



# Host-pathogen interaction using *Dictyostelium* *discoideum* as host model

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**Ida Lindström**

Uppsala 2011

Degree project 15 hp

Department of Molecular Biology

SLU, Sveriges Lantbruksuniversitet  
Department of Molecular Biology

**Author:**

Ida Lindström

**Title:**

*Dictyostelium discoideum* som modellorganism för bakteriella infektioner

**Keywords:** *Dictyostelium discoideum*, *Salmonella typhimurium*, non-coding RNAs, infection.

**Supervisor:**

Fredrik Söderbom, Department of molecular Biology.

**Examiner:**

Jerry ståhlberg, Department of molecular biology

**Course description:**

Independent project in biology C, 15 hp, EX0689.  
Självständigt arbete i biologi C, 15 hp.

**Publication:**

Uppsala, Juni 2011

## **Abstract**

*Dictyostelium discoideum* has long been used as a model organism to study many different biological processes. The amoeba is very similar to macrophages present in higher animals, for example humans. Furthermore, several genes in *D. discoideum* have shown to be homologous with human genes. Due to these facts, *D. discoideum* have also been used to study host-pathogen interactions with many different human pathogens. In this project, the aim was to survey the literature and find conditions where the pathogen *Salmonella typhimurium* can infect *D. discoideum* and further try to explain how this host-pathogen system can be used to study how non-coding RNAs can influence the infectious process.

*S. typhimurium* is a pathogen that is known to infect humans and other animals. When infection occurs it causes gastroenteritis that gives diarrhea and vomiting. The pathogen causes infection by entering host cells, such as macrophages or epithelial cell. Inside the host cells the pathogen can find ways to replicate and persist. ncRNAs are important molecules that regulate many different processes in the cell by either induce degradation of mRNA or inhibit translation. Two of the most important groups of ncRNAs are microRNAs and small interfering RNAs. These are the two groups that would be studied in order to try to elucidate their role and function during the infectious process.

How the project would be set up will be described in this literature project. The first thing that would be done is to try to find conditions where *S. typhimurium* can infect *D. discoideum*; to do this a microtitre plate assay would be used to try to find the right conditions. This allows screening of a large number of different conditions simultaneously. Total RNA would then be extracted from both non-infected and infected *D. discoideum* cells. Both small RNAs and mRNA would be analyzed using high throughput sequencing methods. The results would then be used to create mutants where the identified ncRNAs are knocked out or over-expressed in order to elucidate their function. ncRNA target genes would also be mutated, in order to understand their function. Which role ncRNAs have in *S. typhimurium* during the infectious process would also be analyzed. By studying these factors, the biology behind the infection of *S. typhimurium* could be identified.

**Keywords:** *Dictyostelium discoideum*, *Salmonella typhimurium*, non-coding RNAs, infection.



## Table of Contents

Abstract .....	3
Introduction .....	7
<i>Dictyostelium discoideum</i> .....	7
Life cycle .....	8
A model organism.....	9
Host for bacterial infections.....	9
<i>Salmonella typhimurium</i> .....	10
Infection in mammalian cells.....	11
Infection in <i>Dictyostelium</i> .....	11
Non-coding RNA.....	12
Non-coding RNA in bacteria .....	14
Regulation of non-coding RNAs as response to infection.....	16
Project description.....	16
Infection.....	16
Strains and growth conditions.....	16
<i>Salmonella</i> infection .....	18
Isolation of infected cells and RNA extraction .....	18
Identification of ncRNAs and mRNA .....	21
Functions of ncRNAs and their target genes .....	23
Discussion .....	24
References .....	28



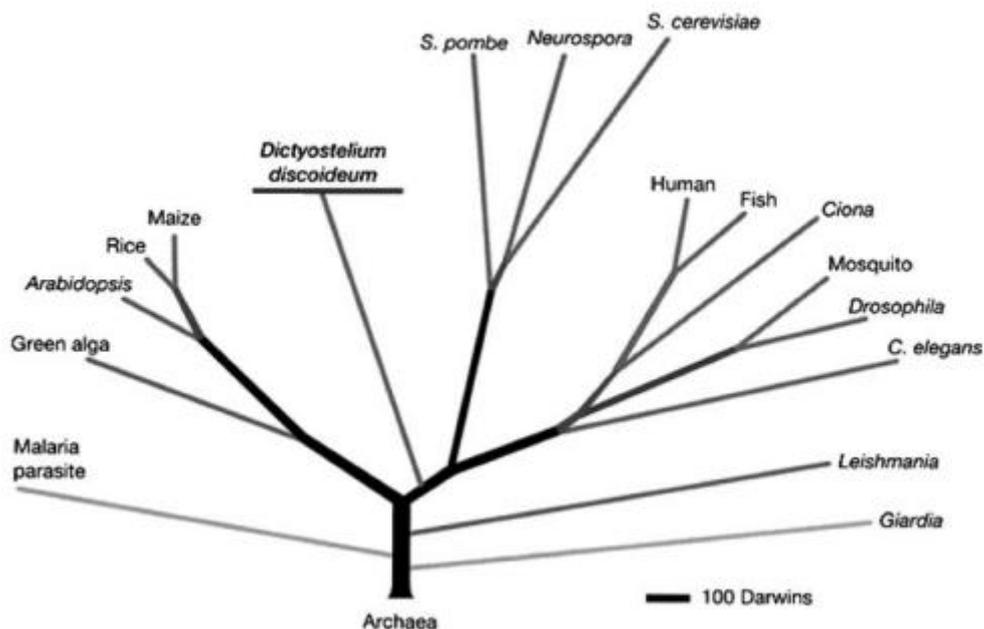
## Introduction

In this literature project the aim is to find conditions where *Salmonella typhimurium* can infect *Dictyostelium discoideum*. This will further be used to study how non-coding RNAs (ncRNAs) influence the infectious process, both in the host and bacteria. Hopefully, this can help us to understand the biology behind the infectious process.

### *Dictyostelium discoideum*

*Dictyostelium discoideum* or the social amoebae as it often is called was discovered 1935 and since then it has been a widely used model organism [1, 2]. In 2005 the complete genome was sequenced [3]. The amoeba has an A+T rich genome (~78%) of the size 34 Mbp and in total the genome consists of six chromosomes with around 12,500 genes [4, 5].

Through molecular phylogeny studies it was shown that *D. discoideum* branched off after plants and before fungi (figure 1). Though, with the aid of bioinformatics tools, it has been shown that *D. discoideum* have more homologous genes with humans than for example *S. cerevisiae* [5].



