**Master's Thesis**

# Effect of temperature change on bacterial virulence

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Abiotic factors such as temperature can influence the evolution of the pathogens, but empirical evidence on this is very scarce. The pathogen *Serratia marcescens*, which had evolved under three different temperature treatments for 1 month, and ancestor (ATCC 13880) of the evolved strains, were tested for their virulence in *Galleria mellonella*- wax moth. Bacteria injected larvae were incubated in two different temperatures to explore phenotypic plasticity in virulence. The mortality of the hosts was recorded and analysed. Survival analysis with linear mixed-effects model in R revealed that temperature change affected the bacteria virulence. Ancestor and those strains that had evolved in constantly high 38°C temperature were found to exhibit high virulence whereas strains evolved in benign 31°C, constant or fluctuating (daily fluctuations between 24-38°C) were found to have attenuated virulence. Whole genome sequencing of evolved strains n=28 revealed putative and new virulence factors. My results show that high temperatures are able to maintain and select for genes increasing virulence. If my result reflects things in the wild and also in other pathogens results mean problems associated with increasing mean temperatures due to global change .

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Hakusanat: evoluutio, virulenssi, mutaatio, lämpötilanmuutos

Patogeenien evoluutio voi olla herkkä lämpötilojen muutokselle, mutta tätä kysymystä on tutkittu yllättävän vähän. Tutkin *Serratia marcescens* -bakteerilla kuinka kuukauden evoluutio erilaisissa lämpötilaympäristöissä vaikuttaa taudinaiheutuskyvyn (virulenssin) evoluutioon. Virulenssi testattiin ymppäämällä bakteeria vahakoisa -hyönteiseen kahdessa eri lämpötilassa. Tilastoanalyysit paljastivat että korkeassa lämmössä muuntuneet bakteerikloonit olivat säilyttäneet alkuperäkannan korkean virulenssin, kun taas vaihtelevassa ja alhaisemmassa lämpötilassa muuntuneet kloonit olivat vähemmän virulentteja. Virulenssikokeessa käytettyjen bakteerikloonien genomin sekvensointi paljasti myös virulenssiin vaikuttavia geenejä. Mikäli tässä laboratoriokokeessa havaittu yhteys korkean lämpötilan ja virulenssin yhteydestä pitää paikkansa luonnossa sekä muilla lajeilla kasvihuoneilmiön myötä nouseva lämpötila voi lisätä tautiriskiä.

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# 1 INTRODUCTION

Climate change is greatly impacting, in numerous ways, both biotic- and abiotic environment (Lovejoy 2010). Both historical and experimental evidence suggest that climate along with other factors have impact on infectious disease. Disease triangle refer to the interplay between host, pathogen and environment (Grulke 2011). Environmental fluctuations are believed to cause reverberating changes in microbial species and lead to infectious disease emergency (Smirnova et al. 2011, Blanford et al. 2003, Wichman et al. 1999), with poses pressure on understanding the influences of thermal environments on the food production as well as human health threatened pathogens. The evolution of virulence in thermal environment has been extensively investigated and the research shows different results and conclusions for different species. Both the increase and decrease in temperature can drive the evolution of virulence (Blanford et al. 2003, Ketola et al. 2013, Maurelli et al. 1984). In addition, temperature fluctuations could increase the possibility of host infection (Shapiro & Cowen 2012). Host-pathogen interactions such as transmission, pathogen variation and infection, might be affected also by within -host dynamics (Gallana et al. 2013, Lafferty 2009, Mihaljevic et al. 2007, Mougi et al. 2011). Overall, changing thermal environment could affect infectious disease through disease triangle.

## 1.1 Climate Change

Climate change refers to the variations in overall climatic conditions, including temperature, wind patterns, humidity and precipitation that might be caused by varying levels of disturbance of the Earth's climate system (Lovejoy 2010). Selection of genotypes might be one of the consequences of environmental fluctuation. In many cases, temperature acts as a significant environmental element that influences protein secretion or gene expression in the bacteria and enables the bacteria to adapt to the changing environment (Smirnova et al. 2011). In the nature, organisms possibly undergo the variation in surrounding temperature caused by seasonal factors or global warming (Shapiro & Cowen 2012). The increasing mean temperature results from greenhouse effect and the increasing temperature fluctuation (daily, seasonality), and extreme weather events might lead to outbreak or evolution of infectious disease (Lafferty 2009, McMichael 2015,

Rohr & Raffel 2010). Evolutionary changes in these situations may involve altered ways of detecting and responding to environmental conditions, leading to changes in life history traits, physiological resistance levels and migration (Price et al. 2003). Phenotypic plasticity can be a trait optimized by natural selection to buffer individuals and populations against environmental heterogeneity (Brown et al. 2012, Price et al. 2003). Climate change could affect infectious disease by changing pathogen-host-(vector) interactions (Blanford et al. 2003, Engering et al. 2013, Laine 2004, Parratt et al. 2016).

Temperature plays a significant role in influencing the birth, growth and etiopathogenesis of many microorganisms such as viruses and bacteria (Shapiro & Cowen 2012). Environmental fluctuations such as daily temperature fluctuations are supposed to cause reverberating changes in microbial species in terms of both populations and individuals (Smirnova et al. 2011, Blanford et al. 2003, Wichman et al. 1999, Ketola et al. 2013). In previous studies (Blanford et al. 2003, Ketola et al. 2013), the results suggested that thermal fluctuations driven by the climate change could affect both species' invasiveness and virulence both of which are important traits dictating spread and emergence of diseases. In addition, Friman et al. (2011) also found that the increase in mean temperature could increase the motility and bacterial virulence of *Serratia marcescens.* Blanford et al. (2003) studied the resistance and toxicity of a host-parasite system in a certain range of temperatures (18–25 °C, 18–28 °C, 18–21 °C) and suggested a potential cross-resistance induced by the variation in environmental temperature.

The adaptive response to strong selection such as changing temperature may need the changes at the molecular level (Wichman et al. 1999). For instance, a bacteriophage $\phi X$ 174 was tested to have amino acid changes genome-wide in each replicate as a consequence of adaptation to high temperature (Wichman et al. 1999). Deatherage et al. (2017) found genome evolution in *E. coli* at the temperature of 20 - 42°C. Although recent research has increased understanding of links between genes and traits and how genes vary across environmental gradients, our understanding of the genetic basis of evolutionary responses to changes in climate is still quite limited (Franks & Hoffman 2012). Therefore, an experimental research on climate change in required.

## 1.2 Evolution of Virulence

Virulence describes the severity of a disease. Commonly, virulence is measured as the failure or death of host brought about by pathogen (Casadevall & Pirofski 2003). Using mortality to measure virulence enables us to compare virulence across different systems better than other surrogates of virulence (Bull & Lauring 2014). Environmentally growing opportunistic pathogens are pathogens that have the ability to develop and multiply both inside and outside a host (Brown et al. 2012).

There is a trade-off between rate of transmission and duration of infection. A virulent strain of parasite may increase in frequency, if, in the process of killing its hosts, it sufficiently increases its chance of being transmitted (Gallana et al. 2013, Lafferty 2009). However, environmentally growing opportunistic pathogens can reproduce without a host. In these cases, the selection for outside host survival and development rather than transmission could change the development of virulence (Mikonranta et al. 2015). The evolution of bacterial thermal tolerance could also affect the ability to inhabit other environments either in positive or negative way (Grulke 2001). Fluctuations in temperature may enable pathogen to response to tackle extreme variation or might induce relevant cellular responses and the successful infection on host (Shapiro & Cowen 2012).

The resistance from host and the virulence of parasite are affected by a plurality of coupled factors in a complicated natural system. The virulence capabilities can be affected by the temperature-induced conformational changes in proteins which are essential for virulence (Maurelli et al. 1984). Stress can act as a driving force for genome reorganization which influenced the pathogenicity (Arnold et al. 2007). Free-living environment and within-host environment should both be considered when trying to reveal the virulence evolution of the environmentally-growing opportunistic bacteria.

