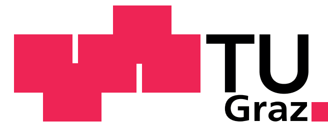

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Optimization strategies for RNA isolation and transcript-level expression analysis from plant growth promoting bacterial strain *Stenotrophomonas* spp.

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To my parents

Gerda and Josef

I am what I am
because you loved me

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Abstract

The ubiquitous plant-associated bacteria *Stenotrophomonas rhizophila* and *Stenotrophomonas maltophilia* have been determined to positively affect health and growth of a series of host plants. These effects are attributed to the production of phytohormones and osmoprotectants or through the inhibition of soil borne plant pathogens that are omnipresent in the environment.

Due to increasing interest for biotechnological applications, a better understanding of the genetic mechanisms is demanded. Transcriptome analysis represents a valuable approach to elucidate the interaction between *Stenotrophomonas* and the host plants. In preparation of whole transcriptome shotgun sequencing (RNA-Seq), appropriate cultivation conditions and RNA extraction protocols were established for the bacterial strains *S. rhizophila* P69, *S. rhizophila* ep17 and *S. maltophilia* ep3. Furthermore, the bioinformatic workflow for processing RNA-Seq data were compiled for *S. rhizophila* P69.

Gene expression profiles were generated for cultures of *S. rhizophila* P69 grown on LB complex agar medium, in LB complex liquid medium, Casamino acid minimal salt medium, and in presence of root exudates from maize plants.

To find out more about the differential expression of genes in response to changing conditions, the following factors were compared. Comparison between Casamino acid containing maize root exudates and Casamino acid minimal salt medium revealed a total of 34 significantly differentially expressed genes (0.15% of the transcriptome). The second comparison between LB complex liquid medium and LB complex agar medium indicated a differential expression of 919 genes (15% of the transcriptome). Finally, the complex and the minimal salt media were compared to each other and indicated a differentially gene expression of 920 (15% of the transcriptome).

The results revealed that the choice of the culture condition has an great impact on the transcriptome of *S. rhizophila* P69.

Zusammenfassung

Die ubiquitären, pflanzen-assoziierten Bakterien *Stenotrophomonas rhizophila* und *Stenotrophomonas maltophilia* können die Gesundheit und das Wachstum bei einer Reihe von Wirtspflanzen positiv beeinflussen. Diese positiven Effekte werden durch die Produktion von Phytohormonen und osmoprotektiven Substanzen oder durch die Hemmung von pathogenen Mikroben erzielt.

Aufgrund des zunehmenden Interesses für biotechnologische Anwendungen ist ein besseres Verständnis der genetischen Mechanismen dieser Effekte erforderlich. Eine Transkriptomanalyse ist ein wertvoller Ansatz zur Aufklärung der Wechselwirkung zwischen *Stenotrophomonas* und den Wirtspflanzen. Als Vorbereitung einer Gesamt-Transkriptom-Shotgun-Sequenzierung (RNA-Seq) wurden geeignete Kultivierungsbedingungen und Protokolle zur RNA Extraktion für die Bakterienstämme *S. rhizophila* P69, *S. rhizophila* ep17 und *S. maltophilia* ep3 festgelegt. Darüber hinaus wurde eine bioinformatische und RNA-spezifische Pipeline zur Verarbeitung von RNA-Seq-Daten für *S. rhizophila* P69 angewendet.

Es wurden Genexpressionsprofile von *S. rhizophila* P69 erstellt, die auf LB Agar Komplexmedium, in LB flüssig Komplexmedium, Casaminosäuren Minimalmedium und in Gegenwart von Wurzelexudaten aus Maispflanzen kultiviert wurden.

Um mehr über die differentielle Expression von Genen als Reaktion auf sich ändernde Bedingungen herauszufinden, wurden mehrere Faktoren verglichen. Der Vergleich von *Casaminosäuren Minimalmedium* und *Casaminosäuren Minimalmedium mit Mais Wurzelexudaten* ergab eine Anzahl von 34 signifikant unterschiedlich exprimierten Genen (0.15% des Transkriptoms). Der zweite Vergleich zwischen *flüssigem LB Komplexmedium* und *LB Agar Komplexmedium* ergab eine differentielle Expression von 919 Genen (15% des

Transkriptoms). Ebenfalls wurden Komplex- und Minimalmedium miteinander verglichen, wobei sich eine differentielle Expression von 920 Genen (15% des Transkriptoms) ergab. Die Ergebnisse dieser Studie haben gezeigt, dass verschiedene Kulturbedingungen einen signifikanten Einfluss auf das Transkriptom von *S. rhizophila* P69 haben.

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1. Thesis Introduction

In recent times agriculture has being faced to the great challenge of different biotic and abiotic stresses [51, 32] resulting in increasing harvest losses (20% yield loss in crops) worldwide [17]. Due to the ever-growing global population, food supplies and consequently agricultural productivity must be ensured [51]. Soil with high pathogen pressures and salt content are the major stress factors that agriculture is facing nowadays which makes the cultivation of crops more sophisticated [4]. Moreover, there is a social trend to demand production systems which are less harmful to the environment and more predictable, consistent and sustainable [37]. As a consequence of all these factors, many chemicals (e.g. pesticides) are reported to be withdrawn from the market in the next few years, but coincidentally the use of genetically modified crops as an alternative is still prohibited in many countries and not yet accepted among the general public. In order to alleviate this problematic situation, adequate and reliable alternatives need to be found, one of which may be the application of plant microbiota [47].

Plants are inhabited by microorganisms which leads to the formation of relationships that can be symbiotic, commensalistic, mutualistic or trophobiotic each with its own set of effects and consequences on the host. The rhizosphere of plants is a "microbial hot-spot" due to the provision of nutrients in high concentrations, thus increasing the abundance of antagonistic bacteria [5, 12, 43]. The term endophytes encompasses all bacteria and fungi, including beneficial bacteria as well as active and latent pathogenic bacteria, that colonize the inner tissues of plants [30].

The genus *Stenotrophomonas* is a prominent representative of plant-associated bacteria [46]. *Stenotrophomonas* spp. includes at least eight species [44] which are of increasing environmental and clinical importance [24]. *Stenotrophomonas* are aerobic and gram-negative bacteria which can be taxonomically

ranked in the class of gamma-proteobacteria [52, 25]. The natural occurrence of *Stenotrophomonas* spp. appears to be predominantly in plants and in the soil [44]. *Stenotrophomonas* spp. includes, inter alia, the species *S. maltophilia* and *S. rhizophila* which are closely related both ecologically and phylogenetically [10]. Both strains are of biotechnological interest due to their potential of conducting bioremediation, their plant-growth promoting properties and the ecological part in the element cycle occurring in nature [52, 11, 48].

1.1. Characteristics of the plant-associated rhizobacteria *S. rhizophila* and *S. maltophilia*

The species *S. rhizophila* is a plant-associated rhizobacterium which can be mainly detected in both the rhizosphere and phyllosphere of plants [5]. The bacterium is of increasing interest due to its application in the biotechnological field as a biocontrol agent, because it is omnipresent, versatile and has no pathogenicity to humans [45, 44, 41]. *Stenotrophomonas rhizophila* (*S. rhizophila*) exhibit a positive affect on health and plant-growth, when applied to the roots of a series of host plants. These positive effects are attributed to the production of phytohormones and osmoprotectants or through the inhibition of soil-borne plant pathogens that are omnipresent in the environment [44]. In addition, *S. rhizophila* can respond quickly to changes in environmental conditions. For example, the bacterium is able to tolerate higher salt content in the soil and as consequence it can protect crops against increasing soil salinity [4, 22]. In response to the elevated salt content in the soil, the bacterium accumulates a comparably high amount of the protecting agents trehalose and glucosylglycerol (GG) without interfering with the cellular metabolism [22].

The species ***Stenotrophomonas maltophilia* (*S. maltophilia*)**, previously known as *Pseudomonas maltophilia* and later renamed *Xanthomonas maltophilia* [24, 14], can be found in various kinds of environments, ranging from untouched nature to clinical environments [6, 40]. In nature, *S. maltophilia* is usually seedborne, but can also be found in a endosymbiotic relationship

with a plant [6]. Due to its endophytic lifestyle, *S. maltophilia* has also a great potential to be used in the biotechnological field - for bioremediation and as a biological control agent against fungal plant pathogens [9, 34]. In the last two decades, however, *S. maltophilia* emerged as an opportunistic nosocomial and multi drug-resistant human pathogen [20] that is particularly harmful for immunosuppressed and attenuated patients causing very high fatality ratios [9].

1.2. Distinguishability between closely related *S. rhizophila* and *S. maltophilia* species

It is possible to easily distinguish between the *S. maltophilia* and *S. rhizophila* strains, because only the latter is able to synthesize GG (which is encoded by the *ggpS* gene) when exposed to high salt contents [41, 4]. Conversely, only *S. maltophilia* possesses a specific multi-drug efflux pump SmeDEF encoded by *smeD* gene [41]. Additionally, the genome of *S. rhizophila* possesses no heat shock genes thus *S. rhizophila* is unable to grow at 37°C (the human body temperature) which indicates that this strain is harmless to humans [10]. However, the genomes of *S. rhizophila* P69 and *Stenotrophomonas rhizophila* ep17 (*S. rhizophila* ep17) have a high degree of sequence similarity thus it is not possible to easily distinguish between these bacterial strains. Despite the significant genome similarity, both strains can have a different mode of interaction with plants and they can differently adapt to environmental changes. A transcriptome analysis represents a valuable approach to understand the strain-specific mode of action, the mechanisms of adaptation and microbe-plant interaction. A transcriptome analysis is an approach which identifies all levels of RNA transcripts that are expressed by the bacterial genome under certain circumstances [33] and it can help to find significant differences between the *Stenotrophomonas* strains [5].

1.3. Objectives of this study

The production of biological control agents for biotechnological applications in agriculture is often unpredictable and volatile [8]. To control and optimize biotechnological applications one must understand the microbe-plant interactions, how the bacterial strains adapt to environmental changes and how the lifestyle of the bacteria can change during a biotechnological process.

For this reason, the biotechnologically relevant plant-associated rhizobacteria *S. rhizophila* P69, *S. rhizophila* ep17 and *Stenotrophomonas maltophilia* ep3 (*S. maltophilia* ep3) were exposed to five different cultivation conditions. These bacterial strains are of great interest because they are able to respond quickly to changes in environmental conditions, they are versatile and omnipresent. These properties make them very popular as a biocontrol agent in the biotechnological field [44].

In order to explore the interaction between the bacterial strains and their host plants, root exudates from maize and tomato plants were generated, which were then utilized in a set of cultivation experiments. The goal of these experiments was to find out how cell density and number of viable bacteria relate to the availability of nutrients containing root exudates. Additionally, the mechanisms associated with colonization and biofilm formation were studied using LB complex agar medium and LB complex liquid medium. For this purpose, a differential gene expressions analysis was conducted and a full transcriptome profile of the biotechnological essential *S. rhizophila* P69 was established. This analysis method was used to identify significant up or down regulated genes in response to changing conditions.

2. Materials and Methods

2.1. Bacterial strains

In this study, the bacterial strains ***Stenotrophomonas rhizophila* P69 (*S. rhizophila* P69)**, ***Stenotrophomonas rhizophila* ep17 (*S. rhizophila* ep17)**, ***Stenotrophomonas maltophilia* ep3 (*S. maltophilia* ep3)** were used (see 2.1). Stock cultures for cultivation were used from collection at the Institute of Environmental Biotechnology[1] (stored at -70°C). The bacterial isolates were streaked out on LB complex agar medium (LBP) and subsequently incubated for two days at 30°C. The agar plates were then stored in the refrigerator at -4°C until further usage. For reactivation, the strains have been streaked out on new LBP once a month.

2.2. Cultivation conditions

For growth experiments and RNA-based studies the strains *S. rhizophila* P69, *S. rhizophila* ep17, *S. maltophilia* ep3 were cultivated under five different conditions using LB complex liquid medium and Casamino acid minimal salt medium with or without root exudates from maize- or tomato plants (see table 2.1).

[1]Graz University of Technology, Graz, Austria

	S. rhizophila P69	*S. rhizophila* ep17	*S. maltophilia* ep3
RNA extraction			
LB	✓	✓	✓
LBP	✓	✓	✓
CAT	✓	✓	✓
CAM	✓	✓	✓
CAN	✓	✓	✓
Growth Parameters			
LB	✓	✓	✓
LBP	X	X	X
CAT	✓	✓	✓
CAM	✓	✓	✓
CAN	✓	✓	✓
Transcriptome Analysis			
LB	✓	X	X
LBP	✓	X	X
CAT	X	X	X
CAM	✓	X	X
CAN	✓	X	X

Table 2.1.: **Overview of performed experiments with included cultivation conditions and *Stenotrophomonas* strains.** Inclusion is marked with a check mark and exclusion with a cross (X). Cultivation conditions of *Stenotrophomonas* strains: LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid containing tomato root exudates (CAT), Casamino acid minimal salt medium (CAN). Growth parameters included the creation of growth curves and the determination of the colony-forming unit (cfu/ml).

2.3. Root exudates preparation from maize and tomato plants

2.3.1. Surface-sterilization of maize and tomato seeds

Surface-sterilization of a batch of maize[2] and tomato[3] seeds with 30 ml of 4% NaClO for 5 minutes was conducted. After the NaClO treatment the seeds had been washed seven times with distilled water (dH_2O) for 2 minutes each time. The surface-sterilized seeds were transferred into sterile petri dishes for drying. The process of drying was implemented under sterile conditions in a laminar flow.

2.3.2. Pre-germination of maize and tomato seeds and plant root exudates production

One litre of vermiculite[4] and 480 ml of Gamborg B5[5] media were added to a six litre plastic pot. Subsequently, the pots and lids were autoclaved separately at 121°C for 15 minutes.

For the pre-germination of maize and tomato seeds, sterile filter papers were moistened with sterilized H_2O and placed in sterile petri dishes. The seeds were positioned between two filter papers and the petri dishes were sealed with parafilm. The pre-germination of seeds was achieved with storage at room temperature for 5-7 days under the exclusion of light.

[2]LG 3258, production year 2016
[3]Runde Resi, risp tomatoes, production year 2017
[4]Ratioform, Austria
[5]Duchefa Biochemie B.V, Haarlem, The Netherlands, Prod. No.: G0209.0050

2.3.3. Pre-germinated seeds sowing and root exudates production

The pre-germinated seeds were sowed into the autoclaved vermiculite pots and 200 ml of Gamborg solution was added. Three pots with 25 seeds per pot were used for tomato and two pots containing 16 seeds per pot were used for maize root exudates production. The arrangement of the tomato seeds were done in five rows with five pre-germinated seeds per row. The maize seeds were sown in four rows with four seeds per row. An autoclaved pot containing the additional 200 mL Gamborg solution (without seeds) was used as negative control system. Plant growth was carried out under greenhouse conditions at 22 to 23 °C at a 16/8 hour (h) day/night cycle, respectively.

The tomato plants were watered with 50 millilitre (ml) autoclaved Gamborg media per pot and the maize plants with 100 ml after one week of growth. Additionally, the lids from the maize pots have been removed under laminar conditions and the pots were transferred into sterile plastic bags instead. The bags were sealed thoroughly with a tape.

2.3.4. Harvesting

Harvesting of tomato and maize plants was carried out after 16 days of plant growth. The trunks and leaves of plants were removed by cutting them off with sterile scissors and then discarded. The vermiculite from each pot containing the roots was transferred into sterile bags. The bottom of the plastic bag was cut open and the root exudates were squeezed onto large petri dishes. Subsequently, the liquids were pipetted into centrifuge tubes and centrifuged (5000 g, 4°C) for 10 minutes. From this point on, the root exudates were continuously kept on ice for the rest of the process.