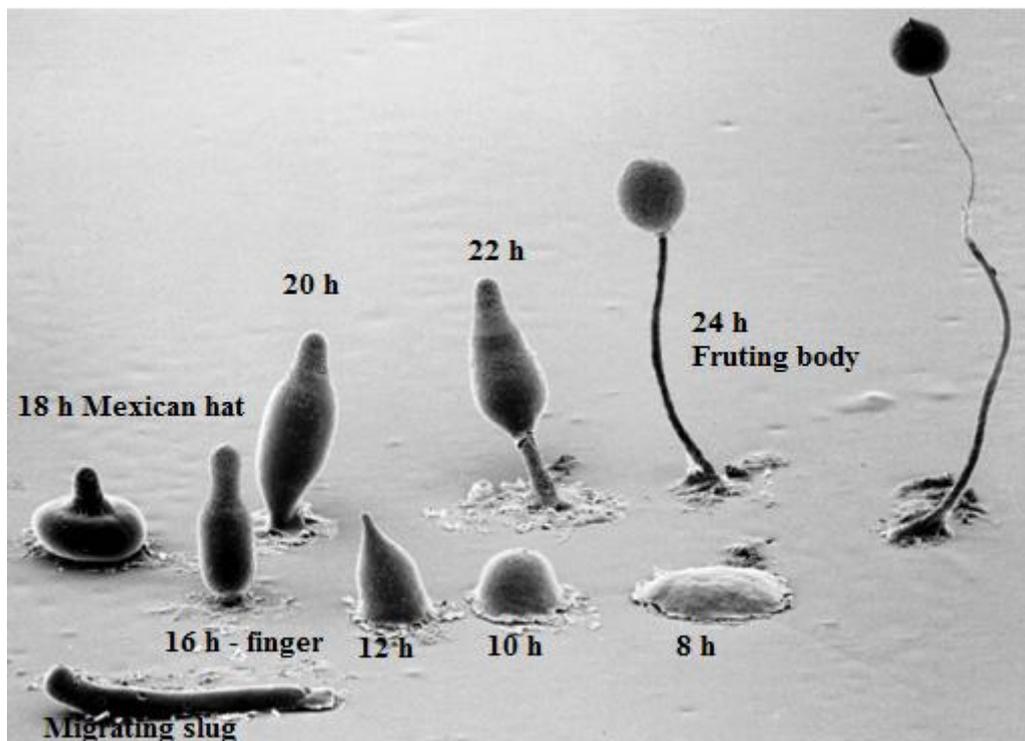
**Figure 1:** *Dictyostelium discoideum*s position in a eukaryotic phylogenetic tree. [3]

It has also been shown that several genes related to different diseases in humans also are present in *D. discoideum* [5]. This together with *D. discoideum* fascinating life cycle (see

figure 2 below) and the many genetic tools available makes it a perfect model organism for many different purposes.

### Life cycle

The amoeba can be found in forest soil where it hunts different bacteria chemotactically and engulf them by phagocytosis. The amoebae have a very unusual life cycle that both contain a unicellular and a multicellular phase [1]. During the unicellular stage *D. discoideum* feeds on nearby bacteria and grow by mitotic division. As long as the amoebae can find bacteria it will stay in this unicellular phase, but when the amoebae cannot find bacterial pray anymore, single cells stops growing and genes involved in growth and division are turned off. At the same time, starvation induces a new set of genes. These new genes are important for e.g. cAMP chemotaxis and cells with these induced genes will be able to release and detect cAMP. This will eventually result in that single cells will start to aggregate and go into the multicellular developmental phase [2, 6]. This phase is shown in figure 2.



**Figure 2.** The developmental phase in *Dictyostelium discoideum* (M.J. Grimson & R.L. Blanton).

The aggregated mass will create a tip that will elongate into a structure called finger, due to its finger-like structure. Depending on where the finger is located it can now take two pathways. Either the finger can fall to its side and develop into a slug that can respond to light and temperature and are able to migrate into better conditions. But if the finger structure already is

in favorable conditions, the structure will directly continue to develop into the fruiting body. This structure consists of two types of differentiated cells, stalk and spore cells, these will determine their fate in the final fruiting body structure. The stalk cells will undergo programmed cell death while the spore cells will be able to germinate again if they are put in better conditions and the life cycle will begin all over again [1, 6]. This entire developmental stage occurs during a time frame of 24 h and can easily be induced in lab environments. The spores will fully go back to the unicellular growth phase as soon food e.g. bacteria is available again [7].

### **A model organism**

*Dictyostelium discoideum* has for many years been used as a model organism for many different areas. It has for example been used to study chemotaxis, cell motility, signaling transduction and phagocytosis [2, 7]. There are several different things that make the amoeba a good model system; first of all it is haploid making it easy to study mutants since the phenotype directly becomes visible [4]. There are also several molecular techniques available for studying *D. discoideum*, it is possible to perform homologous recombination to knock out genes [8], do multiple gene deletions [9], perform random insertional mutagenesis called REMI – restriction-enzyme-mediated integration [10] and to use RNAi technologies to silence genes [11, 4]. Furthermore, the amoeba can easily be grown in large amounts, it can be grown on agar plates with bacteria present (generation time ~ 3h), but it can also be grown in nutrient broth without bacteria (generation time ~ 9h) [1]. Strains can also be frozen away and stored, which enables work with different genotypes over a long time [4].

Due to the many molecular tools available and the fact that they are similar to macrophages in many ways, *D. discoideum* have also become a model organism to study bacterial infections that can cause several diseases in animals, for example human [7].

### **Host for bacterial infections**

The first study where *D. discoideum* served as the host for a pathogen was with the bacteria *Legionella pneumophila* [12]. Thereafter, it rapidly became evident that *D. discoideum* could be suitable as host for several other pathogens as well. Today a large number of studies have been carried out using this system, where several different pathogens have been studied using *D. discoideum* [13]; these can be seen in table 1.

**Table 1:** Bacteria that are known to infect *D. discoideum*

<b>Bacteria</b>	<b>References</b>
Cryptococcus neoformans	Steinert et al. 2006 [14]
Yersinia pseudotuberculosis	Vlahou et al. 2009 [15]
Mycobacterium avium	Salah et al. 2010 [16]
Neisseria meningitidis	Clarke.2009 [13]
Salmonella typhimurium	Jia et al. 2009 [17]
Burkholderia cenocepacia	Clarke.2009 [13]
Pseudomonas aeruginosa	Clarke.2009 [13]
Mycobacterium marinum	Solomon et al. 2003 [18]
Legionella pneumophila	Steinert et al. 2008 [19]
Mycobacterium tuberculosis	Clarke.2009 [13]
Vibrio cholerae	Pukatzki et al. 2010 [20]
Klebsiella pneumoniae	Steinert. 2010 [7]

Several features make the amoeba an interesting model organism for studying bacterial infections. As mentioned earlier *D. discoideum* hunts and engulf bacteria. These processes are in many ways similar to how macrophages in the human body destroy invading bacteria. During evolution pathogenic bacteria have found ways to avoid being killed by the amoeba [7]. For example, some bacteria have evolved ways of reside inside the amoebae and use it for their benefit [13]. Since the bacteria have evolved mechanisms to resist predators in nature, the bacteria can also use these strategies on several phagocytic cells that are present in higher animal's innate immune system and hence find ways to become opportunistic human pathogens [7].

### **Salmonella typhimurium**

The genus *Salmonella* consist of enteric bacteria that can infect both humans and other animals and are a common cause of many different infectious diseases around the world. They are Gram-negative and classified in the family Enterobacteriaceae. The species *Salmonella enteric* is known to infect humans and are divided into six subspecies and they are in turn divided into serovars. In group 1 of the different subspecies we find the serovar *Salmonella typhimurium* [21]. This pathogen causes gastroenteritis in humans, which gives abdominal pain, vomiting and diarrhea [23, 22]. Normally, the treatment for Salmonella-infections is just fluid replacement. It is recommended not to give antibiotic treatments since this can prolong the infection and also increase risk for antibiotic resistance. But in some cases antibiotic can be needed, for example for old or very young people, or if you already have a serious disease,

for example HIV. Today, there is no vaccine for salmonella, but good hygiene and good treatment of food decreases the risk of infection [25, 24].