## 1.3 Putative Virulence Factors

Virulence factors are defined by the elements that enable a microorganism to establish itself on or within a host. It also increases the possibility to lead to a disease by this microorganism (Chen et al. 2005; 2012; 2016). Virulence factors involve bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect bacteria, and hydrolytic enzymes that may contribute to the

pathogenicity of the bacterium are specific adaptations that allow pathogen to attach selectively to host tissues, gain access to nutrients by invading or destroying host tissues and avoid host defends (Chen et al. 2005; 2012; 2016). For example, Salmonella made its survival and rapid growth in the host by signals for the expression of Salmonella plasmid virulence (Spv) which is specific against growth constraint, decrease in nutrient providing and reduced pH value (Marshall et al. 1999). Increasing temperature might give rise to accelerated enzymatic activities which would influence the gene expression in organisms (Smirnova et al. 2011).

Most of the previous research reveals that the coding of genes has been exhibiting increasingly more influence in virulence factors. Opportunistic bacteria are better able to grow in complex environments than obligate bacteria as a result of larger genomes, and they show stronger resistance to the functions of antibacterial agents (Sadikot et al. 2005). Opportunistic pathogens make the secretion of Avr proteins via Hrp system in a temperature-dependent manner, for example *E. amylovora* (Smirnova et al. 2011). Also, Smirnova et al. (2011) stated that reduced temperature can lead to an increased secretion of proteins. Moreover, some of the key players in bacterial colonization are effector proteins that are secreted by pathogenic bacteria into the cells of their host through type III (T3SS) secretion system or type IV secretion system (T4SS) (Arnold et al. 2007). Effectors usually help the pathogen to invade host tissue, damaging immune system, and promoting pathogen survival (Mattoo et al. *2007*). They are often critical for virulence. The point mutations, and the insertions and deletions of base have been reflected in the process of losing a specific function of virulence for plant pathogens which were observed in the process of bean-*P. syringae* interaction (Arnold et al. 2007). When pathogenicity to one host is established, exposure to eukaryotic defences can activate the deletions and rearrangements that maintain virulence and broaden host range (Arnold et al. 2007).

### 1.4 Aim of the Study

The aim of this thesis is to explore how climate change will affect environmental opportunistic bacterial virulence with a primary focus on temperature. This study is based on the data obtained from the evolution experiment performed on *S. marcescens* (Ketola et al. 2013), i.e. 28 strains isolated from experiment where strains were evolving at three different temperatures, and the subsequent host infection experiment performed on *G. mellonella*. Virulence could be affected by temperature in different ways. Thus, studying

the effects of temperature on virulence can reveal the evolutionary potential of bacterial virulence under the condition of increasing mean temperature on the earth.

In this research I ask first if temperature during evolution (constant 38°C and 31°C, daily fluctuation of 24-38°C) affects bacterial virulence. Based on the previous literature I expect that virulence will be sensitive to temperatures (Ketola et al. 2013, Friman et al 2011). Second, I ask if temperature during virulence assay affect bacterial virulence. Since bacterial growth rates are higher in high temperature I expect virulence to be higher at high temperature. Third, I resolve how genetic changes affect virulence.

# 2 MATERIALS AND METHODS

## 2.1 Study species

### 2.1.1 *Serratia marcescens* and the evolution

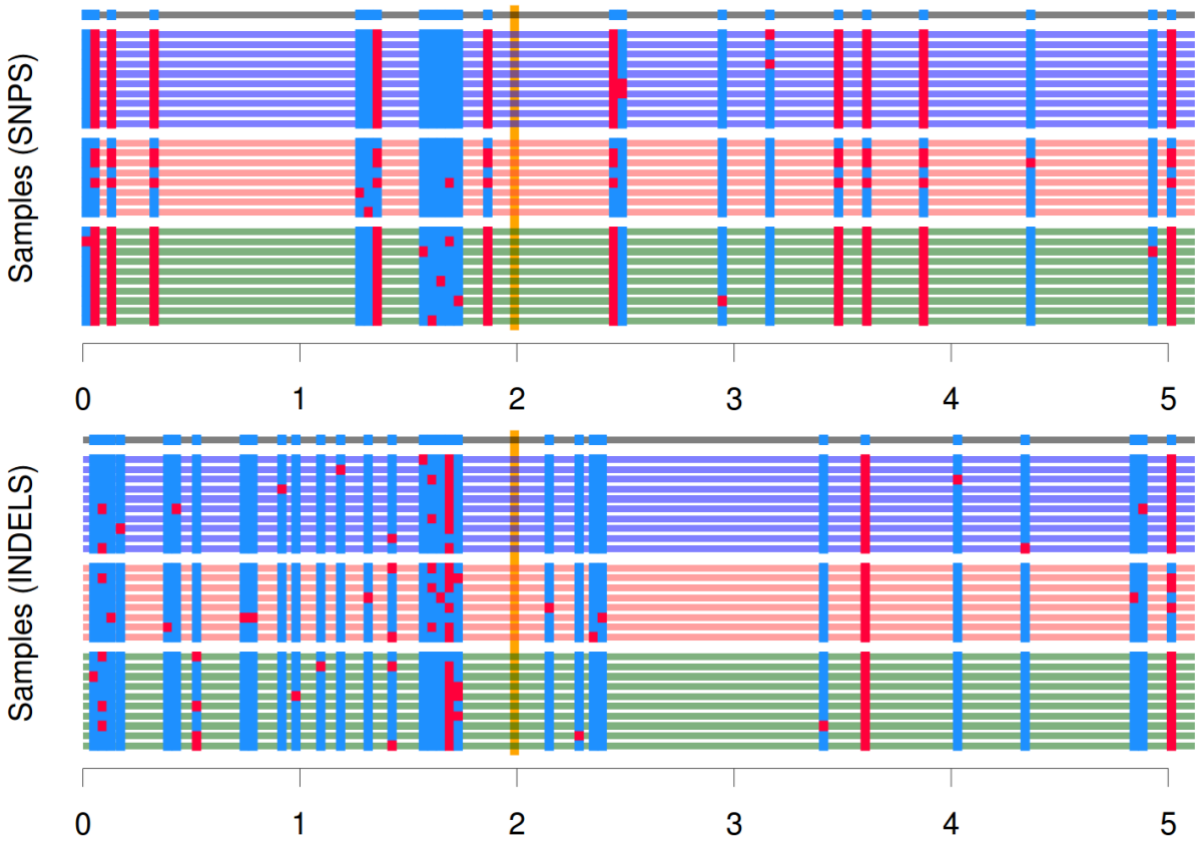
*Serratia marcescens* is a bacterial species of the genus *Serratia.* It is a gram-negative facultative pathogenic bacterium which is widely spread as a free-living form in the nature. It can survive in many different environments such as freshwater, marine ecosystems and soil (Mahlen 2011, Grimont & Grimont 2006). *S. marcescens* can also survive in different hosts, e.g. plants, invertebrates and vertebrates, and cause disease in hosts (Mahlen 2011, Grimont & Grimont 2006).

Among the various *Serratia* species, S. *marcescens* has showed a predominance in the infection of insects (Grimont & Grimont 2006). S. *marcescens* was found in sugar beet root-maggot development stages, suggesting an insect-microbe symbiosis, as well as a nutritional interdependence (Iverson et al. 1984). *Serratia* proteinase and chitinase are very toxic when injected into the hemocoel (Kaska 1976; Lysenko 1976). It can cause a lethal septicemia after penetrating into the hemocoel. However, there is still little information on the exact mechanism of *S. marcescens* pathogenesis inside the host.

One S. *marcescens* strain (ATCC 13880), which was isolated from pond water, was used as an ancestor in evolutionary experiment. Thirty replicate populations were established.