The supernatant from the centrifuged exudates was collected and sterile filtrated twice starting with the filtration through 0.45 μm pore filters. The second filtration was implemented through 0.2 μm pore filters. Filtered exudates were stored in 50 ml Sarstedt tubes at -20°C under the exclusion of light until further use.

100 μL of filtered maize and tomato exudates as well as the negative control were plated onto LB complex agar medium and incubated at 30°C for between one and two days to corroborate sterility.

2.4. Culture and growth media

2.4.1. Preparation of LB liquid medium, LB agar plates and CAA minimal salt medium

In this study, the bacterial strains were grown either on LB complex liquid medium (LB)[6] or on Casamino acid minimal salt medium.

LB complex liquid medium (LB) is composed of trypton (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L) with pH 7. LB is available as a pre-mixed commercial off-the-shelf product and it is mainly used for bacterial growth [26]. 20 g of LB are dissolved in dH_2O to a total volume of 800 ml. In case of LB complex agar medium production it was necessary to add 14.4 g agar-agar[7]/ 800 mL H_2O to the LB medium.

One litre of Casamino acid minimal salt medium consists of 5.0 g Casamino acid minimal salt mediums (CAAs), 1.54 g $K_2HPO_4 \cdot 3H_2O$ and 0.25 g $MgSO_4 \cdot 7H_2O$ [4].

2.4.2. Preparation of overnight cultures in LB and CAA media

Unless otherwise stated, overnight cultures (ONCs) were implemented in two different ways. The bacterial isolates *S. rhizophila* or *S. maltophilia* were either inoculated in 20 mL sterile LB complex liquid medium or in 20 mL Casamino acid minimal salt medium (CAA). The inoculated ONCs were incubated for 16 hours at 30°C at 110 rpm. Additionally, agitation was required if the bacterial isolates were cultivated in CAA medium.

[6] Luria Bertani medium; Carl Roth GmbH + Co KG, Karlsruhe, Germany
[7] Carl Roth GmbH + Co.KG, Karlsruhe, Germany

2.5. Experimental setup for bacterial growth experiments

Bacterial growth analyses were conducted to generate empirical knowledge about the growth behaviour of the different *Stenotrophomonas* strains under various conditions and how quantity increasing over time. The same main cultures have been used for both the bacterial growth experiment and the colony-forming unit trial and both experiments were conducted in two duplicates.

2.5.1. Experimental determination of bacterial growth and the colony forming unit (cfu/mL)

To study bacterial growth and determine the colony-forming unit, a main culture for each strain was prepared containing 50 ml LB complex liquid medium which was inoculated with a pre-culture to an OD_{600} of 0.1. Then, the OD_{600} was measured once every hour for 20 hours. Additional measurements were carried out after 34, 35, and 36 hours. The main cultures have been incubated at 30°C (110 rpm).

The bacterial growth analyses of *S. maltophilia* and *S. rhizophila* with Casamino acid minimal salt medium containing maize or root exudates and control liquid were implemented as well. In order to thaw the control liquid and the maize and tomato root exudates gradually, the frozen samples were placed on ice. The main culture was prepared by inoculating 40 mL CAA (supplemented with 10 mL maize or tomato root exudates and the control liquid) with the pre-culture (ONC) to an OD_{600} of 0.1. The bacteria inocula were cultivated under agitation for 16 hours at 30°C. The optical density was measured at the same time points as the *Stenotrophomonas* strains cultivated in LB complex liquid medium. As a result it was possible to create a bacterial growth curve for each strain and condition by using the collected data obtained from the OD_{600} measurements.

The colony-forming unit determination was implemented at the same time as the bacterial growth experiment. To establish the colony-forming unit a

tenfold serial dilution from 10^0 to 10^{-6} was conducted in a 96-well plate. Therefore, 180 μL of NaCl (0.85%) was loaded to the wells of the plate. Subsequently, 20 μL of the bacterial inoculum (for each strain and condition) was added and the serial dilution was implemented by transferring 20 μL of the mixture from the well (containing the bacterial inoculum) to a second well and successive transference was continued through the well for the 10^{-6} dilution. Every two hours (for a total of 20 h), 10 μL of the various diluted suspensions were plated onto LB plates and incubated at 30°C for 24 hours. After incubation the bacterial colonies were counted and as a result it was possible to determine the colony-forming unit (cfu/mL) using the data from the agar plates.

2.6. Total RNA isolation from *Stenotrophomonas* strains

The total RNA extraction was performed according to instructions based on Qiagen RNeasy Mini Kit[8] with slight adjustments [42].

2.6.1. Precautionary measures

Provision of a RNAse free environment was obligatory thus precautionary measures had been taken preventing degradation of the RNA.

- A laminar flow serving only for the purpose of RNA isolation was used. RNAse free environment was cleaned with RNAse Erase[9] spray and UV light. RNAse Erase is used to inactive RNAse enzymes which are present in the environment, used utensils and water [16]
- It was necessary to prepare 0.1% Diethyl pyrocarbonate (DEPC) treated dH_2O by pipetting 1 ml of DEPC into 1 litre H_2O. Then it was kept at 37 °C overnight after that it was autoclaved at 121°C for 15 minutes.

[8]Qiagen, Hilden, Germany (Cat No./ID: 74104)
[9]MP Biomedicals™, fisher scientific part of Thermo Fisher Scientific, Germany

- The pipettes were dismantled and soaked in 0.5% SDS for 24h. Subsequently, the pipettes were washed with 0.1% DEPC treated dH_2O and dried in a RNAse free environment. All glassware was treated with DEPC treated dH_2O and kept at 37°C overnight.
- For the samples a maximum of $1*10^9$ cfu/ml as starting material was used.

2.6.2. Sample preparation

One mL of cell main culture was pipetted into microcentrifuge tubes and centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was carefully discarded and the pellet was re-suspended in 1 ml of RNA Later solution[10]. Subsequently, the samples were kept at room temperature for one hour for optimal incorporation of the RNA later solution. At this point the sample were either flash frozen and stored at -70°C or the RNA isolation was continued.

2.6.3. Cell lysis

The cells were thawed (if flash frozen) and centrifuged for five minutes at 5000 g. After that, the supernatant was discarded. 10 μL of QIAGEN Proteinase K (20 mg/mL) was added to 200 μL of TE buffer, containing 15 mg/mL of lysozyme. This mixture was added to the pellet and re-suspended by carefully pipetting up and down. Then, incubation at room temperature for 10 to 20 minutes was performed. In the meantime, the samples were vortexed for 10 seconds every two minutes. Following this, 700 μL buffer RLT was added and the samples were vortexed vigorously for 5 to 10 seconds.

Two mechanical cell lysis approaches were tested: In the first approach the lysate was transferred to FastPrep™Lysing Matrix E[11] (maximum capacity 1 mL) and homogenized with a FastPrep™-24 Sample Preparation System[12]

[10] Qiagen Cat Nr. 76104
[11] MP Biomedicals™, fisher scientific part of Thermo Fisher Scientific, Germany
[12] MP Biomedicals™, Germany

in three repeats for 30 seconds each at 6 m/s. The samples were cooled on ice between the runs. Up to 700 μl of lysed cells were pipetted into new microcentrifuge tubes. It was important that as little matrix material as possible was pipetted off to avoiding contamination.

In the second approach the lysate was transferred to homogenizer tubes[13] and centrifuged at 12.000 rpm (3 min, room temperature). This variant of mechanical cell lysis has proved to be the more promising approach. Henceforth, the homogenizer tubes were applied at all total RNA extractions.

2.6.4. Washing steps

A volume of 70% ethanol was added to the samples and up to 700 μL of sample was then transferred to RNeasy spin column which was placed in a 2 mL collection tube. The samples were centrifuged for 15 seconds at $\geq$ 8000 g ($\geq$ 10,000 rpm). The flow-through was discarded and the collection tube was reused.

The following step was performed twice. 500 μL of the buffer RPE was added to the RNeasy spin column and the samples were centrifuged for 15 seconds at $\geq$ 8000 g ($\geq$ 10,000 rpm). After the second centrifugation the collection tube was not reused and the spin column was placed in a new 2 mL collection tube. The samples were then centrifuged for one minute at full speed. This step was necessary to remove residual buffer RPE.

2.6.5. Elution

The RNeasy spin column was placed in a 1.5 ml collection tube which was supplied by QIAGEN. 30 μL of RNase-free water was added directly to the spin column membrane. The columns were then centrifuged for one minute at $\geq$ 8000 g ($\geq$ 10,000 rpm) to elute the isolated RNA.

[13] Invitrogen Cat Nr. 12186-026

10x FA buffer stock	per 1 Litre
200 mM MOPS	41 g
50 mM NaOAc	4.1g
10 mM EDTA	3.8 g

Table 2.2.: **Preparation of 10x Formaldehyde (FA) buffer stock solution** which is composed of (3-(N-Morpholino)propanesulfonic acid (MOPS)), (Ethylenediamine-tetraacetic acid (EDTA)), sodium acetate (NaOAc) and FA. FA was added only during the preparation of the running buffer

2.6.6. Gel Electrophoresis

The gel chamber components have been treated with 0.5% SDS followed by a washing step with 0.5% DEPC treated water. The electrophoresis was performed by preparing 1.2% agarose gel in FA buffer (1x) which was then equilibrated in the running buffer[14] for 30 minutes. It must be mentioned that FA (37%) was applied only during the preparation of the running buffer. The concentration of total RNA was measured via Nano-Drop™Spectrophotometer[15], as well as Qubit 4 Fluorometer RNA assay[16] to determine the amount of RNA applying to the gel. In these experiments 300 to 400 ng of total RNA has been utilized for gel electrophoresis. Each RNA sample was mixed with 5x loading dye which were then boiled at 70°C for 10 minutes and subsequently transferred onto ice for 3 minutes. The samples and the standard were then loaded into the gel slots. RNA Ladder[17] was used as a standard which was mixed 2 μL RNA Loading Dye (2x). The electrophoresis was implemented with 90 Volt for 1.5 h, respectively. The gel was stained with ethidium bromide for at least 45 minutes and the RNA fragments were visualized through UV trans-illumination.

[14] 1x FA buffer prepared from 10x FA stock solution. See table 2.2
[15] Thermo Fisher Scientific
[16] Thermo Fisher Scientific
[17] Thermo Scientific RiboRuler High Range RNA Ladder (♯SM1821)

2.6.7. Quality and Quantity assessment of the total RNA isolations

The quantity and quality of the isolated total was measured via NanoDrop™Spectrophotometer[18], as well as Qubit 4 Fluorometer assay[19]. The 260/280 ratio assesses the purity of DNA and RNA, whereas values between 2.0 to 2.2 are accepted as pure RNA. A ratio between 1.8 and 2.0 may indicate the presence of and values lower than 1.8 indicate the presence of proteins. The 260/230 ratio provides information on whether contaminants in the samples are present in the form of e.g. EDTA, carbohydrates or phenolic compounds. A ratio between 1.8 and 2.0 are accepted as pure RNA. Values lower than 1.8 may indicate the presence of contaminants which absorb at a wavelength of 230 nm [1].

2.7. RNA-Seq data processing

Total bacterial of *S. rhizophila* P69, *S. rhizophila* ep17 and *S. maltophilia* ep3 have been isolated. Purified mRNA and rRNA of *S. rhizophila* P69 cultivated in LB complex liquid medium, LB complex agar medium, Casamino acid containing maize root exudates and Casamino acid minimal salt medium was sent to Eurofins Genomics[20] company which offers a sequencing service.

Once mRNA and rRNA of *S. rhizophila* P69 was isolated and sequenced, a differential expression analysis was performed through the use of software packages selected from the many packages available on the market. In this study, the RNA-Seq data of *S. rhizophila* P69, which was cultivated under five different conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid minimal salt medium (CAN) and Casamino acid containing maize root exudates (CAM)), were analysed by using the freely available software applications Hisat2 (short read aligner), StringTie (transcriptome assembly) and Cuffdiff (differential expression analysis). An

[18] Thermo Fisher Scientific
[19] Thermo Fisher Scientific
[20] Eurofins Genomics Germany GmbH, Ebersberg, Germany

Ubuntu linux virtual machine was used from the command line[21] to run all the applications.

The analysis of the RNA-Seq data was performed according to instructions based on Pertea et al. [39] and Trapnell et al. [50]. The workflow of the differential expression analysis is based on the illustration in figure 2.1. After the index was built it was possible to map the raw reads of the LB, LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM) and Casamino acid minimal salt medium (CAN) samples to the *S. rhizophila* P69 reference genome, whereby SAM files for each sample were generated. After sorting, the resulting alignment files were then fed to StringTie and in the next step the assembled transcripts from LB, LBP, CAM and CAN samples were merged to conduct a final transcriptome assembly through the use of *StringTie -merge*. Abundance of transcripts fragments per kilobase million (FPKM) in all samples was calculated with Cuffdiff. This allowed for detection of differences in gene expression level and testing of statistical significance [49]. Subsequently, the Cuffdiff output was then explored and visualized in different ways using an R package called CummeRbund [50, 29].

In order to obtain information that the up or down regulation of particular genes was systematically or randomly an analysis with Kyoto Encyclopedia of Genes and Genomes (KEGG) was implemented. KEGG is an integrated repository of biological data with added information about chemicals, genomes and systemic functions. It is used as a reference data source to aid in linking genome data to living organisms by using the process of PATHWAY mapping. The result of this process are reference pathways that allow to derive general behaviours that are typical for the living organism [36]. A KEGG_ID list was created for all available genes before starting the KEGG analysis. Each list contained GeneID (KEGG) and log(2) of the pairwise conditions and subsequently was saved as text file. This file was then fed to the online tool *the pathview* [38]. The species was set to "ko-KEGG Orthology-N" and pathway selection to auto. In a further attempt, the pathway selection was set to manual. Subsequently, it was possible to search for specific functions.

[21]For a listing of the used commands, see listings A.1 and A.2 in the Appendix

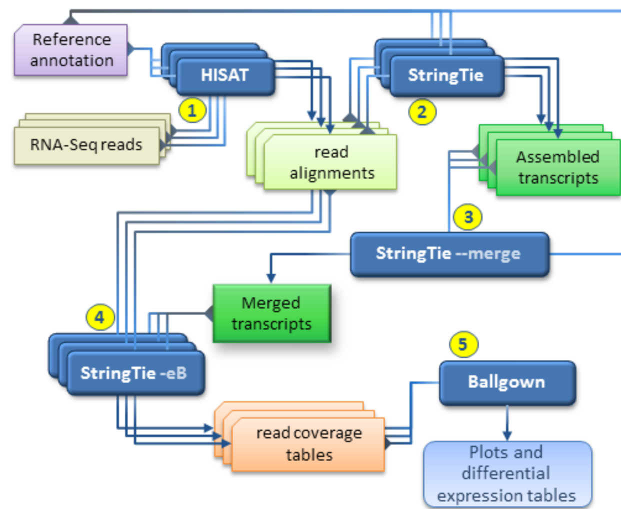

Figure 2.1.: Illustration of the differential expression analysis workflow according to instructions based on Pertea et al. [39]. Two slightly abbreviations from the protocol were the use of the software tool Cuffdiff (Step 4) instead of *StringTie –eB* as well as visualisation of Cuffdiff output through the use of CummeRbund [50] instead of Ballgown.

3. Results

3.1. Growth behaviour of *S. maltophilia* ep3, *S. rhizophila* P69 and ep17

Growth behaviour of *S. maltophilia* ep3, *S. rhizophila* P69 and *S. rhizophila* ep17 in relation to the availability of exudates was examined in two experiments, using two different methods. One experiment used OD_{600} measurements, the other determined colony-forming unit (cfu/ml) over time.

Results of the OD_{600} measurements can be seen in table 3.1. These results were used to create a growth curve for each strain and each cultivation condition (LB complex liquid medium, LB complex agar medium, Casamino acid minimal salt medium (CAN), Casamino acid containing tomato root exudates (CAT), Casamino acid containing maize root exudates (CAM)). The curves showed that the bacterial populations of *S. maltophilia* ep3 (see figure 3.1), *S. rhizophila* ep17 (see figure 3.2) and *S. rhizophila* P69 (see figure 3.3) exhibited similar growth behaviour under the cultivation conditions of the experiment. Furthermore, it was possible to determine that each *Stenotrophomonas* strain reached the stationary phase (in every cultivation condition) after approximately 10 hours of incubation.