### **Infection in mammalian cells**

A *Salmonella* infection is normally acquired from the environment, for example through intake of water or food that is contaminated. After ingestion some of the bacteria are able to survive the low pH in the stomach and after entering the intestine the bacteria cross the mucous layer and gain access to the epithelium. There the pathogen can enter several cell types, for example epithelial cells, microfold cells and different phagocytic cells, like macrophages [22, 21]. Once inside the host cells, it can disturb the normal formation of phagosomes by forming what is called Salmonella-containing vacuoles (SCV) [21, 17]. Using this compartment the bacteria creates a protected place where it can survive and replicate [27, 26]. Infection with this type of strain (*S. typhimurium*) are normally only restricted to the intestine where the bacteria will induce an inflammatory response, while other strains (e.g. *S. typhi*) can be spread to lymph nodes, liver and spleen [22]. To be able to infect different cell types, *Salmonella* relies on several virulence factors. In *S. typhimurium* these virulence factors are encoded by so called Salmonella pathogenicity islands (SPIs). Two examples of such are SPI-1 and SPI-2. These two SPIs encode a special system called type 3 secretion system (T3SS), which is a system that consists of several different proteins that are important for the pathogens ability to invade the host cell (SPI-1) and replicate and survive inside it (SPI-2) [28, 21].

### **Infection in Dictyostelium**

Some studies have been made where *D. discoideum* have been infected with *S. typhimurium*. During these studies it has been noticed that the pathogen is taken up by the amoeba but fails to replicate within the wild-type strain [28]. In several studies [7, 17] were knock-out mutants in autophagy (atg) genes were used (atg1, atg6 and atg7), it was noticed that intracellular replication increased. The autophagic pathway is a lysosomal degradation pathway that degrades bacterial pathogens. Then by using these mutant strains, the bacteria could, instead of being targeted by this lysosomal degradation pathway, use the Salmonella-Containing Vacuoles (SCV) and create a place where the pathogen could replicate [17, 7]. In mammals, *Salmonella* invade non-phagocytic cells and macrophages where they reside in SCVs. They are also able to avoid degradation of the lysosomal pathway. Due to the findings that the pathogen in *D. discoideum* are degraded only in wild-type but not in atg-mutants, it is thought that the autophagic machinery is used by *D. discoideum* to deliver *Salmonella* to the

lysosome. It is also believed that in mammalian cells *S. typhimurium* are able to in some way avoid the lysosomal pathway, since they are able to replicate intracellular. This could also further explain why atg-mutants of *D. discoideum* are mimicking pathogenesis of *Salmonella* in mammalian cells [17].

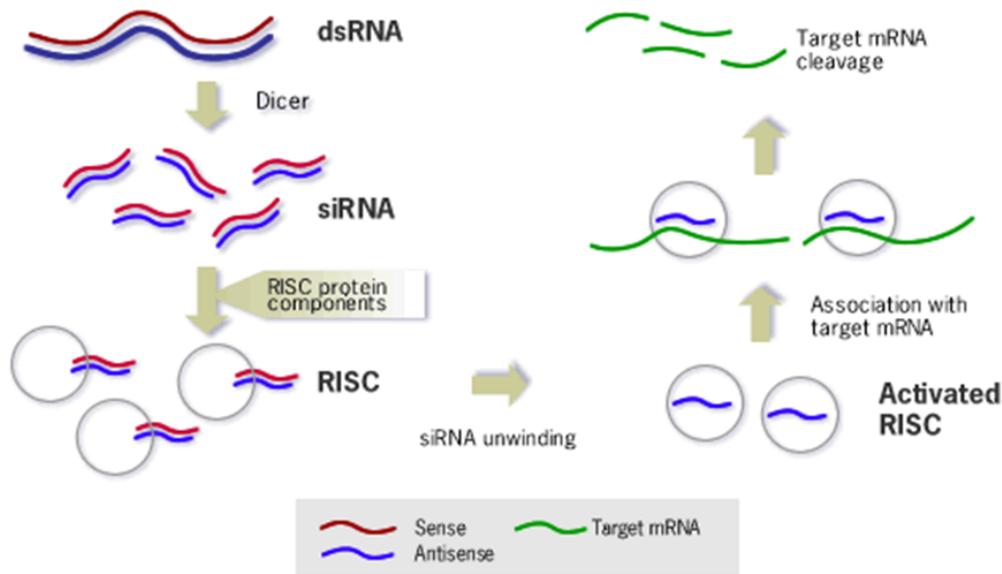
### **Non-coding RNA**

Small non-coding RNAs (ncRNAs) are very important molecules that regulate many different processes in the cell. These molecules are of the size 18-30 nucleotides (nt) and can be divided into several groups, depending on for example their origin, how they execute their functions and which proteins they require [30, 31]. Two of the most well studied groups are microRNAs (miRNAs) and small interfering RNAs (siRNAs). These small ncRNAs do not encode any proteins, as the name implies, but they do have important functions as RNAs. In the cell, these small RNAs can regulate many different functions, for example apoptosis, antiviral defense and development [30, 32].

siRNAs and miRNAs have been discovered in many different eukaryotes, where they target mRNAs with the aid of a protein complex called RISC (see below). This complex can, with the aid of ncRNAs, target both chromatin and mRNA transcripts. When targeting chromatin, the complex can modify it and this will in turn influence transcription. When instead the protein complex targets different mRNAs, they can either inhibit translation or degrade the mRNA totally [33, 32]. Some siRNAs are also known to be able to promote DNA methylation on target genes; this will also influence the transcription [31]. The result from all these mechanisms is that the target gene will have reduced protein expression or the expression will be completely turned off.

Concerning siRNAs, this type of small RNA is derived from double stranded RNA (dsRNA). These dsRNAs can originate from different endo- or exogenous sources, for example viral RNA or transposons [31]. When dsRNAs enters the cell, a process called RNA interference (RNAi) is induced, dsRNA will be recognized by an RNase III-type protein called Dicer. This ribonuclease cleaves the longer dsRNA into shorter 21 nt long dsRNAs, now called siRNAs [33]. These molecules will in turn be incorporated into another protein complex called RISC – RNA induced silencing complex. [34] This protein is built of several different proteins, one of them being the Argonaute proteins [35], which are the proteins that actually accomplish the regulation, for example degradation of target mRNA [31]. The double stranded siRNAs are

unwound and one of their strands is retained in RISC. This strand will then guide the complex to its target and once there the complex will exert its function [34].