These populations evolved at three different temperatures for one month: high temperature 38°C (eight populations, as two ouf of ten contaminated during experiment), constant 31°C (ten populations) and fluctuating temperature from 24 to 38 °C (ten populations). The bacteria strains were cultured in hay extract medium (1 g of cerophyll powder (Ward's natural science, Rochester, NY) were boiled for 10 min in 1 l of dd$H_2O$ to obtain a final concentration of 2.15 mg $l^{K1}$, and then filtered through a glass fibre filter (GF/C, Whatman). After sterilization, buffer with pH 7.5 (0.5 g of ($NH_4$) $2SO_4$,1.5724 g of $K_2HPO_4{\cdot}3H_2O$, 0.1 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of NaCl, 0.4 g of $KH_2PO_4$ and 0.0228 g of $CaCl_2{\cdot}2H_2O$ in 1 l of $dH_2O$) was added to the hay extract medium (Friman et al. 2008). For each replicate population, a single clone was sequenced (Figure 1).

Figure 1. Sequencing results of 28 evolved strains (blue-31 °C, red-38 °C, green- 24~38 °C) and ancestor. The mutations are highlighted with different colour (blue alleles: ancestral state; red alleles: new state). There were 54 mutations including 23 SNPs and 31 indels.




2.1.2 *Galleria mellonella*- wax moth

Wax moth has been used as a host in many host models. Wax moth is distributed all over the world, and its distribution is mainly restricted by the duration of cold period of the year.

The survival of the wax moth is related to environmental temperature, and the growth of eggs and larvae of wax moth requires a temperature ranging from 29 ℃ to 33 ℃ (Kwadha et al. 2017). Extreme temperature will lead to a decrease in growth rate and ultimately, rapid death of the wax moth. Wax moth has many enemies including viruses, bacteria, protozoa and insects (Kwadha et al. 2017). It is known that the important natural enemies of wax moth include S. *marcescens*, nematodes and nuclear polyhedrosis virus, etc. (Flyg et al. 1980, Grimont & Grimont 2006, Kwadha et al. 2017).

**2.2 Experimental design**

2.2.1 Bacteria growth

All 28 evolved-replicates of S. *marcescens* were thawed and transferred into a bioscreen plate with 400 µl 1% SPL per well using the heat-sterilized cryoreplicator (Enzyscreen B.V., Haarlem, The Netherlands). The ancestor stored at -80 ℃ was thawed and inoculated into 1% SPL in a 15 ml tube with loose cap. All together 29 strains were put in a mini incubator (Labnet International, Inc. I5110-230), and regrew at 31℃ for 24 hours.

2.2.2 Infecting the hosts

In order to measure the virulence of the evolved bacteria 5 µl bacteria were injected by Hamilton syringes (Figure 2) into the hemocoel of the hosts. Each of the bacterial samples were repilicated in 20 hosts. These operations were conducted with all 28 strains and the ancestor strain as one block. Order of the injections were randomized within each block. dd$H_2O$ , 1%SPL were used as control groups for each block, injected into hemocoel in similar manner. The larvae were kept on petri-plate separately to record the death time.

After injections, the infected hosts (2480) were put into the incubator. For each block, ten replicates of each strain were incubated at 31 ℃ and ten replicates were incubated at 24 ℃. The mortality was examined with an interval of 1 to 3 hours. The death time points of all samples were recorded. The virulence of the bacteria was measured by the death rate of the hosts.

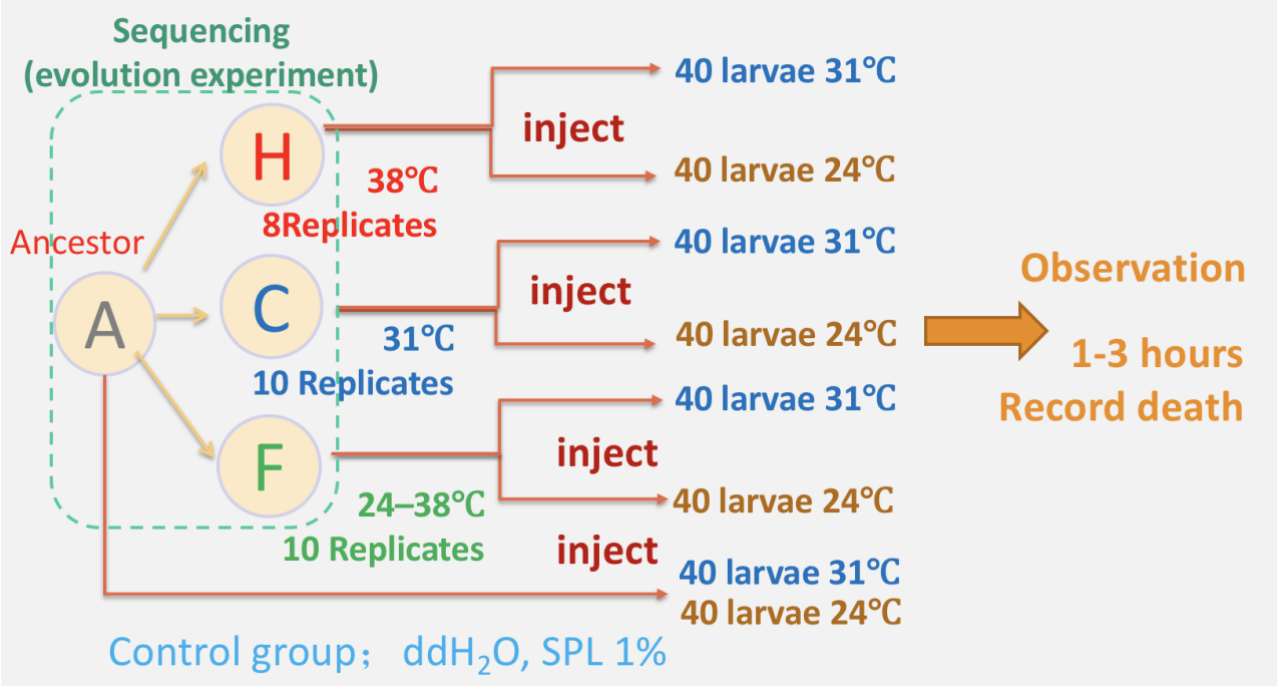



Figure 2. Bacterial clones, tested for their virulence, were obtained from previous experiment examining the effects of environmental fluctuations on bacterial evolution (Ketola et al. 2013) that started from one ancestor clone, altogether 30 replicates (2 replicates in 38 °C were contaminated and discarded) were evolved at three different temperatures for one month. The ancestor and 28 evolved clones which were chosen by the following way: 10 clones from constant 31°C, 10 clones from fluctuating 24-38 °C, 8 clones from constant 38 °C were sequenced). Clones were from different independenently evolved populations.

**2.3 Statistical Analyses**

The survival analyses were performed with Linear Mixed-Effects models in R studio (version 1.0.153, RStudio Inc). The fixed factors in the model were temperature during evolution (38 °C, 31 °C, 24-38 °C), temperature during infection (24/31 °C), block number (1-4) and bodymass (mg). Interactions between temperature during evolution and infection was also fitted to the model. The identity of the population was fitted as a random effect.

Analysis on the effects of mutated genes on virulence at different temperatures was performed in Linear-mixed effects model with the mutated genes and temperature during infection as fixed factors (interactions included) and identity of a population as a random factor. The body mass and block were considered as fixed effects. The adjustment for

multiple corrections was performed with Benjamini-Hochberg method ($p<0.05$, in all tests). The experiment of this study adopted a large sample size, which also indicates a potential risk of overestimation.

The preliminary data analysis indicated the existence of 4 linkage groups in which the clones share several allelic changes such that their independent effects cannot be separated from each others. These linkage groups are denoted in results as Group 1 etc , and in practice only single mutation from this linkage were subjected to statistical analysis. Naturally gene functions for alleles representing whole linkage are not shown.

# 3 RESULTS

## 3.1 Effects of evolution treatments and host treatments on bacterial virulence

The results show that the bacterial virulence was influenced by different evolution treatments (Table 1). The clones that had evolved at constant temperature of 38°C ($t=-3.149$, $p=0.004$, Global test for differences between all) were found to exhibit high virulence whereas the clones that had evolved at benign 31°C, constant or fluctuating (daily temperature fluctuations of 24-38 °C) were found to show attenuated virulence (see statistics below, Figure 3a).