The colony-forming unit (cfu/ml) for *S. maltophilia* ep3, *S. rhizophila* P69 and ep17 cultivated in LB, CAT, CAM or CAN can be seen in table 3.2 and table 3.3. The average achieved cell number of *S. rhizophila* and *S. maltophilia* were of the scale of 10^{10} cfu/ml.

Table 3.1.: Results of the OD_{600} measurements of the *Stenotrophomonas* strains cultivated in LB complex liquid medium (LB), Casamino acid containing maize root exudates (CAM), Casamino acid containing tomato root exudates (CAT) and Casamino acid minimal salt medium (CAN).

											$\mathbf{OD_{600}}$
	0h	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**8h**	**9h**	**10h**	**24h**
***S. maltophilia* ep3**												
LB	0.100	0.154	0.202	0.351	0.569	0.789	0.938	1.079	1.230	1.367	1.539	2.796
CAN	0.100	0.130	0.135	0.195	0.253	0.352	0.468	0.605	0.713	0.839	0.999	2.020
CAM	0.100	0.144	0.145	0.193	0.264	0.332	0.452	0.613	0.724	0.878	1.112	2.179
CAT	0.100	0.125	0.138	0.190	0.253	0.348	0.465	0.591	0.688	0.808	0.961	1.961
***S. rhizophila* ep17**												
LB	0.100	0.196	0.261	0.237	0.592	0.763	0.884	1.013	1.099	1.214	1.349	2.533
CAN	0.100	0.103	0.125	0.434	0.400	0.525	0.647	0.770	0.859	0.951	1.085	2.194
CAM	0.100	0.196	0.260	0.418	0.598	0.731	0.847	0.967	1.044	1.131	1.236	2.327
CAT	0.100	0.203	0.260	0.422	0.567	0.689	0.776	0.886	0.994	1.062	1.163	2.242
***S. rhizophila* P69**												
LB	0.100	0.299	0.364	0.547	0.784	1.001	1.170	1.339	1.468	1.634	1.779	2.815
CAN	0.100	0.253	0.271	0.402	0.519	0.663	0.791	0.894	0.992	1.098	1.254	2.347
CAM	0.100	0.263	0.265	0.406	0.521	0.663	0.777	0.888	0.989	1.101	1.262	2.401
CAT	0.100	0.259	0.279	0.397	0.534	0.655	0.761	0.883	0.980	1.083	1.246	2.328

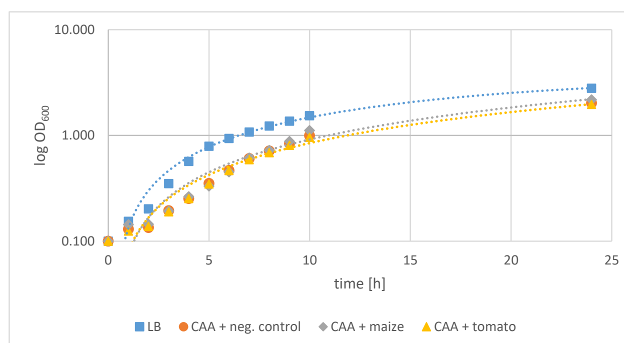

Figure 3.1.: **Results of OD_{600} measurements from *S. maltophilia* ep3** cultivated under four conditions (LB complex liquid medium (LB), Casamino acid containing maize root exudates (CAM), Casamino acid containing tomato root exudates (CAT), Casamino acid minimal salt medium (CAN)). Experiments have been conducted in 50 ml of LB (blue square) as well as in 40 ml of Casamino acid minimal salt medium which was supplemented with 10 ml of either maize (grey square) or tomato (yellow rectangle) root exudates. The graph was generated using the data from table 3.1.

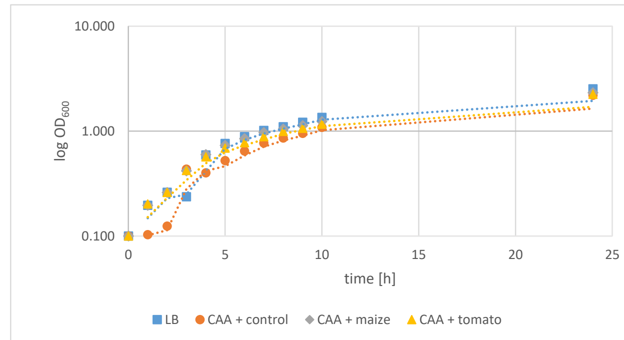

Figure 3.2.: **Results of OD_{600} measurements from *S. rhizophila* ep17** cultivated under four conditions (LB complex liquid medium (LB), Casamino acid containing maize root exudates (CAM), Casamino acid containing tomato root exudates (CAT), Casamino acid containing maize root exudates (CAM)). Experiments have been conducted in 50 ml of LB (blue square) as well as in 40 ml of CAA which was supplemented with 10 ml of either maize (grey square) or tomato (yellow rectangle) root exudates. The graph was generated using the data from table 3.1.

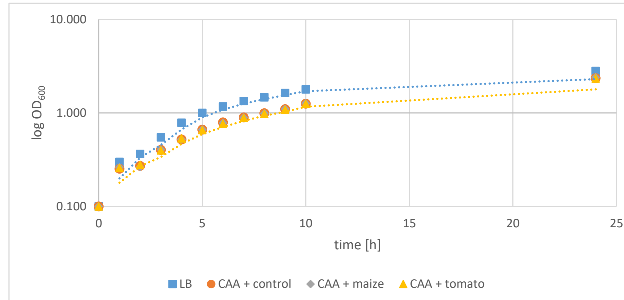

Figure 3.3.: **Results of OD_{600} measurements from *S. rhizophila* P69** cultivated under four conditions (LB complex liquid medium (LB), Casamino acid containing maize root exudates (CAM), Casamino acid containing tomato root exudates (CAT), Casamino acid minimal salt medium (CAN)).Experiments have been conducted in 50 ml of LB complex liquid medium (blue square) as well as in 40 ml of Casamino acid minimal salt medium which was supplemented with 10 ml of either maize (grey square) or tomato (yellow rectangle) root exudates. The graph was generated using the data from table 3.1.

Table 3.2.: Results of the cell count determination given in cfu/mL of *S. maltophilia* ep3, *S. rhizophila* ep17 and *S. rhizophila* P69 cultivated in LB complex liquid medium (LB), Casamino acid minimal salt medium (CAN), Casamino acid containing tomato root exudates (CAT) and Casamino acid containing maize root exudates (CAM). Abbreviation: n/A = not Available

Cell count [cfu/ml]

	0h	2h	4h	6h	8h	9h	10h
***S. maltophilia* ep3**							
LB	$1.17 * 10^8$	$2.14 * 10^8$	$2.45 * 10^9$	$1.01 * 10^{10}$	$4.30 * 10^9$	$2.60 * 10^9$	$2.40 * 10^9$
CAN	$9.65 * 10^7$	n/A	n/A	$1.80 * 10^9$	$1.04 * 10^9$	$4.10 * 10^9$	$2.90 * 10^9$
CAT	n/A	$1.47 * 10^8$	$6.25 * 10^8$	$1.27 * 10^9$	$1.35 * 10^{10}$	n/A	$5.70 * 10^9$
CAM	n/A	$1.33 * 10^8$	$5.80 * 10^8$	$1.37 * 10^9$	$1.20 * 10^{10}$	n/A	$5.20 * 10^9$
***S. rhizophila* ep17**							
LB	$5.90 * 10^7$	n/A	$6.75 * 10^9$	$2.85 * 10^8$	$1.15 * 10^9$	$1.30 * 10^9$	$2.5 * 10^9$
CAN	$5.55 * 10^7$	$1.95 * 10^8$	$3.90 * 10^9$	n/A	$6.35 * 10^9$	$3.10 * 10^9$	$2.15 * 10^9$
CAT	$3.20 * 10^7$	$1.43 * 10^8$	$9.55 * 10^8$	$1.09 * 10^9$	$5.25 * 10^9$	n/A	$3.65 * 10^9$
CAM	$3.35 * 10^7$	$1.68 * 10^8$	$1.12 * 10^9$	$1.18 * 10^9$	$4.55 * 10^9$	n/A	$1.75 * 10^9$
***S. rhizophila* P69**							
LB	$1.87 * 10^8$	n/A	$3.95 * 10^9$	$2.80 * 10^{10}$	$1.10 * 10^9$	$1.50 * 10^9$	$2.30 * 10^9$
CAN	$7.35 * 10^7$	$6.15 * 10^7$	$2.45 * 10^9$	$2.30 * 10^9$	$1.90 * 10^9$	$3.35 * 10^9$	$1.70 * 10^9$
CAT	$5.10 * 10^7$	$1.71 * 10^8$	$6.25 * 10^8$	$8.40 * 10^8$	$8.20 * 10^8$	n/A	$1.25 * 10^9$
CAM	$5.87 * 10^7$	$1.55 * 10^8$	$6.50 * 10^8$	$8.15 * 10^8$	$2.20 * 10^9$	n/A	$1.90 * 10^9$

Table 3.3.: Results of the cell count determination given in cfu/mL of *S. maltophilia* ep3, *S. rhizophila* ep17 and *S. rhizophila* P69 cultivated in LB complex liquid medium (LB), Casamino acid minimal salt medium (CAN), Casamino acid containing tomato root exudates (CAT) and Casamino acid containing maize root exudates (CAM). Abbreviation: n/A = not Available

Cell count [cfu/ml]

	11h	12h	18h	20h	24h	26h	34h	36h
***S. maltophilia* ep3**								
LB	$5.80 * 10^9$	$4.65 * 10^9$	n/A	n/A	$1.77 * 10^{10}$	$2.61 * 10^{10}$	n/A	n/A
CAN	$1.04 * 10^{10}$	$7.95 * 10^9$	n/A	n/A	$2.14 * 10^{10}$	$1.60 * 10^{10}$	n/A	n/A
CAT	n/A	n/A	$1.45 * 10^{10}$	n/A	n/A	n/A	$2.40 * 10^{10}$	n/A
CAM	n/A	n/A	$9.90 * 10^9$	$9.20 * 10^9$	n/A	n/A	$1.95 * 10^{10}$	n/A
***S. rhizophila* ep17**								
LB	$6.10 * 10^9$	$6.55 * 10^9$	n/A	n/A	$1.34 * 10^{10}$	$1.26 * 10^{10}$	n/A	n/A
CAN	$1.73 * 10^{10}$	$6.65 * 10^9$	n/A	n/A	n/A	$1.46 * 10^{10}$	n/A	n/A
CAT	n/A	n/A	$1.75 * 10^{10}$	$7.40 * 10^9$	n/A	n/A	$6.85 * 10^9$	$9.80 * 10^9$
CAM	n/A	n/A	$1.92 * 10^{10}$	$8.30 * 10^9$	n/A	n/A	$7.70 * 10^9$	n/A
***S. rhizophila* P69**								
LB	$2.95 * 10^9$	$4.05 * 10^9$	n/A	n/A	$5.45 * 10^9$	$1.10 * 10^{10}$	n/A	n/A
CAN	$2.50 * 10^9$	$2.85 * 10^9$	n/A	n/A	$7.02 * 10^9$	$1.01 * 10^{10}$	n/A	n/A
CAT	n/A	n/A	$1.25 * 10^{10}$	$6.40 * 10^9$	n/A	n/A	n/A	$8.80 * 10^9$
CAM	n/A	n/A	$8.90 * 10^9$	n/A	n/A	n/A	n/A	$9.60 * 10^9$

3.2. Total bacterial RNA isolation of *S. maltophilia* ep3, *S. rhizophila* P69 and ep17

S. maltophilia ep3, *S. rhizophila* P69 and ep17 have been cultivated on LB complex agar medium (LBP), in LB complex liquid medium (LB), Casamino acid minimal salt medium (CAN), Casamino acid containing maize root exudates (CAM) and Casamino acid containing tomato root exudates (CAT). Subsequently, total bacterial RNA was isolated and the procedure was based on Qiagen RNeasy Mini Kit [42] with small adjustments to the reference procedure to improve the workflow as well as to achieve higher yields and purities. There was only one major change which regards the cell lysis. The cell lysis had a starting version and two improvement steps. At first mechanical disruption only was used, whereas two mechanical cell lysis approaches were tested: In the first approach the lysate was transferred to FastPrep™Lysing Matrix E[1] (maximum capacity 1 mL) and homogenized with a FastPrep™-24 Sample Preparation System[2] in three repeats for 30 seconds each at 6 m/s. In the second approach the lysate was transferred to homogenizer tubes and centrifuged. This variant of mechanical cell lysis has proved to be the most promising approach. Henceforth, the homogenizer tubes were applied at all total bacterial RNA isolations. In the second improvement step cell lysis with lysozyme (15 mg/mL) and Proteinase K (20 mg/mL) were added in addition to the mechanical digestion.

Total bacterial RNA isolation for *S. maltophilia* ep3, *S. rhizophila* P69 and ep17 has proven to be most promising when cell lysis was conducted by using both mechanical disruption and enzymatic cell lysis with lysozyme (15 mg/mL) and Proteinase K (20 mg/mL).

The quantity and quality of the isolated total RNA was measured through the use of a NanoDrop™Spectrophotometer, as well as Qubit 4 Fluorometer RNA assay. The results of the measurements were listed in table 3.4 and table 3.5.

[1]MP Biomedicals™, fisher scientific part of Thermo Fisher Scientific, Germany
[2]MP Biomedicals™, Germany

Table 3.4.: Results of total RNA isolations of *S. maltophilia* ep3, *S. rhizophila* ep17 and *S. rhizophila* P69 cultivated on LB complex agar medium (LBP), in LB complex liquid medium (LB), Casamino acid minimal salt medium (CAN), Casamino acid containing maize root exudates (CAM) and Casamino acid containing tomato root exudates (CAT). In this table the 260/280 and 260/230 ratios, as well as nucleic acid concentrations are listed.

Mechanical disruption

	Condition	**NAC[1] [ng/μl]**	260/280	260/230
S. rhizophila P69	LB_1	286.9	2.04	2.20
S. rhizophila P69	LB_2	216.8	1.97	2.13
S. rhizophila P69	LB_3	159.3	1.98	1.64
S. maltophilia ep3	LB_1	336.1	2.07	2.16
S. maltophilia ep3	LB_2	110.3	2.10	1.69
S. maltophilia ep3	LB_3	124.2	2.03	1.61

Mechanical disruption and enzymatic lysis with lysozyme

	Condition	**NAC[1] [ng/μl]**	260/280	260/230
S. rhizophila P69	LB_1	190.1	2.10	1.30
S. rhizophila P69	LB_2	102.7	2.09	1.07
S. rhizophila P69	LB_3	188.8	2.10	1.50
S. maltophilia ep3	LB_1	418.4	2.10	1.74
S. maltophilia ep3	LB_2	148.0	2.08	1.50
S. maltophilia ep3	LB_3	111.3	2.10	0.95
S. maltophilia ep3	LB_1	346.4	2.05	2.12
S. maltophilia ep3	LB_2	227.8	2.09	1.57
S. rhizophila ep17	LB_1	358.5	2.08	2.16
S. rhizophila ep17	LB_2	615.6	2.11	2.24
S. maltophilia ep3	LB_1	209.0	2.07	2.06
S. maltophilia ep3	LB_2	146.3	2.03	1.99
S. maltophilia ep3	CAN_1	69.4	1.96	1.21
S. maltophilia ep3	CAN_2	71.9	2.01	0.99
S. maltophilia ep3	CAT_1	89.4	2.02	0.96
S. maltophilia ep3	CAT_2	33.6	1.89	1.22
S. maltophilia ep3	CAM_1	29.6	1.90	1.14
S. maltophilia ep3	CAM_2	73.0	2.00	1.59

[1] Nucleic Acid Concentration

Table 3.5.: Results of total RNA isolations of *S. maltophilia* ep3, *S. rhizophila* ep17 and *S. rhizophila* P69 cultivated on LB complex agar medium (LBP), in LB complex liquid medium (LB) and Casamino acid minimal salt medium (CAN), Casamino acid containing maize root exudates (CAM) and Casamino acid containing tomato root exudates (CAT). In this table the 260/280 and 260/230 ratios, as well as nucleic acid concentrations are listed.

