1080-91.
- [44] Boles BR, Thoendel M, Singh PK. Self-generated diversity produces 'insurance effects' in biofilm communities (vol 101, pg 16630, 2004). *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:955-.
- [45] Nowak MA, May RM. *Evolutionary Games and Spatial Chaos*. *Nature* 1992;359:826-9.
- [46] Leigh EG. The evolution of mutualism. *Journal of Evolutionary Biology* 2010;23:2507-28.
- [47] Huston MA. Hidden treatments in ecological experiments: Re-evaluating the ecosystem function of biodiversity. *Oecologia* 1997;110:449-60.
- [48] Doak DF, Bigger D, Harding EK, Marvier MA, O'Malley RE, Thomson D. The statistical inevitability of stability-diversity relationships in community ecology. *American Naturalist* 1998;151:264-76.
- [49] Stachowicz JJ, Fried H, Osman RW, Whitlatch RB. Biodiversity, invasion resistance, and marine ecosystem function: Reconciling pattern and process. *Ecology* 2002;83:2575-90.
- [50] McCloskey D, Palsson BO, Feist AM. Basic and applied uses of genome-scale metabolic network reconstructions of *Escherichia coli*. *Molecular systems biology* 2013;9:661.

S – Supplementary

S1 – Strain List

All strains derive from *E.coli* BW25113. Strains 1, 4-33 are based on Baba *et al.* [3]. Strains 34-57 derive from strains 1, 4-33.

Strain Number	Characteristics	Origin
1	<i>Escherichia coli</i> BW25113 Δ lacI Kan ^R	Pande, Samay
2	<i>E.coli</i> Top Ten PGRG36+EGFP Plasmid PGRG36 with plac-EGFP-cassette between Tn7 ends	Scholz, Sandra
3	<i>E.coli</i> Top Ten PGRG+mCherry Plasmid PGRG36 with plac-mCherry-cassette between Tn7 ends	Scholz, Sandra
4	Δ argH:: kanR ara ⁻	D'Souza, Glen
5	Δ leuB:: kanR ara ⁻	D'Souza, Glen
6	Δ trpB:: kanR ara ⁻	D'Souza, Glen
7	Δ hisD:: kanR ara ⁻	D'Souza, Glen
8	Δ thrC:: kanR ara ⁻	D'Souza, Glen
9	Δ proC:: kanR ara ⁻	D'Souza, Glen
10	Δ lysA:: kanR ara ⁻	D'Souza, Glen
11	Δ metA:: kanR ara ⁻	D'Souza, Glen
12	Δ pheA:: kanR ara ⁻	D'Souza, Glen
13	Δ tyrA:: kanR ara ⁻	D'Souza, Glen
14	Δ ilvA:: kanR ara ⁻	D'Souza, Glen
15	Δ argH:: kanR ara ⁻	D'Souza, Glen
16	Δ argH:: kanR ara ⁺	D'Souza, Glen
17	Δ leuB:: kanR ara ⁺	D'Souza, Glen
18	Δ trpB:: kanR ara ⁺	D'Souza, Glen
19	Δ hisD:: kanR ara ⁺	D'Souza, Glen
20	Δ thrC:: kanR ara ⁺	D'Souza, Glen
21	Δ proC:: kanR ara ⁺	D'Souza, Glen
22	Δ lysA :: kanR ara ⁺	D'Souza, Glen
23	Δ metA :: kanR ara ⁺	D'Souza, Glen
24	Δ pheA:: kanR ara ⁺	D'Souza, Glen
25	Δ tyrA:: kanR ara ⁺	D'Souza, Glen
26	Δ ilvA:: kanR ara ⁺	D'Souza, Glen
27	Δ argH:: kanR ara ⁺	D'Souza, Glen
28	Δ leuB:: kanR ara ⁺	D'Souza, Glen
29	Δ trpB:: kanR ara ⁺	D'Souza, Glen
30	Δ hisD:: kanR ara ⁺	D'Souza, Glen
31	Δ thrC:: kanR ara ⁺	D'Souza, Glen
32	Δ proC:: kanR ara ⁺	D'Souza, Glen
33	Δ lysA :: kanR ara ⁺	D'Souza, Glen
34	Δ argH:: kanR ara ⁻ TN7::Plac + EGFP	this study
35	Δ leuB:: kanR ara ⁻ TN7::Plac + EGFP	this study
36	Δ trpB:: kanR ara ⁻ TN7::Plac + EGFP	this study
37	Δ hisD:: kanR ara ⁻ TN7::Plac + EGFP	this study