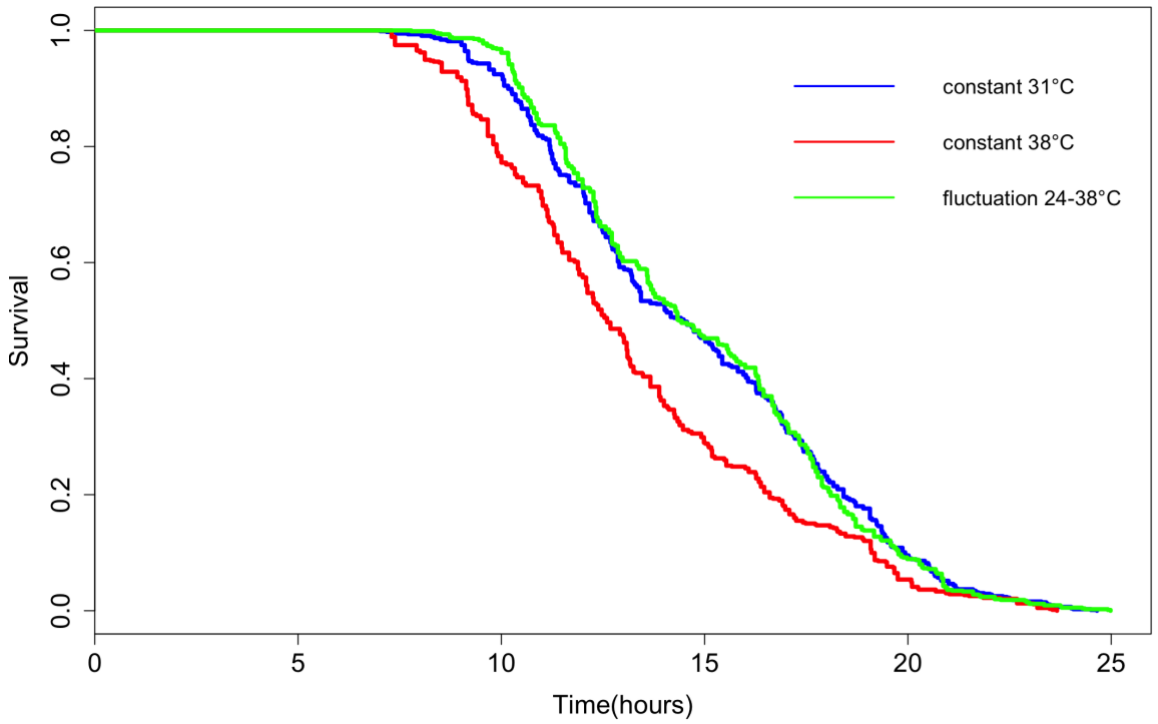
Temperature during infection was found to have strong effect on virulence (Table 1 Figure 3b). Significant interactions were found between evolution treatment and environments during infection ($F_{2,2104.28}=7.8$, $p<0.001$). Strains were found strongly affect the virulence of bacteria when evolved at a constant temperature of 38°C (Figure 3b). Figure 3b show that survival rate was lower with a high temperature during infection (38°C) comparing to a low constant temperature (31°C) during infections. The pairwise test results show that the difference of bacterial virulence is most significant between strains under Evolutionary treatment 38°C and 24~38°C ($p<0.001$). The difference if bacterial virulence is also strong between strains under Evolutionary treatment 31°C and 38°C ($p=0.004$). Block was found to be affect the virulence ($F_{3,2104.70}=8.6$, $p<0.001$). Body mass of the host was found to reduce bacterial virulence ($F_{1,2107.63}=8.6$, $p<0.001$).

Table 1 Liner mixed effects model results of the effects evolutionary treatment (constant 38 ℃ and 31 ℃, daily fluctuation of 24-38 ℃) and environment (constant 24 ℃ and 31 ℃) during infection on the bacteria virulence (survival time) in *S. marcescens*.

| **Survival time** | **F** | **df1, df2** | **P** |
|---|---|---|---|
| **Evolutionary treatment** | 4.2 | 2, 25.01 | 0.027 |
| **Environment during infection** | 3393.8 | 1, 2104.32 | <0.001 |
| **Evolutionary treatment by environment during infection** | 7.8 | 2, 2104.28 | <0.001 |
| **Block** | 8.6 | 3, 2104.70 | <0.001 |
| **Body mass** | 97.4 | 1, 2107.63 | <0.001 |

| **Survival time** | T value | df | Pr(>\|t\|) |
|---|---|---|---|
| **Evolutionary treatment 31°C vs 38°C** | -3.149 | 2.660e+01 | 0.004 |
| **Evolutionary treatment 31°C vs 24~38°C** | -0.325 | 2.670e+01 | 0.747 |
| **Evolutionary treatment 38°C vs 24~38°C** | 31.742 | 4.380e+01 | <0.001 |

a)




b)

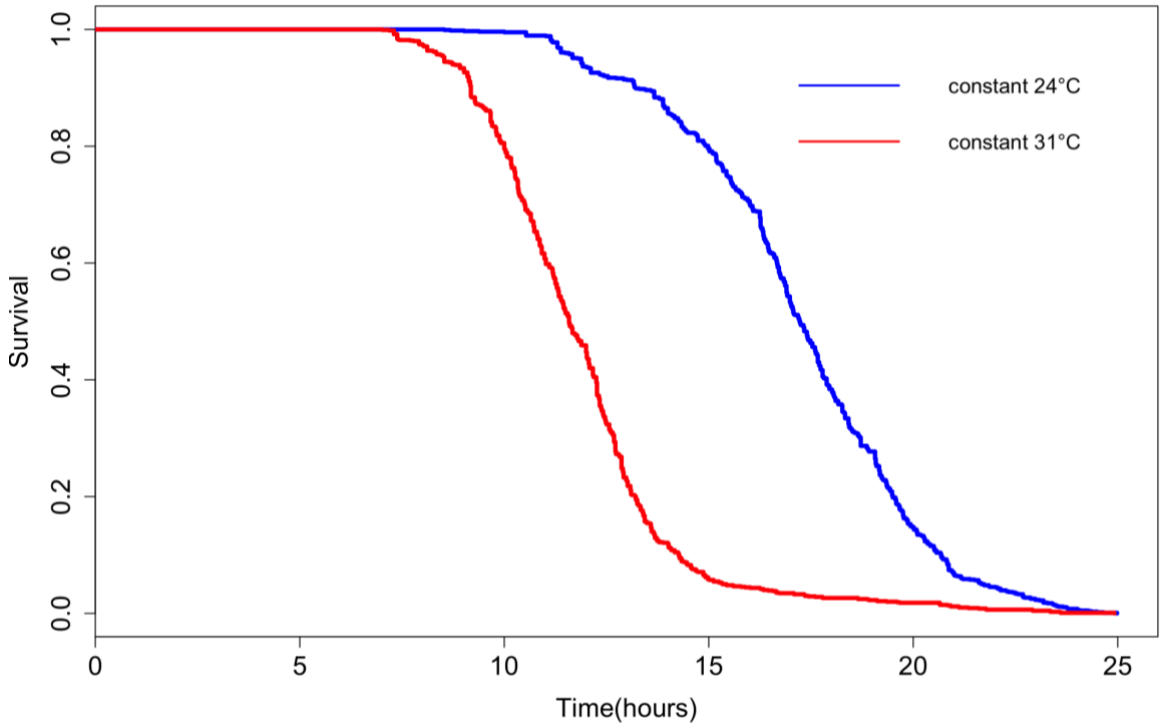



Figure 3. Survival curve of the infected hosts by different treatments. a) survival curve with time(hours) and survival proportion for hosts infected by *S. marcescens* strains that evolved at three temperature treatments. b) survival curve with time(hours) and survival proportion for the infected hosts under different thermal environments.