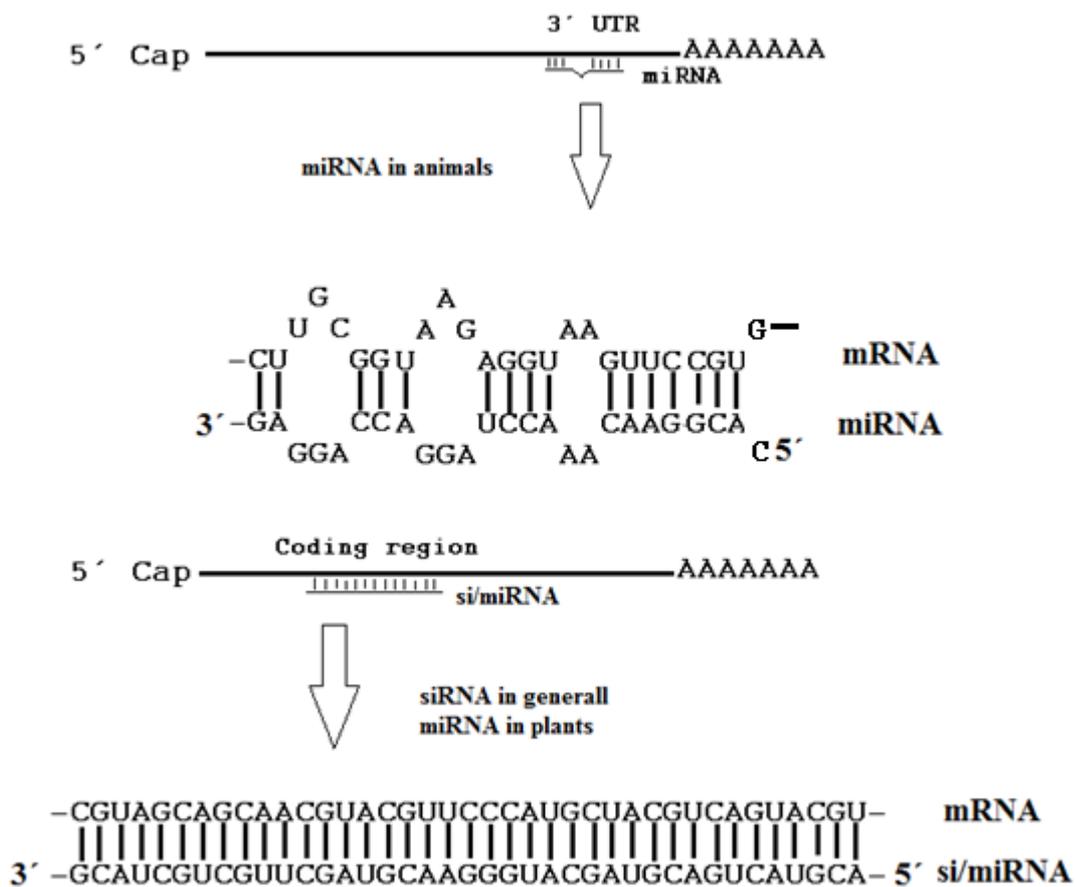


**Figure 3:** RNA interference mechanism [36].

miRNAs constitute a large family of RNAs that functions as post-transcriptional regulators [37]. These miRNAs follow the same pathway as siRNAs do (figure 3), but the steps before they are cleaved by Dicer are a bit different. miRNAs are transcribed from their own genes present in the genome, these transcripts are called primary miRNAs (pri-miRNAs). In animals these pri-miRNAs are then processed by a protein called Drosha, which cleaves the pri-miRNA into a hairpin structure now called pre-miRNA. In plants, it is instead the protein Dicer that processes the pri-miRNA in several steps. The now formed pre-miRNA is transported into the cytoplasm and then it follows the same pathway shown in figure 3 [37].

One more difference between siRNAs and miRNAs is that miRNAs can base-pair to its target with complete complementary, but can also bind with several mismatches [37, 31, 32]. This is shown in figure 4. In animals, the binding site to mRNA for miRNAs is normally located in the 3' UTR region. When the miRNA binds to the mRNA, the first 2-8 nucleotides of the miRNA binds with full complementary, but then several mismatches are allowed. When this occurs the mRNA will be degraded and/or translation will be inhibited [33]. In plants, the miRNAs will instead bind in the coding region of the mRNA and it will also bind with full complementary, as for siRNAs. This will result in that the mRNA will be cleaved by RISC

and degraded [34, 38]. It is today believed that at least 60 % of all protein coding genes in mammals are controlled by different miRNAs and it has also been shown that aberrant miRNAs expression can result in many different human diseases [37, 39].



**Figure 4:** siRNA and miRNA base-pairing with its target mRNA.

### Non-coding RNA in bacteria

Non-coding RNAs are not only present in eukaryotes; studies have shown that they also are present in prokaryotes. In nature, bacteria need to respond to changes in the environment in order to survive, for example when they are introduced to different stress conditions or when pathogenic bacteria enter its host. In the later case, a set of virulence factors need to be induced in order for the bacteria to survive inside the host. Previously, it was thought that this regulation only was performed by several different proteins. But recent studies have shown that non-coding RNAs are a very important regulator in these aspects as well, together with proteins [40].

In bacteria these non-coding RNAs are normally referred to as small RNAs (sRNAs). If they are compared to small eukaryotic ncRNAs, they consist of a very mixed group of RNAs that can vary a lot both in structure and size. In general they are between 50 to 250 nt long [41]. In bacteria small RNAs are divided into two classes, the most abundant class works by modifying the translation or stability of mRNA. The second class of small RNAs is instead known to modify the activity of proteins by binding to them; the RNAs exemplified from this class are for example known to affect the regulation of virulence factors of human pathogens [42].

Extensive studies have been performed on *Escherichia coli*, where over 70 small non-coding RNAs have been found. The majority of these have also been shown to be present in other pathogenic bacteria [40]. For example, in *E. coli* there is a system called CsrA/CsrB (carbon storage regulator) system that controls several functions in the bacteria. CsrA is a protein that binds to different mRNAs and represses their function by degrading them. CsrB is a non-coding RNA that will bind to CsrA and form a complex consisting of 18 CsrA molecules, which will antagonize the function of CsrA [43].

This CsrA/CsrB system has also been found in *S. typhimurium*. In this bacterium, CsrA is an important protein that can bind to several different mRNAs and regulate their translation. It is known that CsrA is important to control genes important for invasion into a host cell [28]. Therefore, it is also evident that CsrA needs to be tightly controlled, for example it has been shown that over-expression of CsrA gives a more attenuated invasion into the host cell. It is here where the small RNA CsrB comes in, this ncRNA is important to regulate CsrA and antagonize its function, making it possible for the bacteria to infect the host cell [28, 44].

There are several more small RNAs that have been found in *S. typhimurium*, today at least 70 are known. Many of them still have unknown functions, but many have also been solved. Those that have been studied have been shown to often regulate outer membrane proteins (OMPs). The outer membrane is very important for pathogenic bacteria, such as *S. typhimurium*. The OMP contains several different proteins, with diverse functions. For example, they can function as barrier for toxins, uptake of nutrients and also in pathogenic bacteria the OMPs are very important for interaction with the host cell and therefore they have direct roles in the pathogens virulence. At least 8 small RNAs have been found to regulate these OMPs in *S. typhimurium*; some examples are InvR, MicA and OmrAB [28, 45].

## **Regulation of non-coding RNAs as response to infection**

Pathogens can upon infection induce ncRNAs that enhance their virulence, but it has also been shown that for example plants can react to the pathogen and induce some ncRNAs as a defense mechanism [46].