Mechanical disruption
+ enzymatic lysis (lysozyme, Proteinase K)

	Condition	NAC[1] [ng/μl]	260/280	260/230
S. rhizophila ep17	LB_1	310.4	2.05	1.84
S. rhizophila ep17	LB_2	307.0	2.07	1.59
S. rhizophila ep17	CAN_1	198.6	2.06	1.38
S. rhizophila ep17	CAN_2	199.9	2.05	2.20
S. rhizophila ep17	CAT_1	157.1	2.04	1.79
S. rhizophila ep17	CAT_2	148.4	2.05	2.08
S. rhizophila ep17	CAM_1	185.5	2.07	2.17
S. rhizophila ep17	CAM_2	120.6	2.04	1.41
S. rhizophila P69	LB_1	338.9	2.09	1.87
S. rhizophila P69	LB_2	155.6	2.10	1.36
S. rhizophila P69	CAN_1	156.3	2.02	2.02
S. rhizophila P69	CAN_2	55.8	2.05	1.10
S. rhizophila P69	CAT_1	61.9	2.04	1.50
S. rhizophila P69	CAT_2	62.8	2.07	1.74
S. rhizophila P69	CAM_1	93.7	2.07	1.96
S. rhizophila P69	CAM_2	115.4	2.04	1.91
S. maltophilia ep3	LBP_1	46.7	2.11	0.88
S. maltophilia ep3	LBP_2	65.4	2.09	1.31
S. maltophilia ep3	LBP_3	164.4	2.12	1.93
S. rhizophila ep17	LBP_1	179.2	2.12	2.24
S. rhizophila ep17	LBP_2	153.9	2.13	1.59
S. rhizophila ep17	LBP_3	83.5	2.11	2.22
S. rhizophila P69	LBP_1	89.0	2.11	1.93
S. rhizophila P69	LBP_2	86.0	2.13	0.66
S. rhizophila P69	LBP_3	102.3	2.11	1.29

[1] Nucleic Acid Concentration

3.3. Data exploration and visualization of the differential gene expression data of *S. rhizophila* P69

Sequencing of the different cDNA samples resulted in 29.1 to 72.3 million cDNA sequencing reads and the overall alignment rate against the *S. rhizophila* P69 reference genome was between 58.2% and 79.9% (see table 3.6). The RNA sequencing data from *S. rhizophila* P69 (cultivated on LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)) was elucidated on a gene and transcript level using a RNA specific sequence analysis pipeline. The differential gene expression data has been visualized with some of the multiple available plotting methods provided by CummeRbund. In order to obtain information about the quality of the Cuffdiff data a *csDensity* plot [29] was used. The generated density plot (which can be seen in 3.4) depicted the differential gene expression patterns between LB, LBP, CAM and CAN. The gene expression of all conditions was distributed in similar FPKM values, except LB exhibited higher density values.

Furthermore, a *dispersionPlot* from CummeRbund was applied which provides an quick and clear overview of the quality of the received data. This plot estimated the dispersion for all four cultivation conditions. The results (which can be seen in figure 3.5) showed a high quality for all cultivation conditions (LB complex liquid medium, LB complex agar medium, Casamino acid minimal salt medium and Casamino acid containing maize root exudates) and in consequence the model fitting was high.

Additionally, a plot was created, which provides information about the relationship between statistical significance (P value) and $\log_2$ (fold-change) [29, 21]. The illustration of the significant differences between the compared conditions can be seen in figure 3.6.

Furthermore, the relationships between the different culture conditions can be explored using a *PCAplot*. This Principal Component Analysis (PCA) clusters all samples due to their similarities in gene expression. The result can be seen in figure 3.7 which indicated a significant difference if *S.*

Table 3.6.: **Statistics of cDNA sequencing reads and the corresponding overall alignment rates (%).**

S. rhizophila P69		
Sample	**Total no. of RNA-Seq reads**	**Overall alignment rate (%)**
LB_1	29,113,300	58.75
LB_2	48,792,500	68.78
LBP_1	45,365,700	78.99
LBP_2	33,776,800	65.35
CAM_1	72,382,435	63.03
CAM_2	67,665,020	55.71
CAN_1	71,513,374	56.47
CAN_2	65,197,623	60.24

rhizophila P69 was cultivated either in LB complex liquid medium or in Casamino acid minimal salt medium, notably, that there was also a significant difference between the LB and LBP samples.

A *csHeatmap* was created in order to cluster the gene expression patterns of *S. rhizophila* P69 which was cultivated in four different cultivation conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid minimal salt medium (CAN) and Casamino acid containing maize root exudates (CAM)). The heatmap relies on the Jensen-Shannon Distance (JS Distance) value which is a statistical value and it gives information about how similar or dissimilar the different comparisons are [29]. The results showed significant differences between the cultivation conditions. This heatmap matrix (which can be seen in figure 3.8) showed that there is a considerable distinction when *S. rhizophila* P69 was grown in complex (LB, LBP) compared to minimal salt medium (CAN, CAM). Additionally, there were noteworthy differences when the bacterial strain was cultivated in either LB or on LBP with a value of 0.0971 JS Distance. However, the slightest difference was found in the pairwise comparison of CAM with CAN which had a value of 0.0334 JS Distance.

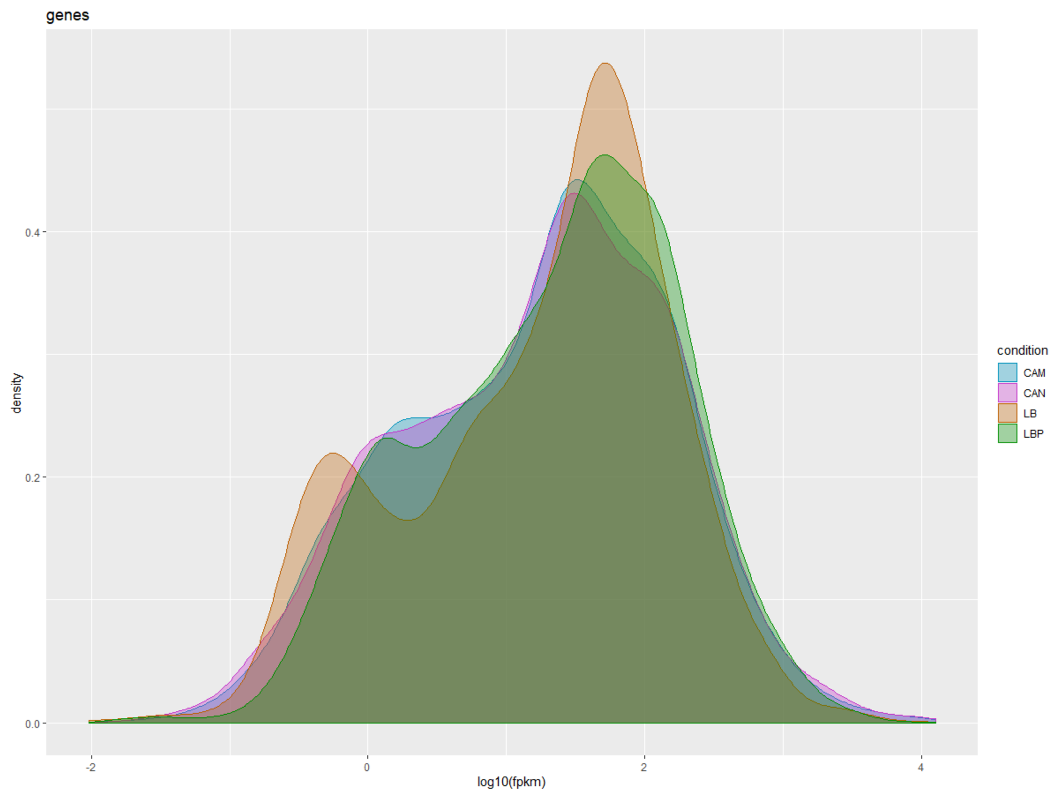

Figure 3.4.: **Density plot rendered from differential gene expression data of *S. rhizophila* P69 cultivated under four conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)).** A density plot assesses the dispersion of FPKM scores across genes [29].

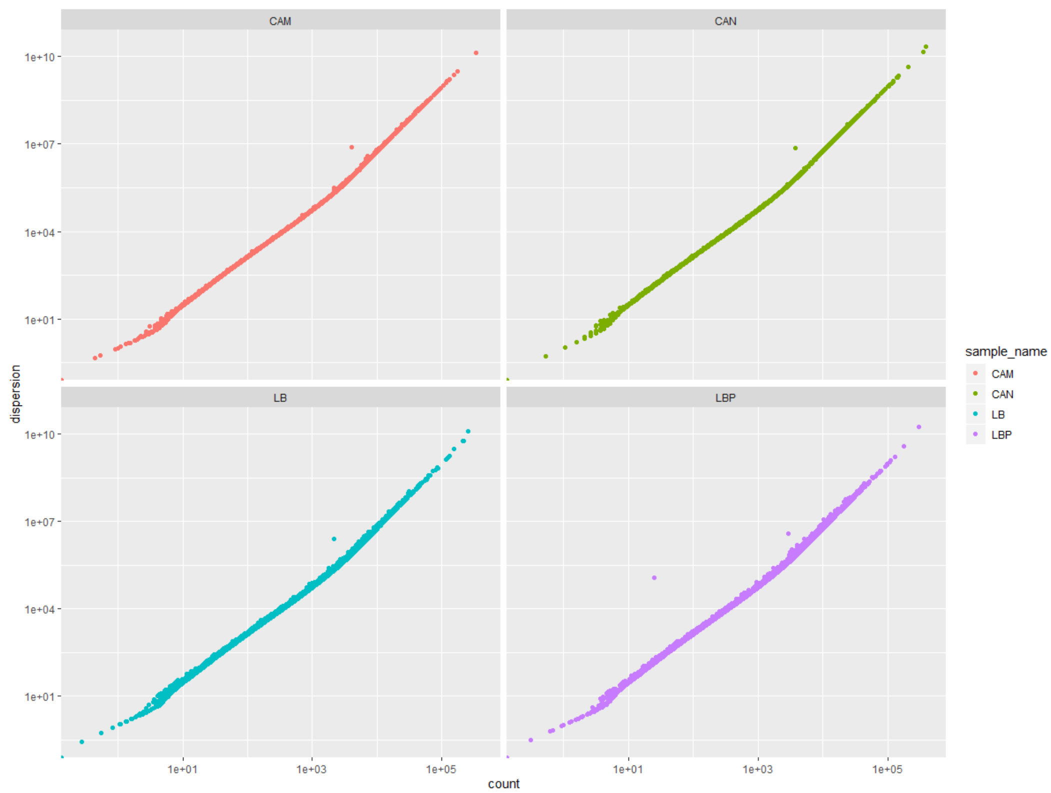

Figure 3.5.: **Dispersion plot created from the differential gene expression data of *S. rhizophila* P69 cultivated under four conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)).** It gives information about the quality of the created data and estimates possible over dispersion for all four cultivation conditions.

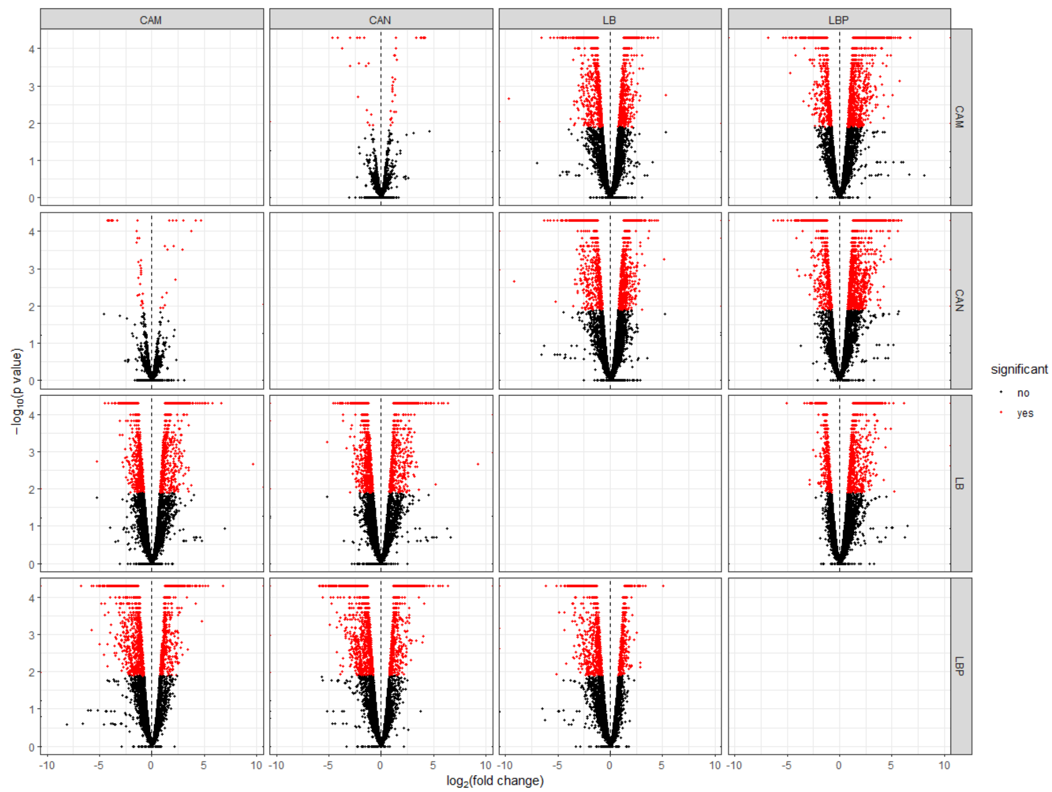

Figure 3.6.: **Volcano plots from the differential gene expression data of *S. rhizophila* P69 cultivated under four conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)).** The plots give information about the relationship between significance and fold-change ($\log_2$) [29]. The statistically significant genes are represented by the red dots. Genes with similar expression levels were represented by the black dots.

Figure 3.7.: **PCA plot rendered from differential gene expression data of *S. rhizophila* P69 cultivated under four conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)).** This plot clusters all samples due their similarities which indicates that there is a significant difference if *S. rhizophila* P69 was cultivated in LB or Casamino acid minimal salt medium, notably, that there is also a difference between LB complex liquid medium, LB complex agar medium.