### 3.2 Effects of putative virulence factors on bacterial virulence

I found out that Gene ID_43 had strongest effect among all mutated genes on virulence (across all models, p<0,001). The function of the gene in which this indel occurred is unknown. Two linkage groups played a clear role in virulence.

Linkage group 1 that had positive effect on virulence consists 7 genes and 5 of them with known product. Gene ID_05 codes for membrane protein, Gene ID_20 codes transcriptional regulator, Gene ID_44 codes hemeABC transporter permease, Gene ID_45 codes for alcohol dehydrogenas and Gene ID_52 codes for deacetylase. The Linkage group 2 which had negative effect on virulence included: Gene ID_07 codes for condensation protein, Gene ID_37 codes for peptidoglycan synthase, Gene ID_42 condes for transcriptional regulator, Gene ID_53 codes foe deacetylase.

Table 2 Linear-mixed effects model results on the effects of putative virulence factors (mutated genes) on the bacteria virulence (survival time) in *S. marcescens*. Only initially signigicant effects are listed and are ordered by t-test statistics. Group 1 to 4 state 4 linkage groups (Appendix 2. Linkage group information).

| Gene ID | Adjust P value (G) | P value (G) | T value (G) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| ID 43 | 0 | 0 | -58.487 | indel | NA |
| Group 1 | <0.001 | <0.001 | -6.012 | | Group 1 |
| Group 2 | <0.001 | <0.001 | 6.012 | | Group 2 |
| Group 3 | 0.038 | 0.005 | 3.095 | | Group 3 |
| Group 4 | 0.061 | 0.010 | -2.764 | | Group 4 |
| ID 27 | 0.061 | 0.011 | 2.737 | indel | NA |
| ID 09 | 0.476 | 0.110 | 1.650 | indel | NA |
| ID 21 | 0.476 | 0.112 | 1.642 | indel | NA |
| ID 38 | 0.567 | 0.150 | 1.480 | SNP | immunity protein |

| Gene ID | Adjust P value (G) | P value (G) | T value (G) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| ID 33 | 0.594 | 0.175 | 1.393 | indel | NA |
| ID 02 | 0.750 | 0.352 | 0.948 | indel | NA |
| ID 03 | 0.750 | 0.485 | -0.707 | indel | NA |
| ID 08 | 0.750 | 0.396 | 0.863 | indel | RNA chaperone Hfq |
| ID 10 | 0.750 | 0.411 | 0.836 | indel | integrase |
| ID 19 | 0.750 | 0.358 | -0.935 | SNP | galactokinase |
| ID 23 | 0.750 | 0.311 | -1.033 | SNP | galactose/methyl galaxtoside transporter ATP-binding protein |
| ID 25 | 0.750 | 0.443 | -0.779 | indel | NA |
| ID 28 | 0.750 | 0.252 | 1.171 | indel | glycosyl transferase |
| ID 31 | 0.750 | 0.473 | 0.728 | SNP | galactosyl transferase |
| ID 35 | 0.750 | 0.358 | 0.935 | indel | fumarate hydratase |
| ID 39 | 0.750 | 0.473 | -0.728 | SNP | NA |
| ID 46 | 0.750 | 0.480 | 0.716 | indel | NA |
| ID 13 | 0.783 | 0.553 | 0.601 | indel | NA |
| ID 30 | 0.783 | 0.543 | -0.616 | SNP | galactosyl transferase |
| ID 00 | 0.833 | 0.612 | -0.513 | SNP | NA |
| ID 34 | 0.900 | 0.715 | 0.369 | indel | NA |
| ID 40 | 0.900 | 0.703 | 0.385 | SNP | nuclease PIN |

| Gene ID | Adjust P value (G) | P value (G) | T value (G) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| ID 16 | 0.928 | 0.764 | -0.303 | indel | hypothetical protein |
| ID 14 | 0.936 | 0.831 | 0.215 | indel | NA |
| ID 29 | 0.936 | 0.853 | 0.187 | SNP | galactosyl transferase |
| ID 47 | 0.936 | 0.835 | -0.210 | indel | NA |
| ID 06 | 0.945 | 0.889 | -0.141 | indel | NA |
| ID 24 | 0.957 | 0.948 | -0.066 | indel | beta-methylgalactoside transporter inner membrane component |
| ID 48 | 0.957 | 0.957 | -0.054 | SNP | NA |

In addition, there was significant interaction between genes and the environments during infection (Table 3). Eight genes/groups were found to greatly affecting the bacterial virulence in *S. marcescens* under two different environments during infections (t- tests $p<0.001$, Table 3). After the adjustment for multiple comparisons, we found environmental sensitivity of the role of genes (gene ID 43, 27, 09, 03 and linkage group 1, 2, 3, 4 (t-tests $p<0.001$, Table 3)) on virulence. The informative genes i.e. not in linkage groups were all indels in genes with unknown function. See above for description of Linkage group 1 (ID 01, ID 05, ID 20, ID 32, ID 44, ID 45, ID 52) , Linkage group 2 (ID 07, ID 37, ID 42, ID 53), Linkage groups 3 (ID 17, ID 26, ID 49) and Linkage group 4 (ID 04, ID 11, ID 12, ID 18, ID 36).

Table 3 Linear-mixed effects model results on the effects of environment by (mutated genes) interaction on bacterial virulence (survival time) in *G. mellonella insect host.* Only initially signigicant effects are listed and are ordered by t-test statistics. Group 1 to 4 state for 4 linkage groups (Appendix 2. Linkage group information).

| Gene ID | Adjust P value (G×E) | P value (G×E) | T value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| ID 43 | <0.001 | <0.001 | 10.038 | indel | NA |

| Gene ID | Adjust P value (G×E) | P value (G×E) | T value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| Group 1 | <0.001 | <0.001 | 5.511 | | Group 1 |
| Group 2 | <0.001 | <0.001 | -5.511 | | Group 2 |
| Group 3 | 0.001 | <0.001 | -3.963 | | Group 3 |
| Group 4 | 0.002 | 0.000 | 3.752 | | Group 4 |
| ID 27 | 0.004 | 0.001 | -3.416 | indel | NA |
| ID 09 | 0.004 | 0.001 | -3.391 | indel | NA |
| ID 33 | 0.014 | 0.003 | -2.973 | indel | NA |
| ID 46 | 0.111 | 0.026 | -2.226 | indel | NA |
| ID 02 | 0.203 | 0.066 | -1.839 | indel | NA |
| ID 31 | 0.203 | 0.072 | -1.802 | SNP | galactosyl transferase |
| ID 39 | 0.203 | 0.072 | 1.802 | SNP | NA |
| ID 13 | 0.203 | 0.070 | -1.814 | indel | NA |
| ID 30 | 0.261 | 0.105 | 1.622 | SNP | galactosyl transferase |
| ID 40 | 0.261 | 0.108 | -1.610 | SNP | nuclease PIN |
| ID 38 | 0.326 | 0.151 | -1.437 | SNP | immunity protein |
| ID 23 | 0.326 | 0.166 | 1.384 | SNP | galactose/methyl galaxtoside transporter ATP-binding protein |
| ID 25 | 0.326 | 0.171 | 1.368 | indel | NA |
| ID 47 | 0.326 | 0.173 | -1.364 | indel | NA |

| Gene ID | Adjust P value (G×E) | P value (G×E) | T value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|
| ID 29 | 0.481 | 0.269 | 1.107 | SNP | galactosyl transferase |
| ID 21 | 0.711 | 0.418 | -0.809 | indel | NA |
| ID 00 | 0.808 | 0.500 | 0.675 | SNP | NA |
| ID 34 | 0.808 | 0.547 | 0.603 | indel | NA |
| ID 16 | 0.808 | 0.531 | -0.626 | indel | hypothetical protein |
| ID 19 | 0.874 | 0.643 | -0.464 | SNP | galactokinase |
| ID 35 | 0.874 | 0.643 | 0.464 | indel | fumarate hydratase |
| ID 08 | 0.886 | 0.703 | -0.381 | indel | RNA chaperone Hfq |
| ID 24 | 0.886 | 0.679 | -0.413 | indel | beta-methylgalactoside transporter inner membrane component |
| ID 03 | 0.907 | 0.747 | -0.322 | indel | NA |
| ID 10 | 0.940 | 0.829 | 0.216 | indel | integrase |
| ID 48 | 0.940 | 0.803 | -0.249 | SNP | NA |
| ID 14 | 0.971 | 0.885 | 0.144 | indel | NA |
| ID 28 | 0.988 | 0.930 | -0.088 | indel | glycosyl transferase |
| ID 06 | 0.997 | 0.968 | 0.040 | indel | NA |