One of the first studies made on this kind of response was performed in *Arabidopsis thaliana*. The plant was infected with a bacterial peptide from a flagellin, which is a protein that builds the flagellum. This flagellum will give the bacterium motility and in that way increase its virulence [47]. When the plant was infected with this peptide, it responded by inducing a special miRNA that gave the plant an increased resistance against infection with the bacterium *Pseudomonas syringae*. It was shown that the miRNA repressed auxin signaling in the plant, which is needed for the plants growth, and instead the plant prioritized defense against the bacteria [48, 49]. Later on it was also shown that when *Arabidopsis* was infected with *P. syringae*, a special class of siRNAs was induced. This class has been referred to as long siRNAs, since their size is around 30- 40 nt in length [46].

These examples demonstrate that plants uses ncRNAs as a defense mechanism against invading pathogens. This raise questions concerning other eukaryotic organisms as well, can for example *D. discoideum* use the same system and induce ncRNAs upon infection by different pathogens, for example *S. typhimurium*?

## **Project description**

### **Infection**

In order to see how the different ncRNAs and the amoebas gene expression are affected by the pathogen during infection, conditions were *S. typhimurium* can infect *D. dictyostelium* need to be found.

### **Strains and growth conditions**

First, both *S. typhimurium* and *D. discoideum* need to be cultured separately.

Concerning *S. typhimurium*, the strains that will be used will contain a plasmid that both express GFP and antibiotic resistance. The reason for this is that it is important to be able to follow the infectious process and with the aid of GFP one can follow it with a fluorescent microscope. The antibiotic resistance is important to be able to select *S. typhimurium* during the infectious process, meaning that other bacteria without antibiotic resistance will be killed

[29]. It will furthermore ensure that the GFP expressing plasmid is maintained in the bacterial population. In previous studies where *S. typhimurium* have been used for infection studies in *D. discoideum*, the bacteria have been grown either in LB-broth with applied shaking at 37°C or on LB-agar plates at the same temperature [17, 29].

For *D. discoideum*, mutant strains will be used. As mentioned before, it was noticed that in infectious studies with *S. typhimurium* and wild-type strains of *D. discoideum* the bacteria failed to replicate intracellular and was degraded by the lysosomal degradation pathway. Therefore, atg - mutant strains will be used to be able to create conditions where *S. typhimurium* actually can infect *D. discoideum* in the same way as it does in for example humans. There are several ways of growing *D. discoideum*, two standard ways are; i) Culture on agar plates using bacteria as food source and ii) Shaking in axenic media. Axenic growth means that you grow the amoeba in absence of any bacteria. In this case, one must have mutants of Dictyostelium, since wild-type strains cannot grow in those conditions. These mutants are called AX (short for axenic) strains and two examples of such strains are AX2 and AX3. When you grow such strains in axenic conditions the doubling time is around 8 - 12 h instead of the usual 4 h. An advantage is also that axenic strains grown on bacteria can be transferred to axenic culture [50, 51].

AX strains of *D. discoideum* will be used of, as mentioned above these can then both be grown on bacterial cultures or in axenic media. When grown in culture, *K. aerogenes* or *E. coli* will be used as food; these are the most common ones. These bacteria are grown in SM-medium [52] to prepare bacterial cultures. Spores of *D. discoideum* are then suspended with a small volume of bacteria and spread on SM-medium agar plates. When grown in axenic media, spores of *D. discoideum* are suspended in a media called HL5 [53] which is a liquid media that is commonly used for axenic growth. The suspension is incubated for a couple of days and then transferred in a new Erlenmeyer flask and left on a shaker. One important thing to remember when growing *D. discoideum* is to keep a temperature below 25°C, the optimal temperature for growth is around 22°C [51]. It is also important to remember that culturing *D. discoideum* they should remain in the exponential growth phase, this means that the cell density should be lower than  $4 \times 10^6$  cells/ml. The reason for this is that at higher cell density, the cells are triggered to go in to developmental stage. Normally you measure the cell density by using a hemocytometer [50, 51]. It is also recommended to after a few weeks make new cultures, since to long culturing can lead to undesired mutations [50, 51]. *D. discoideum* strains can be stored at -80 °C [50].

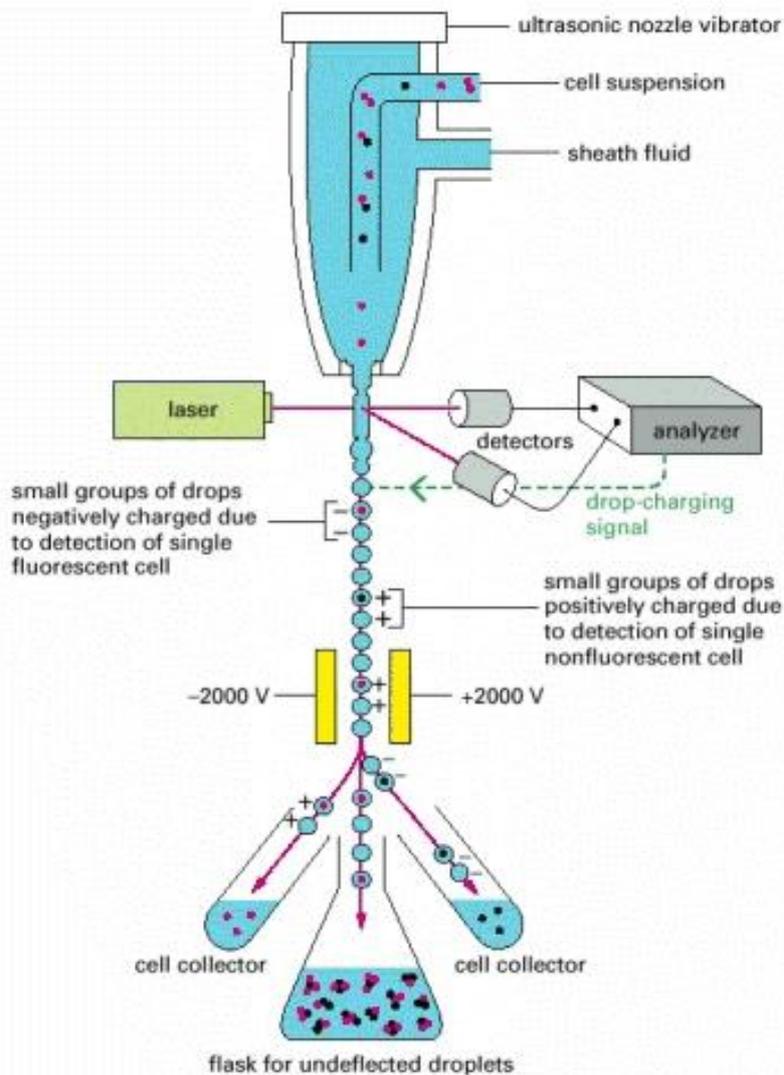
## **Salmonella infection**

When both *S. typhimurium* and *D. discoideum* have been cultured, the infection system can be set up. This will be done in a microtitre plate. Both wild-type *D. discoideum* and mutant strains (agt1<sup>-</sup>, atg6<sup>-</sup> and atg7<sup>-</sup>) of *D. discoideum* will be used and difference in infection will be analyzed. Furthermore, the microtitre wells will be used to test different conditions for infection in order find out which are most beneficial.