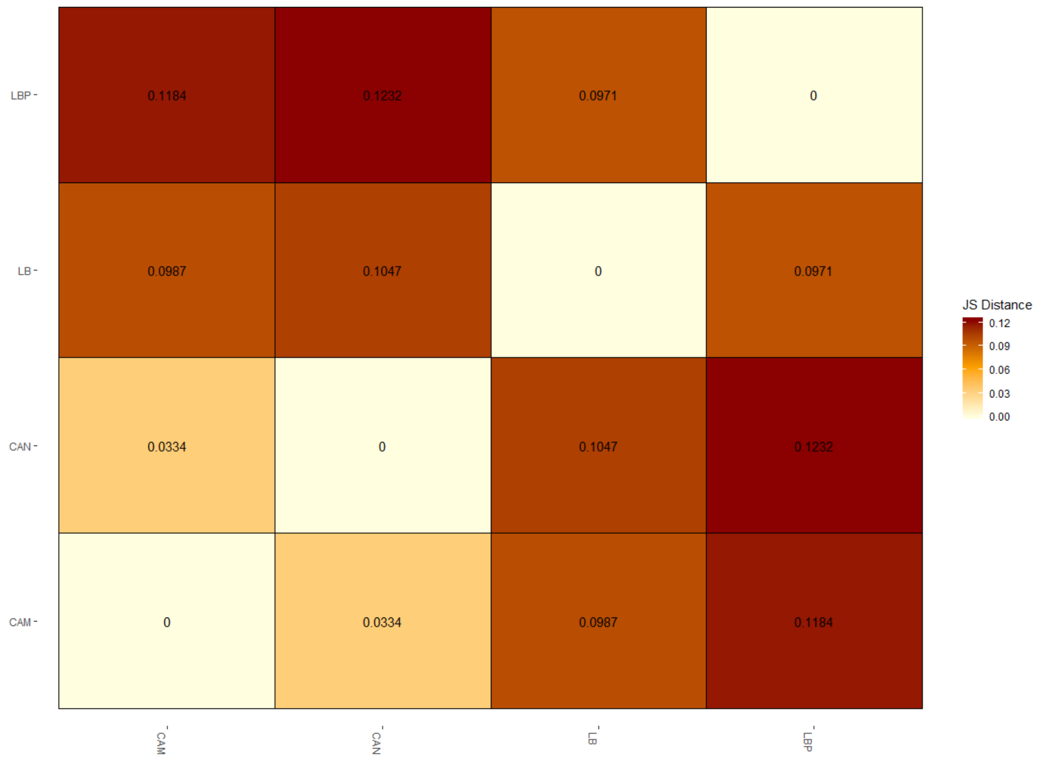

Figure 3.8.: **Distance heat map rendered from differential gene expression data of *S. rhizophila* P69 cultivated under four conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN)).** The distance heat map explores the similarities between the different cultivation conditions.

Table 3.7.: Results of the differential gene expression analysis of *S. rhizophila* P69 cultivated in Casamino acid containing maize root exudates (CAM), Casamino acid minimal salt medium (CAN), LB complex liquid medium (LB) or on LB complex agar medium (LBP). Significant genes are assigned to COG functions [15] which are affected differently in the three pairwise comparisons.

			% affected		
COG Function	**Code**	**Total COG[1]**	**CAM vs. LB**	**LB vs. LBP**	**CAM vs. CAN**
Amino acid transport and metabolism	E	173	16.76	14.45	7.51
Carbohydrate transport and metabolism	G	151	14.57	13.91	0.66
Cell cycle control, cell division, chromosome partitioning	D	28	10.71	7.14	0.00
Cell motility	N	77	12.99	11.69	0.00
Cell wall/membrane/envelope biogenesis	M	184	11.41	11.96	0.00
Coenzyme transport and metabolism	H	76	11.84	7.89	0.00
Defence mechanisms	V	63	9.52	15.87	0.00
Energy production and conversion	C	144	18.75	11.81	1.39
Function unknown	S	204	11.27	10.78	0.00
General function prediction only	R	254	11.42	13.39	0.00
Inorganic ion transport and metabolism	P	149	24.16	16.11	1.34
Intracellular trafficking, secretion, and vesicular transport	U	27	22.22	0.00	0.00
Lipid transport and metabolism	I	109	11.01	13.76	0.92
Nucleotide transport and metabolism	F	44	9.09	18.18	0.00
Post-translational modification, protein turnover, chaperones	O	108	16.67	25.93	0.00
Replication, recombination and repair	L	127	7.09	11.02	0.00
Secondary metabolites biosynthesis, transport and catabolism	Q	43	6.98	16.28	2.33
Signal transduction mechanisms	T	151	17.88	14.57	0.00
Transcription	K	189	19.05	23.28	0.53
Translation, ribosomal structure and biogenesis	J	110	12.73	6.36	0.91

[1] Total number of instances of a particular COG function

CummeRbund can not only be used for the visualization of datasets, but similarly for the creation of a full table containing all significant genes by pairwise comparison of all cultivation conditions. The evaluation of this table showed that a total number of 1608 significant genes have been differently expressed. To find out more about the differential gene expression the conditions were compared in three pairs. The first comparison was between Casamino acid containing maize root exudates and Casamino acid minimal salt medium and the second was between LB complex liquid medium and LB complex agar medium. Finally, the complex and the minimal salt media were compared to each other.

The differential gene expression analysis revealed that particular COG functions have different numbers of instances of that function. This means

that there are more opportunities for significantly expressed genes to belong to that function if the total number for that COG function is higher. For this reason it was necessary to express the number of differentially expressed genes as a percentage of the total number of instances of a particular function (see table 3.7). The first comparison (CAM vs. CAN) the number of differentially expressed genes is mostly at about 1% of possible total genes per COG category, except for *amino acid transport and metabolism* which instead is about 7.5%. The second comparison of LB complex liquid medium and LB complex agar medium showed that the most affected categories were *post-translational modification, protein turnover, chaperones* (with 25.96%) and *transcription* (with 23.28%). The third comparison of Casamino acid minimal salt medium and LB complex liquid medium showed that the most affected categories were *inorganic ion transport and metabolism* (with 24.16%) and *intracellular trafficking, secretion, and vesicular transport* (with 22.22%) (see table 3.7).

3.3.1. Differential expression analysis of *S. rhizophila* P69 cultivated in either Casamino acid containing maize root exudates or Casamino acid minimal salt medium

The first comparison was between Casamino acid containing maize root exudates and Casamino acid minimal salt medium and the differential gene expression data have been visualized with some of the multiple available plotting methods provided by CummeRbund. In order to obtain information about the gene expression level of *S. rhizophila* P69 cultivated either in Casamino acid minimal salt medium or Casamino acid containing maize root exudates a *csScatter* was created which can be seen in figure 3.9. The scatter plot showed that there was a positive correlation in gene expression between the two conditions Casamino acid minimal salt medium and Casamino acid containing maize root exudates which indicated that they have similar gene expression levels.

The evaluation of the differential expression analysis of *S. rhizophila* P69 by pairwise comparison of Casamino acid containing maize root exudates and

Casamino acid minimal salt medium showed a total number of 34 significant genes (see table A.1). 13 of these genes have been classified to encode for proteins with the Clusters of Orthologous Groups (COG) function of *amino acid transport and metabolism*. For 12 (about 35%) of the genes the corresponding COG function of proteins were not available. The remaining genes encode for proteins with the following COG functions: *energy production and conversion*, *inorganic ion transport and metabolism*, *carbohydrate transport and metabolism*, *lipid transport and metabolism*, *secondary metabolites biosynthesis, transport and metabolism*, *translation, ribosomal structure and biogenesis* and *transcription* (see figure 3.11). Additionally, it was interesting that seven of these significant genes in the COG function category of amino acid transport and metabolism encoded for proteins which were part of the histidine metabolism (*hisF*, *hisA*, *hisH*, *hisB*, *hisC*, *hisD* and *hisG*) (see table A.1).

Proteins belonging to the category *amino acid transport and metabolism* showed a positive $\log_2$. These results, which can be seen in 3.10, indicated that these genes were up regulated after growth in CAN. In contrast, proteins of the categories *transcription*, *signal transduction mechanisms*, *carbohydrate transport* as well as the unknown genes (labelled as *not available*) had a negative $\log_2$ and in consequence partly down regulated.

Finally, a KEGG analysis was conducted in order to find out if the differential expression of particular genes was systematic or random. The result can be seen in figure 3.12. It shows the histidine metabolism with up regulated genes (Phosphoribosylpyrophosphat (PRPP) $\Rightarrow$ L-Histidine) in green and down regulated genes (L-Histidine $\Rightarrow$ L-Glutamate) in red.

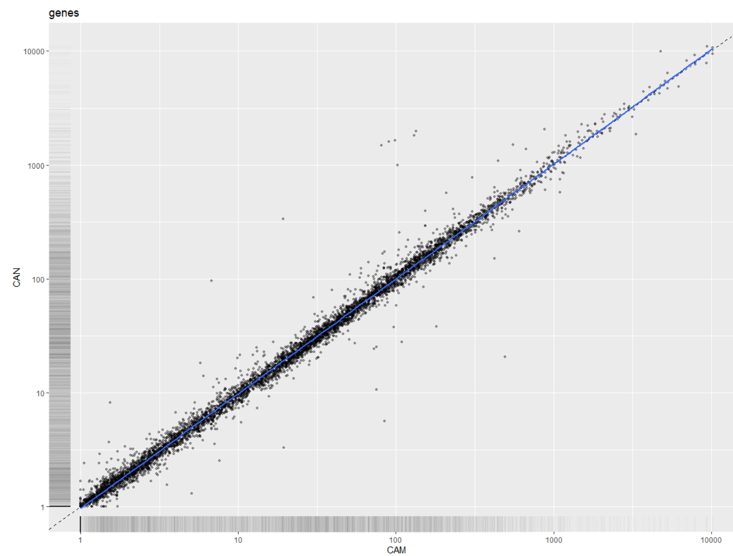

Figure 3.9.: **Scatter plot rendered from differential gene expression data of *S. rhizophila* P69 by pairwise comparison of the cultivation condition Casamino acid containing maize root exudates and Casamino acid minimal salt medium**. The x-axis represents the gene expression values for CAM and the y-axis shows the expression values for CAN. The FPKM values for all transcripts were plotted for each sample. The data of one transcript is represented by a single dot. The cultivation conditions are compared in pairs giving information about the course of a gene expression as well as identifying global changes [29]. Each dot thus shows the expression of one gene under both growth conditions. Genes which show similar expression values are concentrated in the proximity of the dashed diagonal.

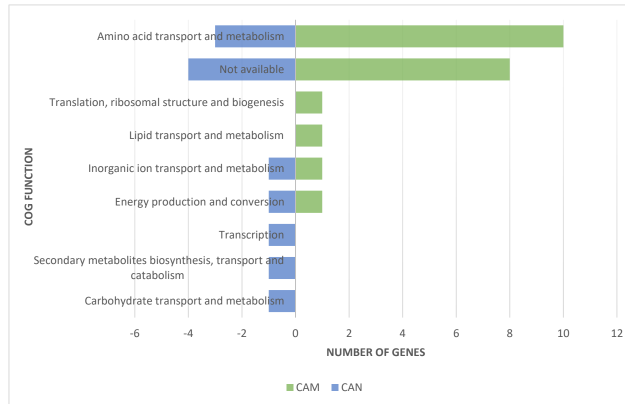

Figure 3.10.: **Results of the differential expression analysis of *S. rhizophila* P69 by pairwise comparison of the cultivation conditions Casamino acid containing maize root exudates and Casamino acid minimal salt medium.** In this graph, the COG function was plotted against the $\log_2$ (fold change) of significantly expressed genes. (The COG functions of proteins with the corresponding codes are listed in 3.8 [15]. 13 out of 34 genes were categorized to the COG function *amino acid transport and metabolism* of which 10 were significant up regulated.

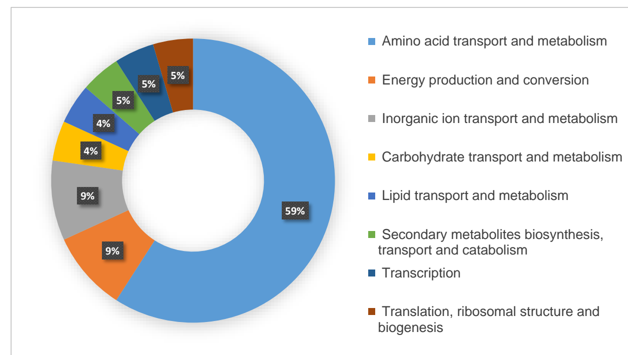

Figure 3.11.: **Number of significant genes of *S. rhizophila* P69 encoding for proteins with the corresponding COG function by pairwise comparison of the cultivation conditions Casamino acid containing maize root exudates and Casamino acid minimal salt medium.** The comparison shows that a total number of 34 significant genes were differently expressed, of which 13 (7.5% in total) encode for proteins which are responsible for *amino acid transport and metabolism*.

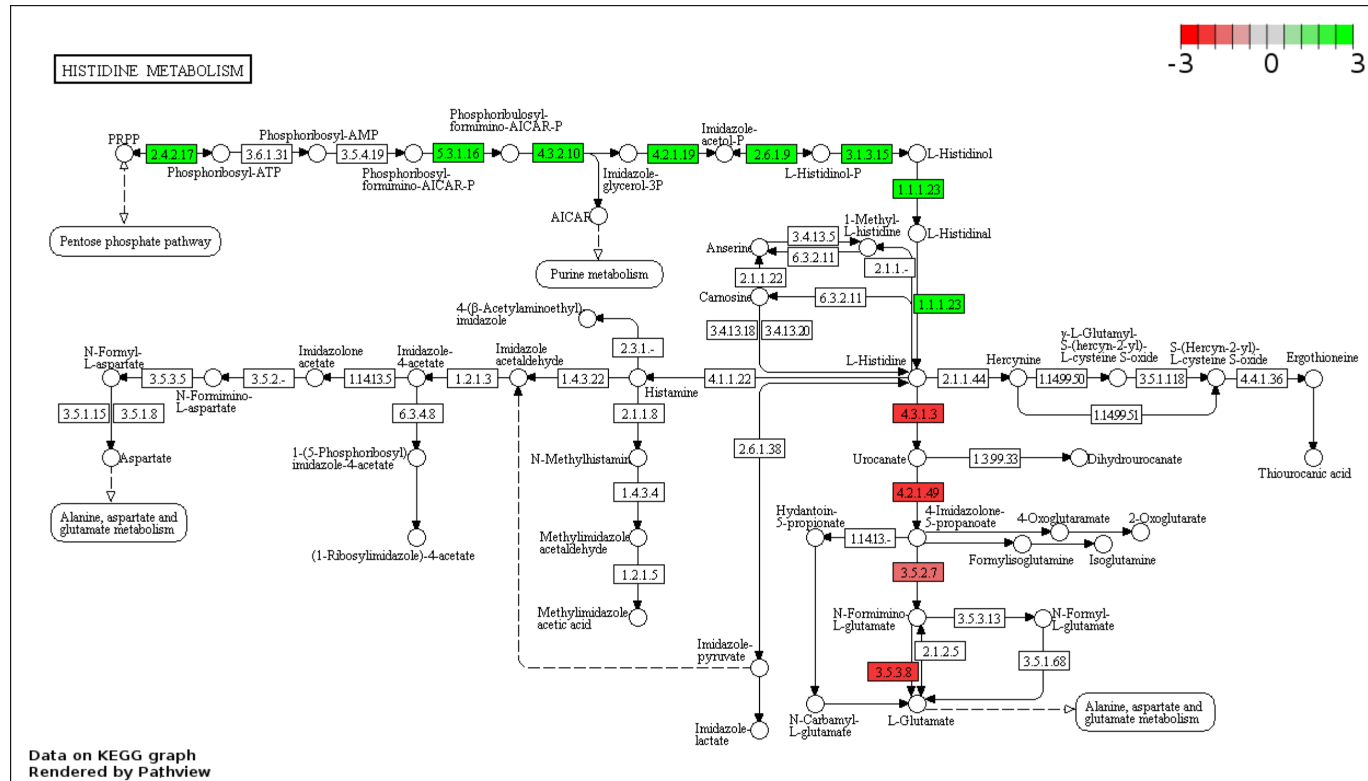

Figure 3.12.: **Results of the KEGG analysis for the differential gene expression of *S. rhizophila* P69 by pairwise comparison of the cultivation conditions Casamino acid containing maize root exudates and Casamino acid minimal salt medium.** This image shows the pathway for up and down regulated genes which express proteins responsible for the histidine metabolism.

3.3.2. Differential expression analysis of *S. rhizophila* P69 cultivated either in LB complex liquid medium or on LB complex agar medium

The second comparison was between the cultivation conditions LB complex liquid medium (LB) and LB complex agar medium (LBP). In order to obtain information about the gene expression level of *S. rhizophila* P69 a *csScatter* was created which shows a relevant distribution of differentially expressed genes. The results indicated that LB complex liquid medium and LB complex agar medium exhibited a relevant amount of difference among each other which can be seen in figure 3.13.