# 4 DISCUSSION

This research investigated the effect of different evolutionary treatments (constant 38 ℃, 31 ℃ and daily fluctuating 24-38 ℃) on the virulence of *S. marcescens* by carrying out a host infection experiment in two ecological treatments (constant 24 ℃, 31 ℃). Using clones from three evolution treatments (constant 38 ℃, 31 ℃ and daily fluctuating 24-38 ℃) to investigate the effects of temperature and evolution, genetics on the bacterial virulence. What's more, phenotypic plasticity in virulence has also been investigated in my study through manipulating experimental environments.

## 4.1 Effects of thermal treatment on the evolution of bacteria virulence

Bacterial virulence of *S. marcescens* was found to be influenced by different evolution treatments and environment during infection. The results of evolution treatment showed that the strains evolved at a constant temperature of 38℃ exhibited high virulence while the strains evolved at a constant temperature of 31°C, or at daily temperature fluctuations of 24-38 ℃ showed attenuated virulence. Oliver et al. (2014) suggested that temperature could affect bacterial virulence through affecting DNA topology, RNA structuring and metabolism. Friman et al. (2011) suggested the virulence of S. *marcescens* was maintained at high level at high temperatures but only in the absence of phage accompanied with the increasing motility in hot temperature.

Fluctuating environments are suggested to select for genotypes that have higher tolerance and can perform well under different environments (Scheiner 1993, Kassen 2002). What's more, fluctuations in temperature may enable the pathogen to response through pathways which would tackle variations in surrounding temperature or might indicate the successful infection on host and start virulence programs (S. Shapiro & Cowen 2012). Increased broadness of tolerance accompanied with reduced virulence was shown in *F. columnare* (Ashrafi 2017), which might suggest the decrease in bacterial virulence at fluctuating temperature.

## 4.2 Effects of thermal treatment on the evolution of putative virulence factors

Wichman et al. (1999) suggested the adaptive response to strong selection such as changing temperature may need changes at the molecular level. Also the expression of

downstream genes might be affected by the disrupted gene (Kurz et al. 2003). In my experiment, 54 mutants, which were suggested to be new virulence factors, were found across all 28 evolved strains, either SNP or indel, by whole genome sequencing. Linkage group 1 and 2 (all together 11 genes) remained the same as the ancestor strain in five evolved strains of constant 38 °C. Linkage group 3 and 4 were mutated under an evolution treatment of constant 38 °C. However, the potential linkage group in the mutants could be biologically meaningful. For example, Gene ID_44 codes for heme abc transporter permease which was also include in the linkage group 1, Gene ID_37 codes for peptidoglycan synthase might influence the formation of bacteria cell wall which is essential to invade host.

The mutations in specific genes are related to the temperature regimes in most of the cases, for example, a *nadR* mutation from 32 °C and an *aceB* mutation from switch treatment in *E. coli* (Deatherage et al. 2017). Virulence capabilities can be affected by the temperature-induced conformational changes in proteins (Maurelli et al. 1984). I found that the translated amino-acid changes between the ancestor and the alternative allele from 13 SNPs in coding region experienced different evolution treatments.

### 4.3 Effects of putative virulence factors on bacterial virulence

I found several virulence factors of *S. marcescens* in the study, yet only part of the virulence factors were coding for known gene products. Also, the different virulence factors were identified to have diverse influences on bacterial virulence without considering the host. However, in most cases, the expression of downstream genes might be affected by the mutants (Kurz et al. 2003). 14 out of 54 mutants showed translated amino-acid changes between the ancestor and the alternative allele. Some indels resulted in a frame shift (Appendix 3) which might be disruptive for protein function. After some potential virulence proteins are lost, the bacteria could possibly avoid being identified by host's immune system (Arnold et al. 2007). It is also possible that enzyme activity changes due to mutations, which would affect regulator system. Moreover, multiple mutations might work together.

Several mutations observed during experimental evolution were related to the structure of lipopolysaccharides present in the outer cell wall of *S. marcescens*, which might have an effect both on bacteria's resistance to phage and on bacteria's own virulence. Such results have been found previously. For example, Poolet & Braun (1988) suggested that hemolytic

activity of *S. marcescens* which decreased at high temperature was affected through the alterations in lipopolysaccharide.

### 4.4 Environmentally-sensitive putative virulence factors

The temperature responsiveness in protein secretion or gene expression can be found in all kinds of bacteria with the role of enabling them to adapt to their habitats (Smirnova et al. 2011). The significant link between putative virulence factors and environments during infection suggested the genetically encoded phenotypic plasticity of the bacterial virulence. In previous study (Brown et al. 2012), increased bacterial virulence on average is favored by natural selection which often accompanied with higher virulence factor expression and higher growth rate in asymptomatic and virulence sites. For distinct environments which might selects for adaptive phenotypic plasticity, the different regulatory investment suggested that it is more benefits to show adaptive plasticity when exposure to these distinct environments (Brown et al. 2012, DeWitt et al. 2011). When pathogenicity in one host is established, exposure to eukaryotic defences can activate the deletions and rearrangements that maintain virulence and broaden the host range (Arnold et al. 2007). As for induced defences, it is a balance between cost and benefit which host need to weigh the benefits of the resistance against the cost under varying environment (Choisy & de Roode 2010, Laine 2011, Taylor et al. 2006). Plasticity is often interpreted as a mechanism of self-optimization which need to square up the cost and benefit under varying environment when considering host-pathogen interactions (Taylor et al. 2006).

The growth rate of the bacteria was highest at a constant temperature of 31°C (Ketola et al. 2013), which might also affect the colonization of *S. marcescens* in the host. Some of the key players in bacterial colonization are effector proteins that are secreted by pathogenic bacteria into the cells of their host through type III (T3SS) secretion system or type IV secretion system (T4SS) (Arnold et al. 2007). Effectors play the role of assisting pathogen to enter host tissue, damaging immune function, and promoting pathogen survival (Mattoo *et al. 2007*). The change in host environment temperature might influence the extent of bacteria DNA (Ye et al. 2007), affect the RNA structuring and metabolism (Morita et al. 1999), and change the processing of proteins (Wichman et al. 1999). Increasing temperature might cause accelerated enzymatic activities which would influence the gene expression of organisms (Smirnova et al. 2011). The results of the significant effects of

putative virulence factors by environment during infections were most likely resulted from the multiple interactions between the defence and the inverness.

### 4.5 Conclusions

Understanding how the environmental cues such as temperature act in the evolution of bacteria is needed. The bacterial virulence of *S. marcescens* evolved in response to different evolution treatments. Putative virulence factors were found to have strong impact on bacterial virulence. Phenotypic plasticity enabled organisms to respond to the variations in environment conditions.

This study revealed that the evolution temperatures drove the evolution of bacterial virulence in *S. marcescens* such as the increased virulence at high evolution temperature. I found that the environmental temperatures influenced the bacterial virulence with the identified putative virulence factors. Also, evidence of climate change especially the increasing temperature support highly virulent strains was shown. As we can see from above, climate change might influence the disease triangle and cause more outbreaks of infectious diseases. This change will endanger the environments and cause harm to human health. Microbe species would be more easily affecting by thermal change. Therefore, more in-depth investigations are required to properly assess the mechanisms of the virulence evolution.