*D. discoideum* grown in HL5 media are harvested by centrifugation and resuspended in infection medium (1x1 HL5 medium and 1x Soerensen buffer (Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, dissolved in ddH<sub>2</sub>O and pH adjusted to 6.0 with KOH)). *D. discoideum* and *S. typhimurium* cells with different concentrations are added to the different microtitre wells. The infection is set up at approximately 24, 5 °C, even though *S. typhimurium* normally infects cells at 37°C. The reason for this is that *D. discoideum* cannot survive at temperatures over 27 °C [50, 29]. After approximately 3 h, *S. typhimurium* cells that did not infect *D. discoideum* are killed by exposing the cells to gentamicin, with the approximate concentration of 100 µg/ml. This is an antibiotic that is commonly used for gram-negative bacteria [54, 29]. Hence, only bacterial cells that have entered inside *D. discoideum* will survive [29]. Then the incubation is continued at 24, 5 °C [29]. As mentioned before, the bacterium is expressing GFP and this allows screening of the infection with the aid of a fluorescence microscope. This makes it possible to see if *S. typhimurium* actually are able to infect *D. discoideum*, i.e. are inside the cells, and to see which conditions are the best for infection. When good conditions are found, a new infection will be done in a larger scale, this will then be performed in dishes instead of microtitre plates.

## **Isolation of infected cells and RNA extraction**

When a large scale infection has been performed it is important to separate those *D. discoideum* cells that have not been infected from those that have been infected by *S. typhimurium*. This is done by a method called FACS – Fluorescence Activated Cell Sorter, the methods is illustrated in figure 5.



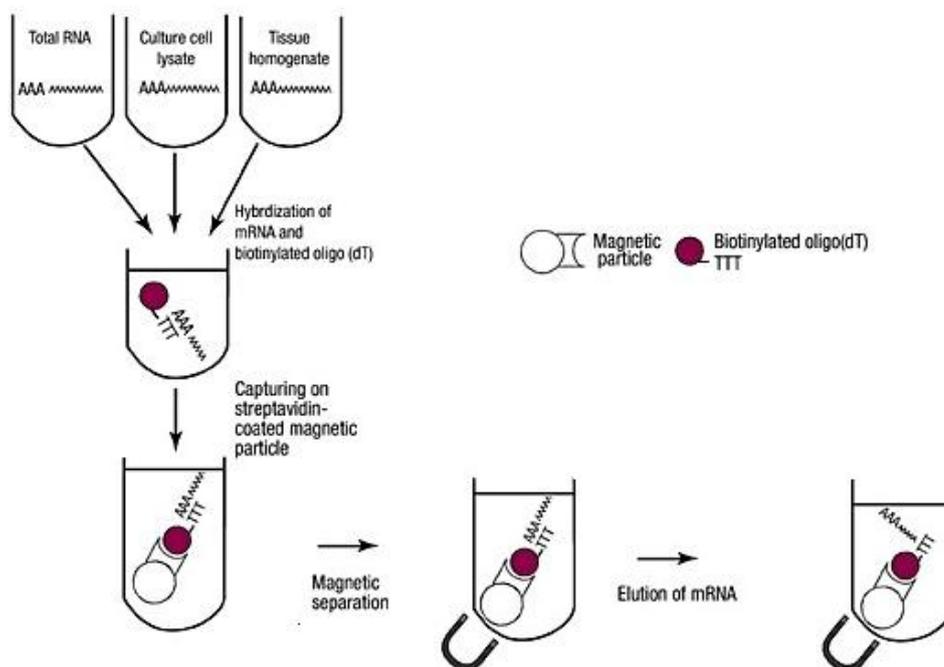
**Figure 5:** Fluorescence activated cell sorter [55].

This method works by sorting cells that contain fluorescent proteins from those that do not. The cell suspension is added and the cells will then pass through a laser where the fluorescence is measured. A little syringe generates small droplets that will contain either one cell or several cells. Those droplets that contain one cell which are fluorescent will get a negative charge, other droplets that contain a cell with no fluorescence will instead get a positive charge. In this way these cells will later on get separated from each other by the help of an electric field. Droplets that contain several cells are gathered in a waste container [55]. With this method, *D. discoideum* cell that have been infected with bacteria expressing GFP can be separated from *D. discoideum* cells that have not been infected.

When infected and non-infected cells have been separated, RNA will be extracted from both cell types. This will be done with TRIzol [56], which is a widely used reagent for extracting RNA.

As previously mentioned one aim with this project is to find out which ncRNAs are induced during infection and also how the host gene expression is affected by the infection. Ideally, ncRNAs which are up- or down-regulated during infection will be identified together with their specific mRNA targets. Therefore both small ncRNAs and mRNAs need to be extracted and separated. When total RNA have been extracted with TRIzol, mRNA and small RNAs need to be separated from each other for further analysis.

mRNA, will be isolated by using biotinylated oligo-dT and streptavidin coated magnetic beads. Biotin can bind strongly to streptavidin; therefore, by using an oligo (dT) that is biotinylated and will bind to the poly-A tail on mRNA, one can easily extract mRNA from total RNA by using magnetic beads that is covered with streptavidin [57]. See figure 6 for an illustration of the method.



**Figure 6:** Extraction of mRNA with biotinylated mRNA and streptavidin coated beads [57].

Small ncRNAs will be enriched by size fractionation using gel electrophoresis. This means that the RNA will be run on a gel that will separate the RNA depending on size. The small RNAs (18-30 nt) will then be excised from the gel [58].

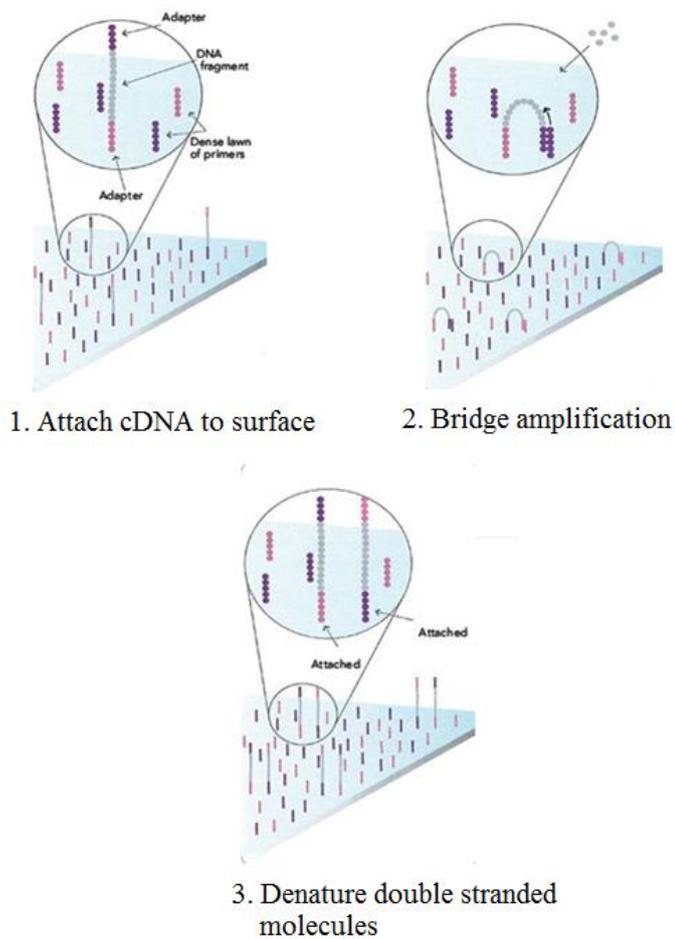
### **Identification of ncRNAs and mRNA**

To be able to see which genes and which ncRNAs that are up-or down-regulated during the infectious process they need to be analyzed. Through every analysis, RNA from both infected and uninfected cells will be used.