The evaluation of the differential expression analysis of *S. rhizophila* P69 by comparison of LB and LBP showed a total number of 919 significant genes, of which 406 were up regulated and 513 were down regulated. These genes encode for proteins which are classified into 19 different COG categories. 582 (approximately 63%) significant genes could not be assigned to a COG category, notably, that 265 of them were up regulated and 317 were down regulated. The remaining 337 significant genes were successfully assigned to a COG function. Looking at the number of significant genes also seen in figure 3.15 it becomes apparent that a big portion of differential expressed genes encode for proteins that were in the areas of *transcription* (with 44 genes), *general function prediction only* (with 34 genes) and *post-translational modification, protein turnover, chaperones* (with 28 genes).

The COG function plotted against the number of genes can be seen in figure 3.14. There was a high number of significant genes exhibiting a negative as well as positive $\log_2$. In general, 196 out of 337 of the significant genes exhibited a negative $\log_2$ (fold change). 141 genes exhibited a positive $\log_2$ and in consequence an increased gene expression. In many categories there was a similar amount of positively and negatively exhibited genes, in particular the genes belonging to COG function *amino acid transport and metabolism* (E), *general function prediction only* (R), *transcription* (K) and *cell wall/membrane/envelope biogenesis* (M).

In addition to plotting the data with CummeRbund, a KEGG analysis was executed, as well. The results can be seen in figure 3.17, which shows the

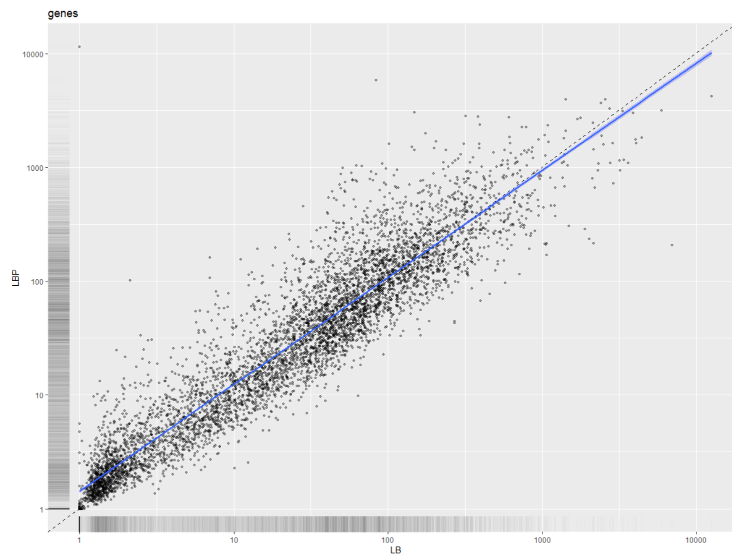

Figure 3.13.: **Scatter plot rendered from differential gene expression data of *S. rhizophila* P69 by pairwise comparison of the cultivation condition LB complex liquid medium (LB) and LB complex agar medium (LBP).** The x-axis represents the gene expression values for LB and the y-axis shows the expression values for LBP. The FPKM values for all transcripts were plotted for each sample. The data of one transcript is represented by a single dot. The cultivation conditions are compared in pairs giving information about the course of a gene expression as well as identifying global changes [29]. Each dot thus shows the expression of one gene under both growth conditions. Genes with similar expression values are concentrated in the proximity of the dashed diagonal.

histidine metabolism. It indicates that there was a differential gene expression level between the cultivation conditions, especially at the conversion from PRPP to L-Histidine and L-Histidine to L-Glutamate which were significantly higher. A second KEGG analysis showed that the differential gene expression corresponding to flagellar assembly and twitching motility was affected and the results can be seen in figure 3.16. The expression of the genes *cheW*, *chpA*, *pilG* and *pilH* seemed to be strongly suppressed after growth on LB complex agar medium.

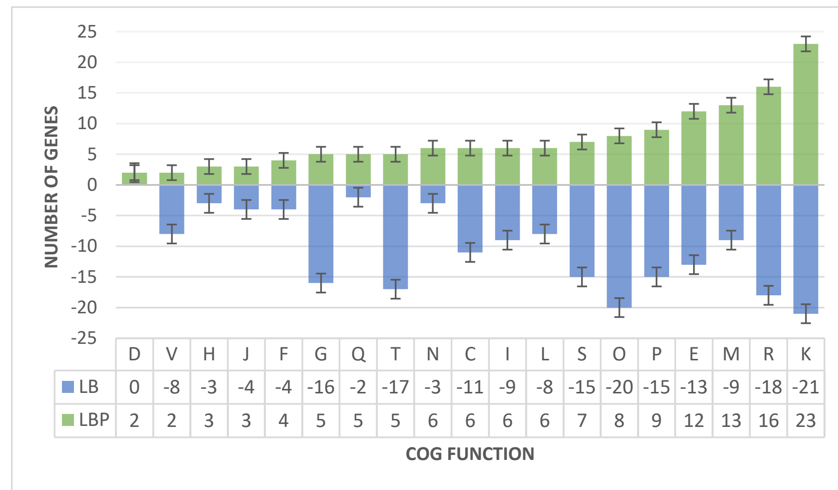

Figure 3.14.: Number of significant up or down regulated genes of *S. rhizophila* P69 encoding for proteins with particular COG functions by pairwise comparison of the cultivation conditions LB complex liquid medium (LB) and LB complex agar medium (LBP). (The COG functions of proteins with the corresponding codes are listed in 3.8 [15].)

	CELLULAR PROCESSES AND SIGNALING		**METABOLISM**
D	Cell cycle control, cell division, chromosome partitioning	C	Energy production and conversion
M	Cell wall/membrane/envelope biogenesis	E	Amino acid transport and metabolism
N	Cell motility	F	Nucleotide transport and metabolism
O	Post-translational modification, protein turnover and chaperones	G	Carbohydrate transport and metabolism
T	Signal transduction mechanisms	H	Coenzyme transport and metabolism
U	Intracellular trafficking, secretion and vesicular transport	I	Lipid transport and metabolism
V	Defence mechanisms	P	Inorganic ion transport and metabolism
W	Extracellular structures	Q	Secondary metabolites biosynthesis, transport and catabolism
Y	Nuclear structure		
Z	Cytoskeleton		
	INFORMATION STORAGE AND PROCESSING		**POORLY CHARACTERIZED**
A	RNA processing and modification	R	General function prediction only
B	Chromatin structure and dynamics	S	Function unknown
J	Translation, ribosomal structure and biogenesis		
K	Transcription		
L	Replication, recombination and repair		

Table 3.8.: Listing of the different COG categories of proteins with the corresponding code. The COG function is categorized into 17 functional classes [15].

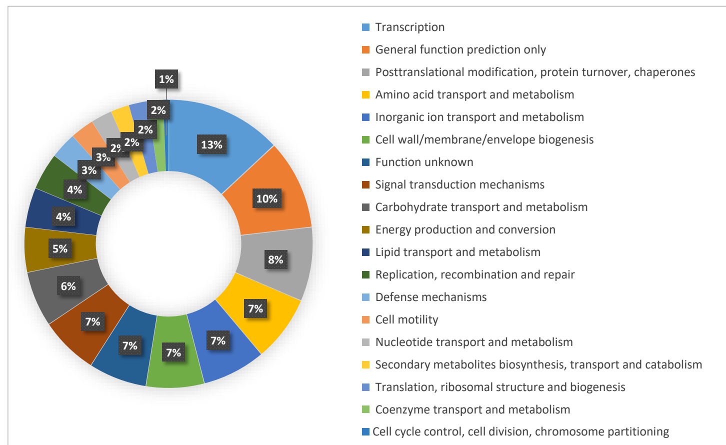

Figure 3.15.: Number of significant genes of *S. rhizophila* P69 encoding for proteins with particular COG functions by comparing the cultivation conditions LB complex liquid medium (LB) and LB complex agar medium (LBP). The differential expression analysis showed that a total number of 339 significant genes were expressed. The majority of genes encoded for proteins classified into the following COG categories, namely, *transcription*, *amino acid transport and metabolism*, *inorganic ion transport and metabolism*.

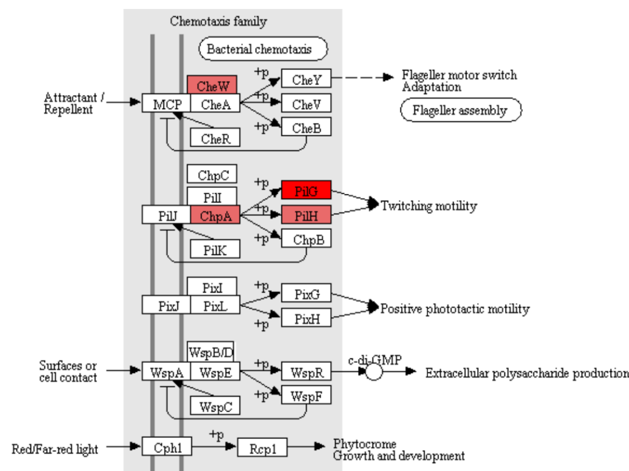

Figure 3.16.: **Results of the KEGG analysis for the differential gene expression analysis of *S. rhizophila* P69 cultivated in LB complex liquid medium (LB) or on LB complex agar medium (LBP).** This image shows the pathway for down regulated genes corresponding for the bacterial chemotaxis.

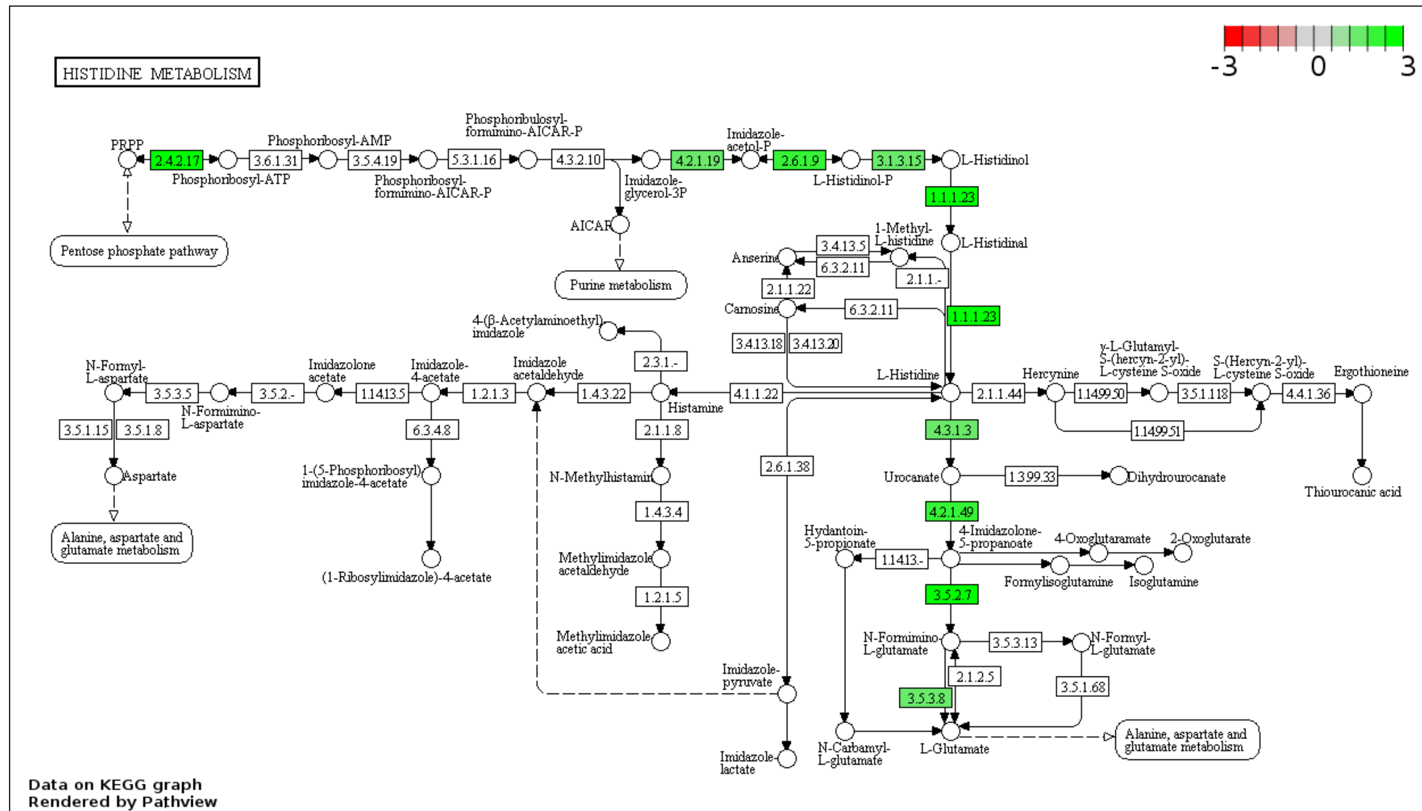

Figure 3.17.: **Results of the KEGG analysis for the differential gene expression of *S. rhizophila* P69 cultivated in LB complex liquid medium (LB) or on LB complex agar medium (LBP).** This image shows the pathway for up and down regulated genes corresponding for the histidine metabolism.

3.3.3. Differential expression analysis of *S. rhizophila* P69 cultivated either in Casamino acid minimal salt medium or in LB complex liquid medium

The third comparison was between the cultivation conditions Casamino acid minimal salt medium and LB complex liquid medium. A created scatter plot (which can be seen in 3.18) revealed that there were significant differences in gene expression due to the growth environments.

The evaluation of the differential expression analysis of *S. rhizophila* P69 with CummeRbund shows a total number of 921 significant genes, of which 421 were up regulated and 500 were down regulated. These differentially expressed genes encoded for proteins which were classified to twelve COG functions (see figure 3.19). For 576 (approximately 60%) the COG function was not available, notably, that 320 of them were down regulated. The remaining 344 significant genes were successfully assigned to a COG function.

Looking at the number of significant genes (also seen in figure 3.19) it becomes apparent that a majority of genes encoding for proteins which are predominantly classified in the area of *transport and metabolism*, mostly of *inorganic ions* (with 36 genes) and *amino acids* (with 28 genes). Similarly, the majority of the genes belonging to the COG function of *amino acid transport and metabolism* encoded for proteins which were part of the histidine metabolism. Additionally, 36 (10%) highly expressed genes belonged to the category *transcription*, while the categories for *energy production and conversion* and *signal transduction mechanism* were represented each with 27 (8%) significant genes.