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## APPENDIX

APPENDIX 1. Linear-mix effects model results on the effects of all pupative virulence factors (54 mutated genes) on the bacteria virulence (survival time) in *S. marcescens*. Only initially signigicant effects are listed and are ordered by t-test statistics.

| Gene ID | Adjust P value (G) | T value (G) | P value (G) | Adjust P value (G×E) | T value (G×E) | P value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|---|---|---|
| **ID_43** | 0 | -58.487 | 0 | 0 | 10.038 | 0 | indel | NA |
| **ID_01** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | NA |
| **ID_05** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | membrane protein |
| **ID_07** | <0.001 | 6.012 | <0.001 | <0.001 | -5.511 | <0.001 | SNP | condensation protein |
| **ID_20** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | transcriptional regulator |
| **ID_32** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | NA |
| **ID_37** | <0.001 | 6.012 | <0.001 | <0.001 | -5.511 | <0.001 | SNP | peptidoglycan synthase |
| **ID_42** | <0.001 | 6.012 | <0.001 | <0.001 | -5.511 | <0.001 | SNP | transcriptional regulator |
| **ID_44** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | heme ABC transporter permease |
| **ID_45** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | SNP | alcohol dehydrogenase |
| **ID_52** | <0.001 | -6.012 | <0.001 | <0.001 | 5.511 | <0.001 | indel | deacetylase |
| **ID_53** | <0.001 | 6.012 | <0.001 | <0.001 | -5.511 | <0.001 | SNP | deacetylase |
| **ID_17** | 0.016 | 3.095 | 0.005 | <0.001 | -3.963 | <0.001 | indel | galactokinase |
| **ID_26** | 0.016 | 3.095 | 0.005 | <0.001 | -3.963 | <0.001 | indel | NA |
| **ID_49** | 0.016 | 3.095 | 0.005 | <0.001 | -3.963 | <0.001 | indel | peptidylprolyl isomerase |
| **ID_04** | 0.027 | -2.764 | 0.010 | <0.001 | 3.752 | <0.001 | indel | NA |
| **ID_11** | 0.027 | -2.764 | 0.010 | <0.001 | 3.752 | <0.001 | indel | DNA polymerase II |
| **ID_12** | 0.027 | -2.764 | 0.010 | <0.001 | 3.752 | <0.001 | indel | NA |

| Gene ID | Adjust P value (G) | T value (G) | P value (G) | Adjust P value (G×E) | T value (G×E) | P value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|---|---|---|
| **ID_18** | 0.027 | -2.764 | 0.010 | <0.001 | 3.752 | ≤0.001 | SNP | galactokinase |
| **ID_36** | 0.027 | -2.764 | 0.010 | <0.001 | 3.752 | ≤0.001 | indel | colicin V secretion protein CvaA |
| **ID_27** | 0.028 | 2.737 | 0.011 | 0.002 | -3.416 | ≤0.001 | indel | NA |
| **ID_09** | 0.252 | 1.650 | 0.110 | 0.002 | -3.391 | ≤0.001 | indel | NA |
| **ID_21** | 0.252 | 1.642 | 0.112 | 0.579 | -0.809 | 0.418 | indel | NA |
| **ID_50** | 0.252 | 1.650 | 0.110 | 0.002 | -3.391 | 0.001 | indel | short-chain dehydrogenase |
| **ID_38** | 0.324 | 1.480 | 0.150 | 0.240 | -1.437 | 0.151 | SNP | immunity protein |
| **ID_33** | 0.363 | 1.393 | 0.175 | 0.007 | -2.973 | 0.003 | indel | NA |
| **ID_28** | 0.504 | 1.171 | 0.252 | 0.965 | -0.088 | 0.930 | indel | glycosyl transferase |
| **ID_02** | 0.585 | 0.948 | 0.352 | 0.125 | -1.839 | 0.066 | indel | NA |
| **ID_19** | 0.585 | -0.935 | 0.358 | 0.771 | -0.464 | 0.643 | SNP | galactokinase |
| **ID_22** | 0.585 | 0.948 | 0.352 | 0.125 | -1.839 | 0.066 | SNP | NA |
| **ID_23** | 0.585 | -1.033 | 0.311 | 0.252 | 1.384 | 0.166 | SNP | galactose/methyl galaxtoside transporter ATP-binding protein |
| **ID_35** | 0.585 | 0.935 | 0.358 | 0.771 | 0.464 | 0.643 | indel | fumarate hydratase |
| **ID_51** | 0.585 | 0.948 | 0.352 | 0.125 | -1.839 | 0.066 | SNP | threonine dehydratase |
| **ID_08** | 0.628 | 0.863 | 0.396 | 0.808 | -0.381 | 0.703 | indel | RNA chaperone Hfq |
| **ID_10** | 0.634 | 0.836 | 0.411 | 0.896 | 0.216 | 0.829 | indel | integrase |
| **ID_03** | 0.639 | -0.707 | 0.485 | 0.841 | -0.322 | 0.747 | indel | NA |
| **ID_25** | 0.639 | -0.779 | 0.443 | 0.252 | 1.368 | 0.171 | indel | NA |

| Gene ID | Adjust P value (G) | T value (G) | P value (G) | Adjust P value (G×E) | T value (G×E) | P value (G×E) | Variation Type | Gene Product |
|---|---|---|---|---|---|---|---|---|
| **ID_31** | 0.639 | 0.728 | 0.473 | 0.125 | -1.802 | 0.072 | SNP | galactosyl transferase |
| **ID_39** | 0.639 | -0.728 | 0.473 | 0.125 | 1.802 | 0.072 | SNP | NA |
| **ID_41** | 0.639 | 0.728 | 0.473 | 0.125 | -1.802 | 0.072 | indel | NA |
| **ID_46** | 0.639 | 0.716 | 0.480 | 0.059 | -2.226 | 0.026 | indel | NA |
| **ID_13** | 0.694 | 0.601 | 0.553 | 0.125 | -1.814 | 0.070 | indel | NA |
| **ID_30** | 0.694 | -0.616 | 0.543 | 0.176 | 1.622 | 0.105 | SNP | galactosyl transferase |
| **ID_00** | 0.735 | -0.513 | 0.612 | 0.658 | 0.675 | 0.500 | SNP | NA |
| **ID_15** | 0.735 | -0.513 | 0.612 | 0.658 | 0.675 | 0.500 | indel | competence protein ComEA |
| **ID_34** | 0.821 | 0.369 | 0.715 | 0.686 | 0.603 | 0.547 | indel | NA |
| **ID_40** | 0.821 | 0.385 | 0.703 | 0.176 | -1.610 | 0.108 | SNP | nuclease PIN |
| **ID_16** | 0.860 | -0.303 | 0.764 | 0.683 | -0.626 | 0.531 | indel | hypothetical protein |
| **ID_14** | 0.902 | 0.215 | 0.831 | 0.937 | 0.144 | 0.885 | indel | NA |
| **ID_47** | 0.902 | -0.210 | 0.835 | 0.252 | -1.364 | 0.173 | indel | NA |
| **ID_29** | 0.903 | 0.187 | 0.853 | 0.382 | 1.107 | 0.269 | SNP | galactosyl transferase |
| **ID_06** | 0.923 | -0.141 | 0.889 | 0.986 | 0.040 | 0.968 | indel | NA |
| **ID_24** | 0.957 | -0.066 | 0.948 | 0.798 | -0.413 | 0.679 | indel | beta-methylgalactoside transporter |
| **ID_48** | 0.957 | -0.054 | 0.957 | 0.885 | -0.249 | 0.803 | SNP | NA |

APPENDIX 2. Linkage group informations according to t-test statistics and the sequenced results.