Concerning mRNA, the analysis can be done in several ways. One method is microarray analysis. With RT-PCR, the purified mRNA is converted into cDNA with a fluorescent tag attached. Onto the microarray plate, known sequences of genes are attached and then the plate is incubated with the cDNA. When complementary sequences are found, they will hybridize with each other and this can later on be detected since the cDNA is fluorescent [59]. Another method that can be used, and most probably will be used here, is a high-throughput sequencing method. The technology that will be used is called Illumina. The purified mRNA is converted to cDNA by reverse transcription. Then to these fragments adaptors with known sequences are ligated and finally the cDNA is amplified using PCR [60].

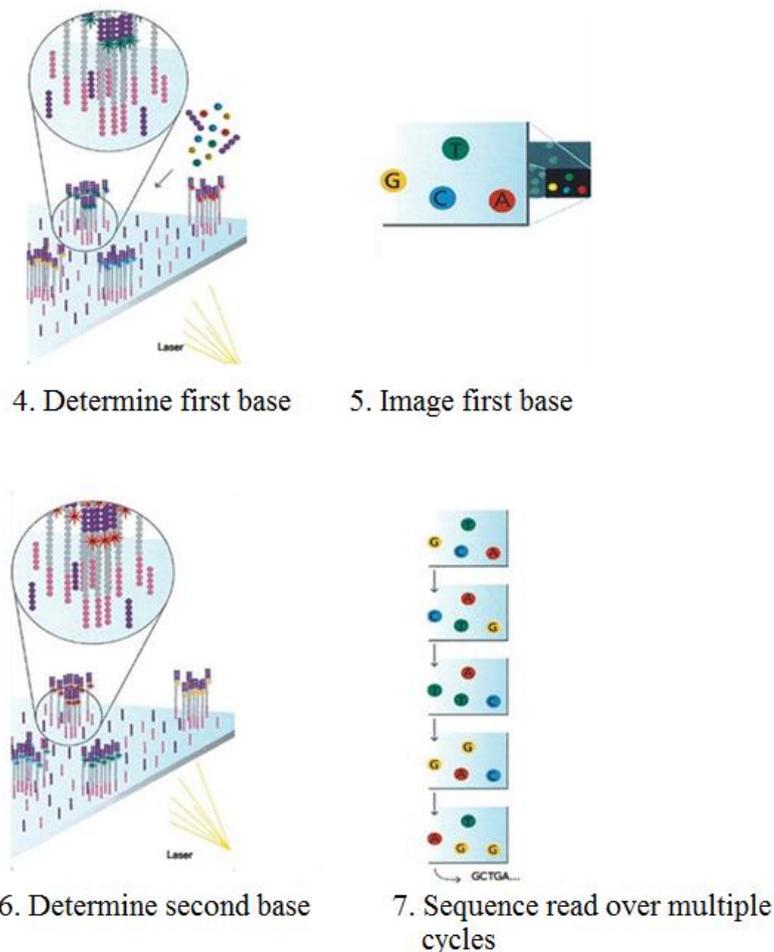
Small RNAs, purified using size fractionation, will also be sequenced using Illumina sequencing technology. These small RNAs are also converted to cDNA, adaptors are ligated and the cDNA is amplified using PCR [58, 60].

The adaptors that are ligated to both small RNAs and mRNA will be used to attach the fragments to the surface in the sequencing machine. When the cDNA is attached to the surface, amplification of the templates is performed during a mechanism called bridge amplification (see figure 7). Once this amplification is finished, the now created double stranded fragments will be denatured, creating two strands from one. This will eventually create around 1,000 identical copies of each template attached to the surface [60].



**Figure 7:** Fragment amplification during Illumina sequencing [60].

Once the fragments are amplified, fluorescently labeled nucleotides are added to the surface where cDNA is attached. When one nucleotide base-pairs with the cDNA, the polymerization is terminated by the fluorescent molecule. After adding a fluorescent nucleotide, the base is imaged by a laser and then it is cleaved off enzymatically so that the polymerization can continue. This is then repeated and finally you will have a whole sequenced cDNA [60]. This mechanism can be seen in figure 8.



**Figure 8:** Base-by base- sequencing by Illumina technology [60].

Once both mRNA and small ncRNAs are identified, the aim is to see which ncRNAs are influenced by the infectious process and also to see which mRNA they target. For example, we expect that when certain ncRNAs are up-regulated, their specific target mRNAs will be down-regulated.

### **Functions of ncRNAs and their target genes**

To be able to understand the biology behind the immunogenic response in *D. discoideum* during infection with *S. typhimurium* and understand how the bacteria regulate different small RNAs in order to infect its host, several mutant strains of both *D. discoideum* and *S. typhimurium* will be created and tested on the host-pathogen set up used above.

When the ncRNAs in *D. discoideum* have been identified and the affected genes are known, several mutant strains of *D. discoideum* will be created. The genes that were found to be regulated by ncRNAs will be knocked-out or over-expressed and then used in the same

infection studies as above to see how the infectious process are influenced. By also using the same strains in normal growth and developmental studies, the function of the genes can be elucidated. Mutant strains of *D. discoideum* will also be created by knocking out or over-expressing the small RNAs that were expressed differently during the infectious process, which will hopefully explain their function.

Concerning *S. typhimurium*, there are several ncRNAs that already are known to regulate different functions in the cell. As mentioned above at least 70 small RNAs have already been identified in *S. typhimurium*. Using these, new strains can be created by knocking-out or over-expressing known ncRNAs. These will then be used in the same infection system as above and their function will be analyzed in order to see if they affect the pathogens ability to invade and replicate inside its host cell.

## Discussion

The aim of the project was to survey the literature and find conditions where *Salmonella typhimurium* infects *Dictyostelium discoideum*. This host-pathogen set up will subsequently be used to identify non-coding RNAs that are influenced by the infectious process and also to reveal which genes these ncRNAs are affecting. The set up will also be used to understand how already known ncRNAs in *S. typhimurium* influence the infectious process.

ncRNAs have recently shown to be a very important part of the regulation of gene expression in both eukaryotic and prokaryotic cells [61]. Earlier it has been shown that ncRNAs are induced in eukaryotic cells upon infection. For example, when *Arabidopsis thaliana* was infected with a bacterial peptide from *Pseudomonas syringae* it responded by inducing a special miRNA. This gave the plant an increased resistance against the bacterium [49, 48]. It was also shown when the plant was infected with *P. syringae* it induced several siRNAs [46]. These examples show that eukaryotic cells are able to induce a response with ncRNAs to increase their resistance against infections of different bacteria. Therefore, when *D. discoideum* are infected with *S. typhimurium*, it is expected that different ncRNAs will regulate several genes that will facilitate the immunogenic response in *D. discoideum*. The question is then, which genes could be expected to be affected by the infectious process?

