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Environmental Factors Influence the Competition Between Different Antibiotic Resistant Mutants of *Escherichia coli*

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ABSTRACT OF THE THESIS

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Varying conditions in the environment can act as factors that influence the emergence and growth of antibiotic-resistant bacteria. To explore the effects of the environmental conditions on different antibiotic resistant mutants, we monitored the growth curves of the wild type and two strongly antibiotic resistant mutants and three mildly antibiotic resistant mutants of E.coli K-12 and conducted competition tests in pairs under different temperature, pH and salinity concentrations. Wild type was dominant at all experimental conditions, while adding antibiotic and pesticides selection stress reversed this overwhelming superiority only when we changed the incubation temperature at pH=7. The strongly resistant mutant gained more growth advantages as the temperature and pH increased and outcompeted the mildly resistant mutants. When we added additional selection pressure under experimental conditions, we found that the antibiotic and pesticide stress made this growth advantage of the strongly resistant mutant more obvious

and further inhibited the growth of the wild type and mildly resistant mutants. These findings provide suggestions for the impact of environmental factors on the growth of various antibiotic resistant bacteria and the effective elimination of strong antibiotic resistant bacteria in certain conditions in the natural environment.

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INTRODUCTION

Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines. It accounts for hundreds of thousands of deaths annually, and has become one of the biggest threats to global health, food security, and development today.[1-2] Antibiotic resistance can occur naturally, but misuse of antibiotics in humans and animals is accelerating the process. Inappropriate use of antibiotics by humans, factories, farms, and sanitation are considered important reasons in the emergence and distribution of antibiotic-resistant bacteria.[3-5] Over the past years, the role of the environment as an important source and dissemination route of antibiotic resistance has been increasingly recognized. Many antibiotic-resistant bacteria will ultimately end up in the environment, such as wastewater, sludge, soil, sea water and river water. There is a growing concern that the varying conditions in the environment can act as factors that influence the emergence and growth of antibiotic-resistant bacteria.

Previous studies showed that for different aquatic systems, such as hospital wastewater, urban wastewater, landfill leachates, and drinking water, antibiotic resistant genes (ARG) abundances did not correlate well with antibiotic levels.[6] This made researchers realize that there may be other factors involved in the selection and development of antibiotic resistance. For example, heavy metals may assist the acquisition and maintenance of ARGs,[7] while oxidative stress may help to eliminate the resistant bacteria. Some researchers show the abundance of ARGs is positively related with wastewater quality.[8]

These findings open an intriguing and profound question on the roles of varying conditions of environments in the growth of antibiotic resistant bacteria.

It has been reported that pH, temperature, salt concentration act additively to affect the growth rate.[9] Conner and Kotrola[10] reported that the growth of *E.coli* was inhibited at pH=4 adjusted with citric acid. Parhad and Rao[11] found that *E.coli* failed to grow and was eliminated within one day when incubated under high pH.

Here, we want to explore what environmental conditions will make an antibiotic-resistant strain outcompeting another. We examined and compared the growth of wild type and five derived antibiotic resistant mutants of *E.coli* K-12 under different incubation temperature, pH and salinity concentrations to find some environmental conditions that may favor the wild-type or mildly resistant mutant, and that could inhibit the growth and survival of antibiotic resistant bacteria.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. The bacterial strains used in this study are listed in Table 1. *E. coli* K-12 resistant mutants with *rpsL, dsbC, glnE, nuoG548, rsmG, yaiW, nuoG10,* and *sbmA* are streptomycin-resistant strains derived from the *E. coli* K-12 wild-type from an exposure experiment under a low level (1/5 minimum inhibitory concentration, 1/5 MIC) of streptomycin together with environmental levels (20-2000 μg/L total) of pesticides for 500 generations. The medium used for this study is Luria-Bertani (LB) broth. *E. Coli* K-12 wild-type and mutants starter cultures were revived from -80 ºC by adding 2uL of thawed aliquots into 2mL of fresh LB medium and

incubated overnight at 37 ºC at 200 rpm on an orbital shaker. These cultures were regarded as G0.