| Group | Adjust P value (G) | P value (G) | T value (G) | Adjust P value (G×E) | P value (G×E) | T value (G×E) | Variation ype | Gene Product |
|---|---|---|---|---|---|---|---|---|
| Group 1 | | | | | | | | |
| ID 01 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | NA |
| ID 05 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | membrane protein |
| ID 20 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | transcriptional regulator |
| ID 32 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | NA |
| ID 44 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | heme ABC transporter permease |
| ID 45 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | SNP | alcohol dehydrogenase |
| ID 52 | <0.001 | <0.001 | -6.0119 | <0.001 | <0.001 | 5.5106 | indel | deacetylase |
| Group 2 | | | | | | | | |
| ID 07 | <0.001 | <0.001 | 6.0119 | <0.001 | <0.001 | -5.5106 | SNP | condensation protein |
| ID 37 | <0.001 | <0.001 | 6.0119 | <0.001 | <0.001 | -5.5106 | SNP | peptidoglycan synthase |
| ID 42 | <0.001 | <0.001 | 6.0119 | <0.001 | <0.001 | -5.5106 | SNP | transcriptional regulator |
| ID 53 | <0.001 | <0.001 | 6.0119 | <0.001 | <0.001 | -5.5106 | SNP | deacetylase |
| Group 3 | | | | | | | | |
| ID 17 | 0.0162 | 0.0045 | 3.0945 | <0.001 | <0.001 | -3.9625 | indel | galactokinase |
| ID 26 | 0.0162 | 0.0045 | 3.0945 | <0.001 | <0.001 | -3.9625 | indel | NA |

| Group | Adjust P value (G) | P value (G) | T value (G) | Adjust P value (G×E) | P value (G×E) | T value (G×E) | Variation ype | Gene Product |
|---|---|---|---|---|---|---|---|---|
| ID 49 | 0.0162 | 0.0045 | 3.0945 | <0.001 | <0.001 | -3.9625 | indel | peptidylprolyl isomerase |
| Group 4 | | | | | | | | |
| ID 04 | 0.0272 | 0.0101 | -2.7640 | <0.001 | <0.001 | 3.7523 | indel | NA |
| ID 11 | 0.0272 | 0.0101 | -2.7640 | <0.001 | <0.001 | 3.7523 | indel | DNA polymerase II |
| ID 12 | 0.0272 | 0.0101 | -2.7640 | <0.001 | <0.001 | 3.7523 | indel | NA |
| ID 18 | 0.0272 | 0.0101 | -2.7640 | <0.001 | <0.001 | 3.7523 | SNP | galactokinase |
| ID 36 | 0.0272 | 0.0101 | -2.7640 | <0.001 | <0.001 | 3.7523 | indel | colicin V secretion protein CvaA |

APPENDIX 3. Gene data frame shift informations. Gene variation type declare the variable locus a SNP (single-nucleotide polymorphism) or an indel (insertion-deletion). Allele Start/Allele End declare the coordinate of the loci in the genome alignments.

| Gene ID | Gene Variation Type | Allele Start | Allele End | Reference Allele in ancestor | Alternative Allele | Gene Product |
|---|---|---|---|---|---|---|
| ID 00 | SNP | 31752 | 31753 | C | T | NA |
| ID 01 | SNP | 40238 | 40239 | T | G | NA |
| ID 02 | indel | 70546 | 70547 | - | T | NA |
| ID 03 | indel | 92160 | 92161 | G | - | NA |
| ID 04 | indel | 108316 | 108317 | - | T | NA |
| ID 05 | SNP | 131842 | 131843 | T | G | membrane protein |
| ID 06 | indel | 173553 | 173554 | - | T | NA |
| ID 07 | SNP | 328603 | 328604 | C | T | condensation protein |
| ID 08 | indel | 391162 | 392421 | - | TAGTGG……ACCATG | RNA chaperone Hfq |
| ID 09 | indel | 431150 | 431151 | - | T | NA |
| ID 10 | indel | 524141 | 525400 | - | CAATGT……ACTACA | integrase |
| ID 11 | indel | 754483 | 754484 | - | T | DNA polymerase II |
| ID 12 | indel | 773055 | 773056 | - | A | NA |
| ID 13 | indel | 917058 | 917059 | - | A | NA |
| ID 14 | indel | 981644 | 981645 | - | T | NA |
| ID 15 | indel | 1096043 | 1096044 | - | T | competence protein ComEA |
| ID 16 | indel | 1187546 | 1187547 | - | T | hypothetical protein |
| ID 17 | indel | 1314190 | 1314208 | GCATC……CCAGC | - | galactokinase |
| ID 18 | SNP | 1314262 | 1314263 | A | T | galactokinase |
| ID 19 | SNP | 1314523 | 1314524 | C | T | galactokinase |
| ID 20 | SNP | 1319872 | 1319873 | T | C | transcriptional regulator |
| ID 21 | indel | 1424407 | 1424408 | C | - | NA |
| ID 22 | SNP | 1612224 | 1612225 | G | T | NA |
| ID 23 | SNP | 1615315 | 1615316 | G | A | galactose/methyl galaxtosID e transporter ATP-binding protein |

| Gene ID | Gene Variation Type | Allele Start | Allele End | Reference Allele in ancestor | Alternative Allele | Gene Product |
|---|---|---|---|---|---|---|
| ID 24 | indel | 1616564 | 1616573 | - | GGCGGTGGT | beta-methylgalactosID e transporter inner membrane component |
| ID 25 | indel | 1652369 | 1652390 | - | ATCTACCATCTACCATCTACC | NA |
| ID 26 | indel | 1652834 | 1652855 | TACCATCTACCATCTACCATC | - | NA |
| ID 27 | indel | 1652855 | 1652876 | - | TACCA…...ACCATC | NA |
| ID 28 | indel | 1669779 | 1669780 | - | A | glycosyl transferase |
| ID 29 | SNP | 1673985 | 1673986 | G | T | galactosyl transferase |
| ID 30 | SNP | 1674194 | 1674195 | C | A | galactosyl transferase |
| ID 31 | SNP | 1674208 | 1674209 | G | A | galactosyl transferase |
| ID 32 | SNP | 1865065 | 1865066 | T | C | NA |
| ID 33 | indel | 2148521 | 2148522 | - | T | NA |
| ID 34 | indel | 2286323 | 2286324 | - | T | NA |
| ID 35 | indel | 2357167 | 2357168 | T | - | fumarate hydratase |
| ID 36 | indel | 2387934 | 2387935 | - | A | colicin V secretion protein CvaA |
| ID 37 | SNP | 2460179 | 2460180 | A | T | peptID oglycan synthase |
| ID 38 | SNP | 2470427 | 2470428 | C | T | immunity protein |
| ID 39 | SNP | 2945725 | 2945726 | C | T | NA |
| ID 40 | SNP | 3165202 | 3165203 | G | T | nuclease PIN |
| ID 41 | indel | 3412436 | 3412454 | CGCTCGCTTTCCCTGCTG | - | NA |
| ID 42 | SNP | 3481207 | 3481208 | A | G | transcriptional regulator |
| ID 43 | indel | 3604351 | 3604352 | A | - | NA |
| ID 44 | SNP | 3611458 | 3611459 | T | C | heme ABC transporter permease |
| ID 45 | SNP | 3873060 | 3873061 | T | C | alcohol dehydrogenase |

| Gene ID | Gene Variation Type | Allele Start | Allele End | Reference Allele in ancestor | Alternative Allele | Gene Product |
|---|---|---|---|---|---|---|
| ID 46 | indel | 4029566 | 4029567 | - | A | NA |
| ID 47 | indel | 4340905 | 4340914 | TTTCAGGGA | - | NA |
| ID 48 | SNP | 4366595 | 4366596 | C | T | NA |
| ID 49 | indel | 4849680 | 4849681 | G | - | peptID ylprolyl isomerase |
| ID 50 | indel | 4876832 | 4876833 | - | A | short-chain dehydrogenase |
| ID 51 | SNP | 4928598 | 4928599 | A | T | threonine dehydratase |
| ID 52 | indel | 5014694 | 5014695 | C | - | deacetylase |
| ID 53 | SNP | 5014711 | 5014712 | A | G | deacetylase |