The COG function plotted against the number of genes can be seen in figure 3.20. There was a high number of significant genes exhibiting a positive as well as a negative $\log_2$. In general, 178 out of 344 of the significant genes exhibited a negative $\log_2$ (fold change) and in consequence a decreased gene expression. 166 significant genes exhibited a positive $\log_2$ (fold change) and in consequence a increased gene expression. In many categories there was a similar amount of positively and negatively exhibited genes, in particular the genes belonging to COG function *amino acid transport and metabolism*(E),

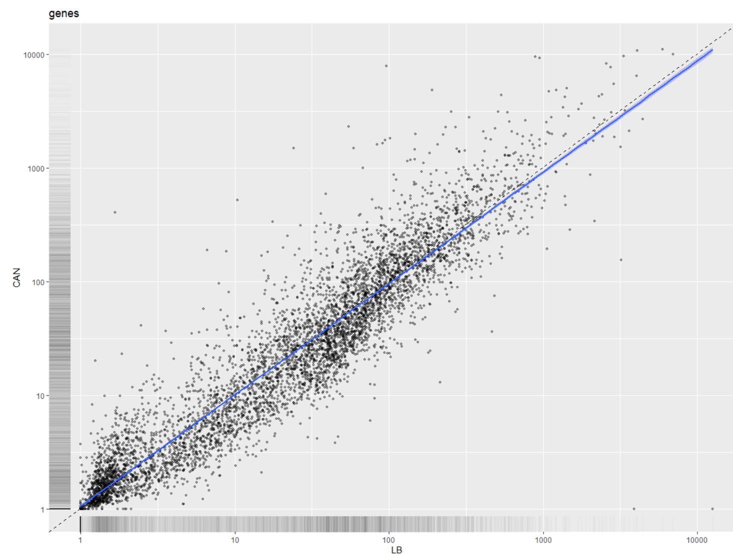

Figure 3.18.: **Scatter plot rendered from differential gene expression data of *S. rhizophila* P69 by pairwise comparison of the cultivation condition Casamino acid minimal salt medium (CAN) and LB complex liquid medium (LB).** The x-axis represents the gene expression values for LB and the y-axis shows the expression values for CAN. The FPKM values for all transcripts were plotted for each sample. The data of one transcript is represented by a single dot. The cultivation conditions are compared in pairs giving information about the course of a gene expression as well as identifying global changes [29]. Each dot thus shows the expression of one gene under both growth conditions. Genes which show similar expression values are concentrated in the proximity of the dashed diagonal.

energy production and conversion(C), *function unknown* (S), *general function prediction only* (R), *inorganic ion transport and metabolism* (P), *signal transduction mechanisms* (T), *transcription* (K).

Furthermore, KEGG showed that the histidine metabolism was notably strong affected (see figure 3.21), in particular the conversion of PRPP to L-Histidine appeared to be strongly suppressed.

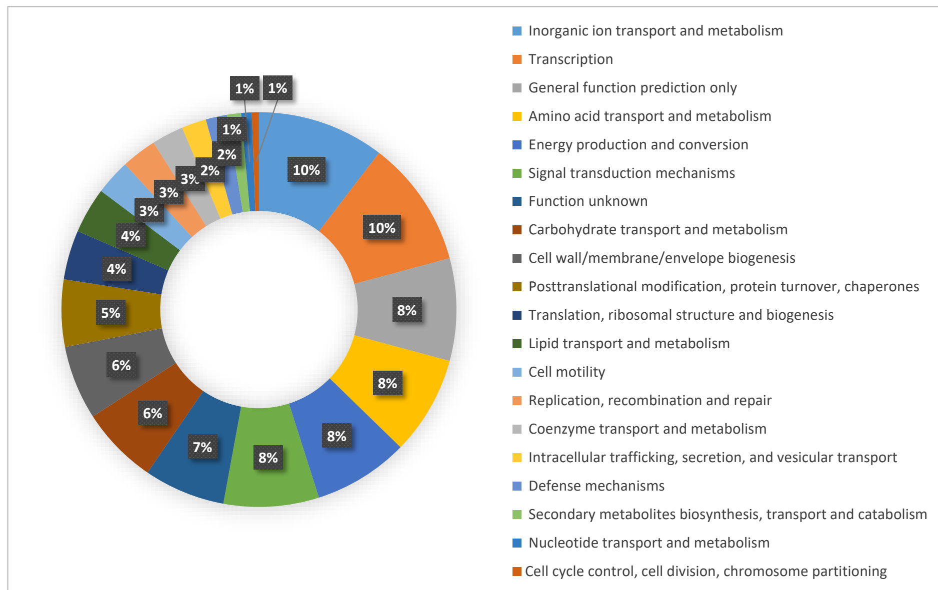

Figure 3.19.: **Number of significant genes of *S. rhizophila* P69 encoding for proteins that are classified into particular COG functions by comparing cultivation in either Casamino acid minimal salt medium (CAN) or in LB complex liquid medium (LB).** The differential expression analysis showed that a total number of 921 significant genes have been differentially expressed. The majority of these genes encoded for proteins belonging to the following COG categories: *inorganic ion transport and metabolism*, *transcription* and *amino acid transport and metabolism*.

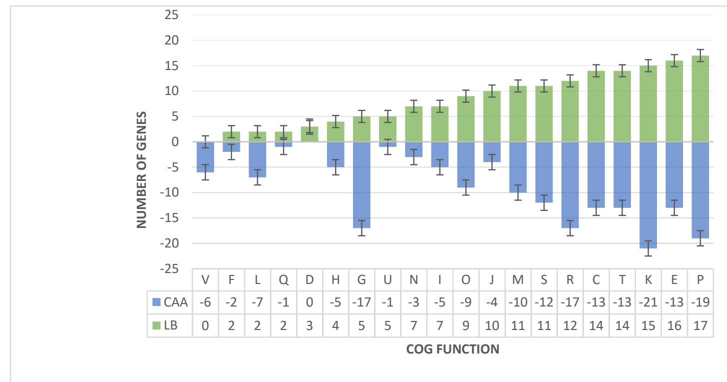

Figure 3.20.: **Number of significant genes of *S. rhizophila* P69 encoding for proteins with a classified COG functions.** In this graph a pairwise comparison of the cultivation conditions Casamino acid minimal salt medium (CAN) and LB complex liquid medium (LB) have been conducted. For each category, the number of up and down regulated genes is indicated. (COG functions with the corresponding codes are listed in 3.9 [15].)

CELLULAR PROCESSES AND SIGNALING		METABOLISM	
D	Cell cycle control, cell division, chromosome partitioning	C	Energy production and conversion
M	Cell wall/membrane/envelope biogenesis	E	Amino acid transport and metabolism
N	Cell motility	F	Nucleotide transport and metabolism
O	Post-translational modification, protein turnover and chaperones	G	Carbohydrate transport and metabolism
T	Signal transduction mechanisms	H	Coenzyme transport and metabolism
U	Intracellular trafficking, secretion and vesicular transport	I	Lipid transport and metabolism
V	Defence mechanisms	P	Inorganic ion transport and metabolism
W	Extracellular structures	Q	Secondary metabolites biosynthesis, transport and catabolism
Y	Nuclear structure		
Z	Cytoskeleton		
INFORMATION STORAGE AND PROCESSING		**POORLY CHARACTERIZED**	
A	RNA processing and modification	R	General function prediction only
B	Chromatin structure and dynamics	S	Function unknown
J	Translation, ribosomal structure and biogenesis		
K	Transcription		
L	Replication, recombination and repair		

Table 3.9.: Listing of the different COG categories of proteins with the corresponding code. The COG function is categorized into 17 functional classes [15].

Figure 3.21.: **Results of the KEGG analysis for the differential gene expression of *S. rhizophila* P69 cultivated in Casamino acid minimal salt medium (CAN) and LB complex liquid medium (LB)** This image shows the histidine metabolism. Up regulated gene expression were highlighted in green and down regulated highlighted in red.

4. Discussion

In recent times agriculture has stepped up to the great challenge of different biotic and abiotic stresses [51, 32] resulting in increasing harvest losses (20% yield loss in crops) worldwide [17]. Due to the ever-growing global population, food supplies and consequently agricultural productivity must be ensured [51]. Soil with high pathogen pressures and salt content are the major stress factors that agriculture is facing nowadays which makes the cultivation of crops more challenging [4]. In addition, there is a social trend to demand biocontrol agents which are less harmful to the environment [47] compared to certain agrochemicals [2]. The plant-associated rhizobacteria *S. maltophilia* and *S. rhizophila* are of biotechnological interest due to their application as biocontrol agents in the biotechnological field [52]. The production of biological control agents for biotechnological applications in agriculture is often unpredictable and volatile [8]. In order to control and optimize biotechnological applications, it is necessary to understand microbe-plant interactions, adaptation of the bacteria to environmental changes and how the lifestyle (planktonic or sessile) of bacteria can change during a biotechnological process. Transcriptome analysis represents a valuable approach to elucidate the interaction between *Stenotrophomonas* and the host plants.

In this study, the bacterial strains *S. rhizophila* P69, *S. rhizophila* ep17 and *S. maltophilia* ep3 have been exposed to five different conditions (LB complex liquid medium (LB), LB complex agar medium (LBP), Casamino acid minimal salt medium (CAN), Casamino acid containing tomato root exudates (CAT) and Casamino acid containing maize root exudates (CAM)). The isolation of total bacterial RNA was performed to detect significant gene expression patterns. For this reason, a transcriptome analysis of *S. rhizophila* P69 has been conducted by pairwise comparison of the mentioned above cultivation conditions whereas the data from Casamino acid containing

tomato root exudates (CAT) were not available and thus not included in this study. A transcriptomic approach can detect significant differences among the *Stenotrophomonas* strains.

4.1. Pairwise comparison of cultivation conditions

Influence of root exudates

The rhizosphere is the plant root surrounding zone influenced through an array of chemical and biological parameters [35]. Plant root exudates are involved in several and important functions as well as in structuring of the bacterial community of the rhizosphere [35]. The roots of a plant exude compounds (e.g. predominantly organic acids, amino acids and monosaccharides) [53] of which the soil microbiota can benefit. The plant also releases signal molecules which serve as a defence mechanism against harmful bacteria [23]. In this study, the response *S. rhizophila* P69 to root exudates was explored by transcriptome analysis comparing the cultivation condition Casamino acid containing maize root exudates (CAM) and Casamino acid minimal salt medium (CAN). In this comparison, only small changes were perceived which could be explained by the fact that CAM only gets slightly more complex compared to CAN. However, 34 genes (0.15% of the transcriptome) were significantly different in expression and were assigned to eight COG categories. Many of the COG categories found in other comparison (LB versus LBP and CAM versus LB) were not detected in the differential expression results of this comparison. This indicates that the change between these two conditions is inherently narrower in spectrum, more specialised and the adaptation requirements are lower than in any of the other comparisons.

Up regulated genes of *S. rhizophila* P69 cultivated in the presence of maize root exudates (CAM) encoded proteins which were predominantly classified in the area of *transport and metabolism*, mostly of *inorganic ion*, *carbohydrate* and, to a lesser extend, *amino acids*, but also the categories *energy production*

and conversion and *transcription*. This observation may indicate that the maize root exudates act as an energy source for the rhizobacteria and this may contribute to the interaction of the rhizobacteria and their host plant [3].

A study by Mark et al. analysed the gene expression pattern of *Pseudomonas aeruginosa* PA01 cultivated in Casamino acid minimal salt medium supplemented with root exudates from sugar beet varieties var [31] which showed that genes with known function were up regulated and encoded for proteins belonging to the categories *amino acid metabolism and biosynthesis*, *compound catabolism* and *energy generation*. In that study, this finding was explained by the fact that the major elements of root exudates are amino acids, organic acids and monosaccharides [31]. All of which are building blocks for the energy and biomass production of rhizosphere microbes [23].

Furthermore, a study by Ben showed that 302 genes (8.2% of the transcriptome) of *Bacillus amyloliquefaciens* FZB42 were differentially expressed in the presence of maize root exudates of which 261 significant genes were up regulated and 41 genes were down regulated [18]. This genes encoded proteins which were involved in three main functions, namely *non-ribosomal synthesis of secondary metabolites*, *bacterial chemotaxis and motility* and *nutrient utilization* [18]. In particular, nutrient utilization seems to be relevant since it aligns with the findings of this study and the study from Mark et al.

Influence of medium composition

The comparison between the cultivation conditions Casamino acid minimal salt medium (CAN) and LB complex liquid medium (LB) exhibited a significant amount of transcriptomic differences among each other which revealed that 921 (15% of the transcriptome) significant genes of *S. rhizophila* P69 were differentially expressed. 421 significant genes were significantly up regulated after the growth in LB in comparison to CAN. 344 out of the 921 differential expressed genes were assigned to 20 different Clusters of Orthologous Groups (COG) categories. A high number of significant genes encode for proteins belonging to the categories *transport and metabolism*, mostly of *inorganic ions* and *amino acids*.

This is one of highest amount of differently expressed genes in any comparison (except Casamino acid minimal salt medium vs. LB complex liquid medium, which had a similar amount of changes in gene expression). This is a significant amount of changes which would indicate that there is a large amount of adaptation that *S. rhizophila* P69 goes through when changing environment between complex and minimal medium.

There are differentially expressed genes encoding for proteins belonging to one COG function in this comparison, which can not be found in the other comparisons. This COG category is *Intracellular trafficking, secretion, and vesicular transport* which is overall up regulated after growth in LB complex liquid medium. Furthermore, this COG function was the most affected category (with 22.22%) in this comparison.

A study by Blair et al. compared the transcriptome of *Salmonella enterica* after growth in MOPS minimal medium and LB complex liquid medium, in order to gain knowledge about the impact of a culture medium on the bacterial gene expression. Blair and colleagues found out that a total of 621 genes were differentially expressed. However, 287 genes were up regulated and 334 down regulated after the growth in MOPS minimal medium in comparison to LB complex liquid medium [13]. 42 out of the 287 up regulated genes were involved in amino acid biosynthesis and metabolism, which was assumed to be due to the reduced availability of amino acids in the minimal medium [13].

A study by Kim et al. compared the transcriptome of *Escherichia coli* BL21 after growth in LB complex liquid medium and minimal modified R medium. The study that the amount of differentially expressed genes in minimal modified R medium was significant higher than in LB complex liquid medium. As expected, a high number of the differentially expressed genes were involved in *transport and metabolism* of *amino acids and nucleotides* as well as *ribosomal biosynthesis* [27]. Similarly, pairwise comparison of LB complex liquid medium and minimal modified R medium revealed that gene expression involved in *amino acid biosynthesis* is increased after growth in minimal medium.

Taking all of the findings into account, it seems to be significant whether the bacteria are cultured in minimal or complex media. In particular, *amino*

acid transport and metabolism seems to be relevant since it aligns with the findings of this study, a study by Blair et al and a study by Kim et al.

Differences in lifestyle on liquid or solid complex medium

In this study, the lifestyle of *S. rhizophila* P69 on liquid and solid complex medium was investigated. The lifestyle of bacteria can be either planktonic or sessile. The lifestyle in nature depends on environmental factors and bacterial characteristics but a sessile lifestyle thus biofilm formation seems to be more relevant in nature [7]. The transcriptomic analysis of the pairwise comparison of LB complex liquid medium (LB) and LB complex agar medium (LBP) revealed significant differences between them. The comparison showed that 919 (15 % of the transcriptome) genes of *S. rhizophila* P69 were differentially expressed. Down regulated genes (513 significant genes) outnumbered highly expressed genes (406 up regulated genes) after growth of *S. rhizophila* P69 on LBP in comparisons to LB. 377 out of 919 genes were assigned to 19 different COG categories including those responsible for *metabolism and transport of inorganic ions, carbohydrates, amino acids and secondary metabolites* as well as *energy production, transcription* and *cell wall/membrane/envelope biogenesis*. This is the highest amount of differently expressed genes in any comparison (except CAM versus CAN, which had a similar amount of changes in gene expression). This is a significant amount of changes which would indicate that there is a large amount of requirements and adaptation that *S. rhizophila* P69 goes through when changing environments between liquid and solid medium.

In addition, a study by Alavi et al found out that *S. rhizophila* P69 (Synonym: DSM14405^{T}[1]) possesses two gene blocks encoding for flagellar apparatus, whereas the first gene block includes 22 genes and the second gene block includes 26 genes. Cell motility through flagella and fimbriae is important for biofilm formation [5]. A down-regulation of flagellar-driven motility would indicate a change in lifestyle from planktonic to sessile [4].

[1] Leibniz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH

In LB liquid medium the lifestyle of bacteria would be expected to become more planktonic, which would mean that genes for flagellar-driven motility would be up regulated. The current data support this expectation and confirmed that genes responsible for *flagellar assembly* and *twitching motility* were up regulated after growth in LB complex liquid medium. In contrast, there were detectable phenotypic differences after the growth on LB complex agar medium which was noticeable by a decreased expression of genes involved bacterial chemotaxis, especially genes for flagellar assembly (*cheW*) and twitching motility (*chpA*, *pilG* and *pilH*) seemed to be strongly suppressed. This findings indicate that the lifestyle of *S. rhizophila* P69 would be expected to become sessile.