This is a hard question to answer, but in previous studies where *D. discoideum* had been infected with *Legionella pneumophila* and *Pseudomonas aeruginosa*, transcriptomic changes e.g. changes in mRNA levels, have been investigated by using microarray technology.

*D. discoideum* were used to study the host response during infection with two different strains of *P. aeruginosa*. With both strains it was noticed that in total 268 genes was down-regulated and 106 genes were up-regulated. These were involved in many different functions in the cell. Concerning down-regulated genes, most genes affected were important for metabolism, translation, cell growth and transport facilitation [62]. Examples of such genes are *gcsA*, glutamylcysteine synthetase, which are important for cell growth and *sgkA*, sphingosine kinase, which is important for cell division [62]. Concerning up-regulated genes, the genes affected were also involved in metabolism but also many other functions such as cell proliferation. These changes were observed using both strains, but the different strains also had a specific response to the infection, where different genes in the host were down- or up-regulated during the infection. This means that when different bacterial strains infect *D. discoideum* they are able to induce a special response [62]. In another study with *L. pneumophila*, it was noticed that many genes in metabolism, protein biosynthesis, fatty acid modification was down-regulated [63], but when a comparison was made with the results from *P. aeruginosa*, they noticed that only 8 genes was regulated in the same manner. Those genes were for example involved in metabolism [62]. This is once again a sign that different bacteria result in different response in *D. discoideum*. But in all different infections, we can find affected genes involved in basic cellular functions such as metabolism, cell growth and cell division. This can probably be due to the fact that bacteria are able to use different mechanism in the host cell, such as metabolism, to its advantage. This will allow the bacteria to grow and replicate inside its host cell. Alternatively, by reducing these factors the host cell can concentrate on creating a response to the infection and try to overcome it instead of concentrating on growing.

*S. typhimurium*s effect on the host has also been studied. In one study a murine macrophage cell line was used to study *S. typhimurium*s effect on the host cell during infection. It was noticed that 34 genes were up-regulated, some important for antimicrobial properties and some for intracellular signaling. It was also noticed that 24 genes were down-regulated, these were negative regulators of macrophage differentiation and different enzymes such as kinases and cyclins [64]. Kinases are a group of enzymes that for example can regulate protein activity, whereas cyclins can indirect regulate the cell cycle by binding to cdk (cyclin-dependent-kinases) enzymes, which in turn regulate the cell cycle. By reducing these two enzymes, maybe some important proteins are overexpressed since they cannot be regulated

anymore and the division of the cell is reduced since the cell-cycle cannot be regulated properly.

During infection of *S. typhimurium* in *D. discoideum*, cellular functions that are important for growth, cell division and differentiation will probably be down-regulated and other genes important for the cells defense against the invading pathogen will be up-regulated. In previous mentioned examples, with *P. aeruginosa* and *S. typhimurium*, genes important for basic functions were down regulated. Therefore, this is probably also going to happen when *S. typhimurium* infects *D. discoideum*. But since specific responses were induced using both different strains of *P. aeruginosa* and *L. pneumophila*, *S. typhimurium* will most probably induce a species specific response in *D. discoideum* as well. When *D. discoideum* are infected with *S. typhimurium* we predict that several different mRNAs will be up and down regulated, some of them will surely not be regulated with the aid of ncRNAs. Therefore it will be important to try to pair a special ncRNAs to a gene, to actually see that the gene in question is regulated by the ncRNAs and not by other factors. This will be done by pairing an up-regulated ncRNA to a down-regulated mRNA searching for complementary sequences, since we expect that when a certain ncRNA are up-regulated its specific target will be down-regulated.

One question that also arises when using this model system is if *D. discoideum* really reflects how *S. typhimurium* infects human cells. One difference with using *D. discoideum* instead of other animal model organisms could be that you set up the infection at lower temperatures compared to what the bacteria meet in the human body. As mentioned before, *D. discoideum* cannot grow at temperatures above 27°C and the ideal temperature is around 25° C. But in the human body, the bacterium is exposed to temperatures of around 37 °C. This could mean that the bacteria behave in a different way than it would in human cells and this could in turn mean that some regulatory functions, important for infection in the human body, are missed. In human cells, *S. typhimurium* can infect several cell types, for example epithelial cells and Microfold cells, these cells are non-phagocytic, which means that the bacteria have found other ways to infect these cell types. The pathogenic island SPI-1 is known to encode several different virulence factors that enable the pathogens entrance into these non-phagocytic host cells [28]. Since *D. discoideum* hunts bacteria and engulf them by phagocytosis [1], they resemble macrophage cells in the human body, which *S. typhimurium* also are able to infect. Therefore, *S. typhimurium* maybe do not need to use the same SPI-1

virulence factors as it does when it enters non-phagocytic cells, since they are engulfed instead. This may mean that virulence factors encoded by SPI-1 during the invasion are not expressed and an important part of the infection that normally occurs in the human body is missed and cannot be analyzed using *D. discoideum*. It is possible that these differences can cause that different ncRNAs are up and down regulated during the infectious process in human cells and *D. discoideum*. This could give results from *D. discoideum* infection that could be insufficient. Also *D. discoideum* may contain different ncRNAs than human cells, this would result in that we could miss some important ncRNAs that normally are present in human cells. If this would be the case, hopefully those different ncRNAs would regulate the same genes and it will be possible to draw some conclusions anyway.

Even though *D. discoideum* have some differences in comparison with human cells, it is still a good place to start to analyze the infection of *S. typhimurium*. By using *D. discoideum* instead of other animal model, you can come around some ethical problems. *D. discoideum* also have the advantage of being really easy to handle and grow in lab conditions, making it more cost efficient to use than other animal models. A large molecular toolbox is also available for *D. discoideum*, for example methods to knock out genes. Further, *D. discoideum* also share environment with many different bacteria, which have allowed bacteria to develop ways to resist being killed by the amoeba. Since pathways involved in engulfment of bacteria are highly conserved through evolution, it is believed that different bacteria have gained ways to infect human cell types, such as macrophages since they contain the same conserved pathways [65].

Further research, which did not fit into the time-plan in this literature project, would be to use the microtitre plate assay and the host-pathogen set up to search for new antibacterial drugs. When the GFP-expressing bacteria are infecting *D. discoideum* in the microtitre plate, it is possible to add different chemicals to screen for drugs. The chemicals will be positively scored if they are able to kill or reduce the bacteria ability to grow intracellular, without harming *D. discoideum* cells. These chemicals could then be used for further analyses to see if they affect *D. discoideum* normal growth and developmental stages and later on also test on different animal models. As a final conclusion, it is important to mentioned that even though this project have been concentrated on *S. typhimurium*, this host-pathogen set up with *D. discoideum* can also be used for several other pathogens in order to study intracellular pathogens. It can help to understand the biology behind different infectious processes and

hopefully, due to *D. discoideum's* resemblance with human cells, help us understand the infectious process in humans as well, at least as a start.

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