38	Δ thrC:: kanR ara ⁻ TN7::Plac + EGFP	this study
39	Δ proC:: kanR ara ⁻ TN7::Plac + EGFP	this study
40	Δ lysA:: kanR ara ⁻ TN7::Plac + EGFP	this study
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50	Δ thrC:: kanR ara ⁻ TN7::Plac + mCherry	this study
51	Δ proC:: kanR ara ⁻ TN7::Plac + mCherry	this study
52	Δ lysA:: kanR ara ⁻ TN7::Plac + mCherry	this study
53	Δ metA:: kanR ara ⁻ TN7::Plac + mCherry	this study
54	Δ pheA:: kanR ara ⁻ TN7::Plac + mCherry	this study
55	Δ tyrA:: kanR ara ⁻ TN7::Plac + mCherry	this study
56	Δ argH:: kanR ara ⁻ TN7::Plac + mCherry	this study
57	Δ lacI Kan ^R TN7::PLAC+EGFP	this study

S2 – Programms for growth conditions in plate reader

S2.1 – Program for experiments described in chapters 2.3.3.2, 2.3.3.3, 2.3.3.4

Program of the Spectramax

Measuring of the OD _{600nm}	each 20 min
Shaking before measuring	5 s
Shaking between measuring	180 s
Temperature	30°C
Time	72h:20min:00s

S2.2 – Program for 2.3.3.5

Program for Tecan F200

Number of Cycles: 192

Kinetic Interval: 00:15:00

Shaking

Duration: 15 sec

Mode: Orbital

Amplitude: 2 mm

Frequency: 280.8 rpm

Absorbance

Measurement Wavelength: 600 nm

Measurement Bandwidth: 10 nm

Number of Reads: 5

Fluorescence Intensity

Excitation Wavelength: 485 nm

Excitation Bandwidth: 20 nm

Emission Wavelength: 535 nm

Emission Bandwidth: 25 nm

ReadingMode: Top

Lag Time: 0 μ s

Integration Time: 20 μ s

Number of Reads: 5

Settle Time: 0 ms

Gain: Manual

Gain Value: 50

Mirror: Automatic

Fluorescence Intensity

Excitation Wavelength: 580 nm

Excitation Bandwidth: 20 nm

Emission Wavelength: 635 nm

Emission Bandwidth: 35 nm

ReadingMode: Top

Lag Time: 0 μ s

Integration Time: 20 μ s

Number of Reads: 5

Settle Time: 0 ms

Gain: Manual

Gain Value: 100

Mirror: Automatic

Label: mcherry

Shaking

Duration: 300 sec

Mode: Orbital

Amplitude: 2 mm

Frequency: 280.8 rpm

S3 – Calculations

First the OD₆₀₀ and the fluorescence intensity was measured (see 2.3.3.5) for each strain growing alone in MMAB medium with supplemented amino acids. Then the OD and the fluorescence intensity were plotted in a diagram and the nonlinear range was excluded. In each sample of the consortia one strain was fluorescence labeled. With the equation the share of this strain at the consortium could be determined.