The L-histidine metabolism

In the current study, the L-histidine metabolic pathway was strongly affected in all three comparisons. L-histidine is a member of the 20 proteinogenic amino acids and can be found as a building block of proteins in all living organisms [28]. It is a precursor for many other metabolites (e.g. histamine, carnosin, glutamate, ergothioneine *et cetera*).

In the first comparison (CAM versus CAN) the conversion from PRPP to L-histidine was significantly up regulated. The genes *hutH* and *hutI* responsible for L-histidine degradation were significantly down regulated in Casamino acid minimal salt medium (CAN) compared to Casamino acid containing maize root exudates (CAM) thus the L-glutamate and ammonia production seems to not have been produced. Furthermore, this result may indicate that L-histidine was accumulated in the cell in high amounts.

The comparison of LB complex liquid medium versus LB complex agar medium revealed that genes encoding for proteins involved in L-histidine and L-glutamate biosynthesis were strongly up regulated. L-glutamate is a non essential amino acid and plays an important role in many biological processes (gluconeognesis, citric acid cycle, glycolysis as well as in protein synthesis) [19]. The third comparison (Casamino acid minimal salt medium and LB complex liquid medium) indicated that production of L-histidine from PRPP was strongly down regulated and L-histidine seems to not have been produced.

5. Conclusion

This instance of total bacterial RNA isolation from *S. rhizophila* P69, *S. rhizophila* ep17 and *S. maltophilia* ep3 was very successful, but the process of isolation and purification has been steadily improved, as well. There was one major change in regards to the process of cell lysis. The variant of mechanical disruption with homogenizer tubes and enzymatic cell lysis with lysozyme and Proteinase K has proven to be the most promising approach. It was possible to increase quantity as well as quality of RNA samples, except for the 260/230 ratio, which indicated the presence of contaminants which absorb at 230 nm [1]. The reasons for this are unknown at the current time, but further research can shine a light on the reasons for this situation.

Due to increasing interest for biotechnological applications, a better understanding of the genetic mechanisms is demanded. This thesis sought to explore the transcriptome of *S. rhizophila* P69 exposed to different cultivation conditions in order to study and identify differential gene expression patterns. Overall, the cultivation condition for *S. rhizophila* P69, especially the choice of the culture media, had a relevant impact on gene expression.

The comparison Casamino acid minimal salt medium (CAN) versus LB complex liquid medium (LB) exhibited the highest amount of differentially expressed genes (15% of the transcriptome) in any comparison (except LB complex liquid medium (LB) vs. LB complex agar medium (LBP), which had a similar amount of changes in gene expression, also 15%). This level of changes would indicate that there is a large amount of adaptation that *S. rhizophila* P69 goes through when changing environment between minimal and complex medium. In addition, up regulated genes were predominantly involved in *amino acid biosynthesis and metabolism* after growth in minimal medium and can be explained due to the reduced availability of amino acids.

Furthermore, the analysis revealed that the environmental change from LB complex liquid medium to LB complex agar medium caused *S. rhizophila* P69 to alter its lifestyle from planktonic to a sessile one after the growth on LBP.

The influence of root exudates (Casamino acid containing maize root exudates (CAM) versus Casamino acid minimal salt medium (CAN)) showed that 0.15% of the transcriptome were differentially expressed. The interaction between *S. rhizophila* P69 and its host plant showed that up regulated genes were predominantly classified in the area of *transport and metabolism*, mostly of *inorganic ion*, *carbohydrates* and *amino acids* which would indicate that maize root exudates act as an energy source for the rhizobacteria. Overall, the interaction between *S. rhizophila* and the host plant indicated that the impact of maize root exudates on *S. rhizophila* is inherently narrower in spectrum, more specialised and the adaptation requirements are lower than in any of the other comparisons.

Appendix A.

Tables and listings

```
# index building
hisat2-build S_rhizo_P69.fasta S_rhizo_P69

# read alignments
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S CAM1.sam \
-U CAM_1_RNA_1_1.fastq.gz,CAM_2_RNA_1_1.fastq.gz
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S CAN1.sam \
-U CAN_1_RNA_1_1.fastq.gz,CAN_2_RNA_1_1.fastq.gz
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S LBP1.sam \
-U LBP_1_3_1.fastq.gz,LBP_1_2_1.fastq.gz,LBP_1_2_2.fastq.gz
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S LBP2.sam \
-U LBP_2_5_1.fastq.gz
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S LB_1.sam \
-U LB_1_3_1.fastq.gz
hisat2 -x S_rhizo_P69_index -p 10 --dta -q -S LB_2.sam \
-U LB_2_3_1.fastq.gz,LB_2_2_1.fastq.gz,LB_2_2_2.fastq.gz

# samtools sort and bam conversion in one
samtools sort -@ 10 CAM1.sam -o CAM1.sorted.bam
samtools sort -@ 10 CAN1.sam -o CAN1.sorted.bam
samtools sort -@ 10 LBP1.sam -o LBP1.sorted.bam
samtools sort -@ 10 LBP2.sam -o LBP2.sorted.bam
samtools sort -@ 10 LB_1.sam -o LB_1.sorted.bam
samtools sort -@ 10 LB_2.sam -o LB_2.sorted.bam

# stringtie
stringtie CAM1.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o CAM1.gtf
stringtie CAN1.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o CAN1.gtf
stringtie LBP1.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o LBP1.gtf
stringtie LBP2.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o LBP2.gtf
stringtie LB_1.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o LB_1.gtf
stringtie LB_2.sorted.bam -p 10 \
-G S_rhizo_P69_gff_neu.gff -m 50 -o LB_2.gtf

find . -type f -name '*.gtf' > assembly_GTF_list.txt

stringtie --merge -p 10 -G S_rhizo_P69_gff_neu.gff \
-o merged.gtf assembly_GTF_list.txt

```

```
45 cuffdiff -b S_rhizo_P69_short.fasta -p 10 \
46 -L CAM,CAN,LBP,LB merged.gtf \
47 -o cuff_diff_results \
48 CAM1.sorted.bam \
49 CAN1.sorted.bam \
50 LBP1.sorted.bam,LBP2.sorted.bam \
51 LB_1.sorted.bam,LB_2.sorted.bam
```

Listing A.1: Command line for the differential expression analysis according to instructions based on Trapnell et al. [50]

```
1 library(cummeRbund)
2 cuff<-readCufflinks(rebuild=T)
3 myDistHeat<-csDistHeat(genes(cuff))
4 disp<-dispersionPlot(genes(cuff))
5 s<-csScatterMatrix(genes(cuff))
6 v<-csVolcanoMatrix(genes(cuff))
```

Listing A.2: Command line used for visualization of the differential gene expression date according to instructions based on Trapnell et al. [50]

Table A.1.: Results of the differential expression analysis of *S. rhizophila* P69 cultivated either in Casamino acid containing maize root exudates or in Casamino acid minimal salt medium . In this table significant genes, their protein product and the COG function of proteins are listed.

Gene ID	Protein name	Gene name	log_2	COG function
BASYS00603	Hypothetical B_A	BASYS00603	1.049	N.A.
BASYS01546	Aconitate hydratase [H]	*acnA*	1.154	C
BASYS01548	Hypothetical	BASYS01548	1.115	N.A.
BASYS01997	Acetylglutamate kinase [H]	*argB*	1.031	E
BASYS01998	GCN5-Related N-Acetyltransferase	BASYS01998	1.028	J
BASYS01999	N-acetyl-gamma-glutamyl-phosphate reductase [H]	*argC*	1.077	E
BASYS02274	Urocanate hydratase [H]	*hutU*	-2.283	E
BASYS02275	N-Formylglutamate Amidohydrolase	BASYS02275	-2.012	E
BASYS02276	Histidine ammonia-lyase	*hutH*	-2.107	E
BASYS02278	Imidazolonepropionase	*hutI*	-1.619	Q
BASYS02717	Uncharacterized MFS-type transporter YPO1221/y2967/YP_0917	ybjJ	-3.763	G
BASYS02718	Hypothetical Protein BASYS02718	BASYS02718	-4.178	N.A.
BASYS02719	Glycerol-3-phosphate regulon repressor	glpR	-4.645	K
BASYS02745	Thioredoxin Domain-Containing Protein	BASYS02745	1.041	N.A.
BASYS02882	Hypothetical Protein BASYS02882	BASYS02882	-2.992	N.A.
BASYS02883	Hypothetical Protein BASYS02883	BASYS02883	-2.943	N.A.
BASYS02884	Sulfite reductase [NADPH] flavoprotein alpha-component	*cysJ*	-1.624	P
BASYS03154	Hypothetical Protein BASYS03154	BASYS03154	1.224	N.A.
BASYS03156	Imidazole glycerol phosphate synthase subunit hisF	*hisF*	3.743	E
BASYS03157	Conserved Hypothetical Protein	BASYS03157	4.044	N.A.
BASYS03158	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	*hisA*	4.203	E
BASYS03159	Imidazole glycerol phosphate synthase subunit hisH	*hisH*	3.887	E
BASYS03160	Histidine biosynthesis bifunctional protein hisB	*hisB*	4.226	E
BASYS03161	Histidinol-phosphate aminotransferase	*hisC*	4.079	E
BASYS03162	Histidinol dehydrogenase	*hisD*	4.164	E
BASYS03163	ATP phosphoribosyltransferase	*hisG*	3.815	E
BASYS03164	Hypothetical Protein BASYS03164	BASYS03164	3.307	N.A.
BASYS03830	Asparagine synthetase B [glutamine-hydrolyzing]	*asnB*	1.290	E
BASYS03921	Hypothetical Protein SmaI	BASYS03921	-1.212	N.A.
BASYS04466	Vitamin B12 transporter BtuB	*btuB*	1.453	P
BASYS05176	Bifunctional protein putA	*putA*	-1.495	C
BASYS05333	Hypothetical Protein BASYS05333	BASYS05333	1.369	N.A.
BASYS05335	3-hydroxypropionyl-coenzyme A dehydratase	*paaF*	1.350	I
BASYS05336	Hypothetical Protein BASYS05336	BASYS05336	1.436	N.A.

[1] Fold Change

List of Terms

abiotic abiotic factors are non-living factors of the environment (e.g. water, temperature, light, radiation and many more) that affect living organsisms. 1, 49

agar-agar a linear polymer used for the stabilization of liquids and frequently used in microbiology. 9

***B. amyloliquefaciens* FZB42** *Bacillus amyloliquefaciens* FZB42. 51

biotic biotic factors are the living factors of the environment (e.g. animals, microorganisms, plants). 1, 49

°C degree centigrade. 5, 7–12, 14

CAA Casamino acid minimal salt medium. ix, 9, 10, 19, 20, 31

CAM Casamino acid containing maize root exudates. v, x, xi, 6, 15, 16, 18–38, 49, 50, 53, 54, 57, 61

CAN Casamino acid minimal salt medium. v, x–xii, 5, 6, 10, 15, 16, 18–38, 44–57, 61

CAT Casamino acid containing tomato root exudates. 6, 18–25, 49, 50

cDNA complementary DNA. 26, 27

COG Clusters of Orthologous Groups. xii, 33–35, 37, 39, 41, 42, 44, 46, 47, 50, 52, 53, 61

colony-forming unit colony-forming unit is used to estimate the number of viable cells in a sample. 6, 10, 11, 18

Cuffdiff a software tool that calculates the abundance of transcripts FPKM in all samples. 15–17, 26, 29

CummeRbund freely accessible software tool for high-throughput RNA sequencing data exploration and visualization. 16, 17, 26, 33, 34, 39, 44

DEPC Diethyl pyrocarbonate. 11, 12, 14

dH_2O distilled water. 7, 9, 11, 12

dsDNA double stranded DNA. 15

E. coli BL21 _Escherichia coli_ BL21. 52
EDTA Ethylenediaminetetraacetic acid. 14
e.g. exempli gratia. 1, 15, 50, 54
ethidium bromide commonly used in molecular biology as fluorescent tag for staining nucleic acids that fluoresce when exposed to ultraviolet light. 14
exudate roots exudates consist of organic compounds (e.g. amino acids, sugars, carbohydrates and many more) which are secreted by the roots of plants. v, ix, 4, 5, 7–10, 18–20, 50, 51, 57

FA Formaldehyde. 14
FPKM fragments per kilobase million. 16, 26, 28, 36, 40, 45, 62

GG glucosylglycerol. 2, 3

h hour. 8, 11, 12, 14
H_2O chemical formula for water. 7, 9, 11
Hisat2 a ultrafast software tool which aligns short sequencing reads to reference sequences. 15

JS Distance Jensen-Shannon Distance. 27

K_2HPO_4 dipotassium phosphate is a salt which is highly water-soluble. 9
KEGG Kyoto Encyclopedia of Genes and Genomes. 16, 35, 38–40, 42, 43, 45, 48

μl microlitre. 24, 25
LB LB complex liquid medium. v, ix–xii, 4–6, 9–11, 15, 16, 18–34, 39–57
LBP LB complex agar medium. v, x–xii, 4–6, 9, 15, 16, 18, 23–34, 39–43, 49, 50, 53, 54, 56, 57
L-histidine L-histidine is a proteinogenic amino acid. 54, 55
$\log_2$ binary logarithm or dual logarithm. 26, 30, 35, 37, 39, 44, 61
lysozyme N-acetylmuramide glycanhydrolase, an enzyme that catalyses hydrolytic cleavages and is found in plants, bacteria, fungi and bacteriophages. 12, 23, 25, 56

$MgSO_4$ magnesium sulfate is an inorganic salt. 9
ml millilitre. 7–13, 19
MOPS 3-(N-Morpholino)propanesulfonic acid. 14, 52

mRNA messenger RNA. 15

NaCl sodium chloride. 11
NaClO sodium hypochloride. 7
NaOAc sodium acetate. 14
nosocomial infection occurring as part of a hospital stay. 3

OD_{600} optical density of a sample measured at a wavelength of 600 nm. xi, 10, 18–20
ONC overnight culture. 9, 10

***P. aeruginosa* PA01** *Pseudomonas aeruginosa* PA01. 51
PCA Principal Component Analysis. 26, 31
Proteinase K Proteinase K is a serine protease. 23, 25
PRPP Phosphoribosylpyrophosphat. 35, 40, 45, 54, 55

RLT lysis buffer prior to RNA isolation. 12
RNA ribonucleic acid. v, vii, 11, 13–15, 25, 26, 49, 64
RNA-Seq transcriptome shotgun sequencing. x, 15, 16
RPE washing buffer that removes remaining salts. 13
rpm revolutions per minute. 9, 10, 12, 13
rRNA ribosomal RNA. 15

S. enterica *Salmonella enterica*. 52
S. maltophilia *Stenotrophomonas maltophilia*. v, vii, ix, xi, 2–6, 9, 10, 18, 49, 64
***S. maltophilia* ep3** *Stenotrophomonas maltophilia* ep3. v, vii, x, xi, 4, 5, 15, 18, 19, 21–25, 49, 56
S. rhizophila *Stenotrophomonas rhizophila*. v, vii, ix, x, xii, 2, 3, 5, 6, 9, 10, 18, 23, 49, 57, 64
***S. rhizophila* ep17** *Stenotrophomonas rhizophila* ep17. v, vii, xi, 3–5, 15, 18–22, 24, 25, 49, 56
***S. rhizophila* P69** *Stenotrophomonas rhizophila* P69. v–viii, x–xii, 3–5, 15, 16, 18–22, 24–34, 36–54, 56, 57, 61
SDS sodium dodecyl sulfate is an organic compound and detergent which is commonly used in hygiene and cleaning products. 12, 14
spp. species pluralis. 1, 2
Stenotrophomonas a gram-negative bacteria predominantly found in plants and soil. v, vii, ix, xi, xii, 1–6, 10, 11, 18, 19, 49, 50, 64

StringTie a software tool that assembles RNA-Sequence alignment into potential transcripts. 16, 17

TE commonly used buffer that consist of Tris and EDTA. 12

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