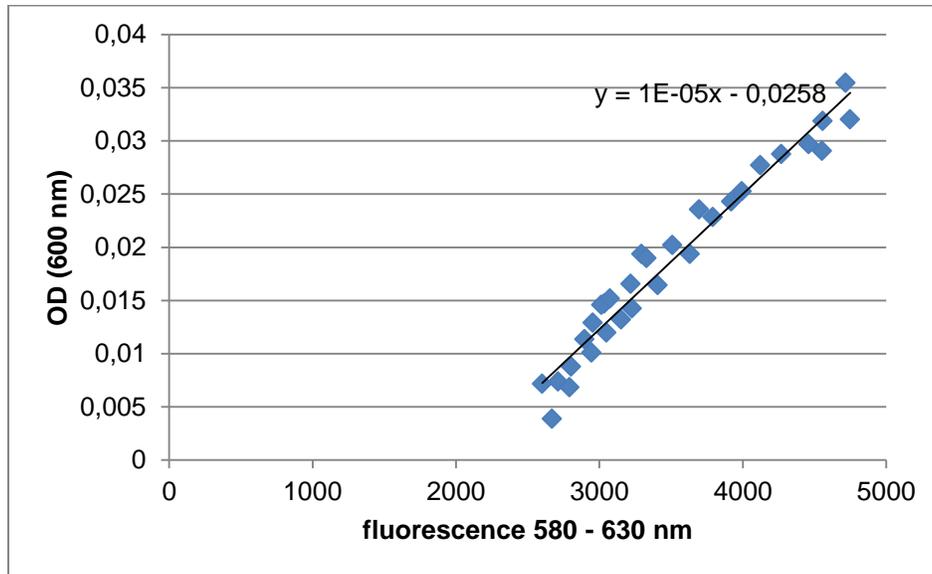


Figure 12: Example for calibration curve.

Strain 49 measured in 2.3.3.5 Experiment to analyze Distribution of the Strains in the Consortia via fluorescently labeled Strains. The equation is calculated for the linear measurement range.

S4 – Advantages and Disadvantages of the used Methods and Troubleshooting

S4.1 – Negative Controlls were positive

In the experiments negative controls were done by growing each single auxotrophic strain in unsupplemented MMAB medium. In some pre-experiments and the experiment of 2.3.3.2 there was growth in almost all wells where actually no growth should be visible. This growth occurred later and with a less MGR. Also co-workers saw this phenomenon. Probably the samples got contaminated or the strains gained auxotrophy again which was observed multiple times by co-workers. Nevertheless it does not contradict the conclusions of the results in 3.1 – the productivity increased with increasing diversity. In the repetition of this experiment with only little changes by Merker, H. this error did not occur again and the results were the same. One error source is suspected in the mixing and pre-cultures in deepwell plates. In the other experiments

glass tubes and 1,5 ml Eppendorf tubes were used instead of deepwell plates for the pre-cultures and the compositioning. No such errors were visible then.

S4.2 – Errors of Consortia of 10 and 11 Strains

After 24 h there the consortia of 10 and 11 strains of the experiment of 2.3.3.2 showed an OD₆₀₀ below 0.05 whereas even the consortia with growth at all of 2 had an OD₆₀₀ of about 0.2 on average. In other experiments the consortia of 10 and 11 showed no such behavior. Wrong compositioning, contamination with antibiotics or other harmful substances can be assumed. Thus, these results were excluded and were not monitored by Merker, H. in his experiments.

S4.3 – Distribution Analyzes: Selective Media and fluorescent Measurement

The cfu measurement on TA agar did not work satisfyingly. This method was not sensitive enough because it was observed that up to 5 % of the ara⁺ strains seemed to have lost its functioning arabinose operon. Another problem was that the specific share of each single strain was not for sure so that probably the right dilution for inoculating the TA agar plates was not chosen. To get significant results the sample size is just too big. The other methods were much better. The use of selective media was probably the best in sensibility because also a very small share could be detected. The disadvantage of this method is the time and effort which is used by plating. Furthermore to measure the distribution over the whole time is even more costly and not possible with such a high resolution as it is by fluorescence labeling. The disadvantage of the fluorescence measurement is the low sensibility for poor growing strains and of course the strains show all different fluorescence behavior. It is also not sure how the fluorescence behaves in the consortia. So a combination of the last two method is recommended.

The labeling of the auxotrophic strains with the pGRG-P_{lacZ}-*egfp* included all strains but unfortunately all strains showed a strong autofluorescence in that spectrum which could not be subtracted satisfyingly in the calculations. This could not be observed with the pGRG-P_{lacZ}-*mCherry*.

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich diese Arbeit selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln durchgeführt und verfasst habe. Ich habe aus anderen Werken entnommene Daten, Abbildungen sowie Zitate mit Quellenangaben gekennzeichnet.

Jena, den 24.06.2013

Florian Rötz