Table 1. Bacteria strains used in this study

Strains	Genotypes	Fold change of MIC_0
WT	Wild-type, gram-negative *Escherichia coli* strain K-12 (ATCC. 10798)	1
S1	*rpsL* (Arg86Ser), *rsmG* (Trp150fs)	25
S2	*rpsL* (Arg86Ser), *rsmG* (Trp150fs), *dsbC* (Val172Glu)	40
M1	*glnE* (Ala423Val), *yaiW* mutations (Phe183Ile, Gln186Asp, His187fs)	4
M2	*nuoG* (Ser548*)	3
M3	*glnE* (Ala423Val), *nuoG* (Glu10*), *sbmA* (Glu282*)	3

Competition Test and Culture Conditions. To do the competition test, we have 9 pairs of strains for competition. They are: WT/S1, WT/S2, WT/M1, WT/M2, WT/M3, S2/S1, S2/M1, S2/M2, and S2/M3. Environmental conditions studied included temperature (20 ºC, 30 ºC, 37 ºC, 45 ºC), pH (5, 6, 7, 8), and salinity (KCl 50 mM, 100 mM, 200 mM, 300 mM). Assays were conducted for each of the single conditions, followed by combining the selected environmental conditions (pH=5, 6, 7, 8; T=20 ºC, 30 ºC, 37 ºC, 45 ºC; and KCl 100 mM, 300 mM) with a combination of Streptomycin (Strep) and pesticides (P) as selection stress. The concentrations of Strep is 1/5 of its original minimal inhibitory concentration (MIC_0 = 8 mg/L). The pesticides used in the study is a mixture of different pesticide species that were detected in the environments (Table S1).[12] The concentration of pesticide is 100 times of environmental concentrations.

Temperature was manipulated by setting the temperature of the incubator to 20 ºC, 30 ºC, 37 ºC, 45 ºC. pH was manipulated by adjusting the pH of LB medium with citric acid - sodium citrate buffer solutions (pH=5 and pH=6), or sodium carbonate - sodium bicarbonate buffer solutions (pH=8). Salinity was manipulated by adding 12.8 uL, 25.6 uL, 51.2 uL and 76.8uL of 4 M KCl into fresh LB medium to generate 50 mM, 100 mM, 200 mM and 300 mM KCl solutions. The combination of environmental conditions with selection stress was carried out by first adding 10.24 uL of the pesticide mixture stock solution in methanol with the concentration of 10^4 times of environmental concentration into the wells, and then adding appropriate volumes of LB medium with designated pH or KCl concentration and 1.64 uL of 1 g/L Strep stock solution after the methanol was completely evaporated.

Cultures of the strains were diluted in fresh LB medium to an equal optical density (OD) measured at 600 nm. And then, every pair of strains that were compared were mixed at the ratio around 1:1 for inoculation. 1 uL of 1:1 mixed cultures were inoculated into LB medium with different experimental conditions to the total volume of 1024 uL, and incubated overnight at the specified experimental temperature at 200 rpm on an orbital shaker. The cultures were serially passaged into LB medium with the same experimental conditions every day and incubated under the same conditions for three days, resulting in approximately 30 generations for each condition tested. Each pair and condition had three replicates. After 30 generations, 700 uL of the cultures were centrifuged to collect cell pellets. The remaining cultures were archived by adding 200 μL of 50% glycerol and stored at −80 ºC.

Growth Curves Determination. *E. coli* strains were grown overnight to stationary phase in 2 ml LB medium at 37 ºC. Culture volumes of 1uL were transferred into 255 uL of LB medium with experimental conditions on a 96-well plate, which was sealed with a cover foil and incubated in a microplate reader (BioTek Instruments, Winooski, VT), and the OD_{600} was automatically measured every 30 min for 24 h.

DNA Extraction and SNP genotyping. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA concentrations were determined on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE). After the concentrations of DNA were determined, we diluted them to a uniform concentration, which is 3 ng/uL, to do SNP genotyping.

We used PCR-based SNP genotyping assay via Custom TaqMan SNP Genotyping Assays (Thermo Fisher Scientific). The assay could target the mutant alleles identified in *rpsL*, *dsbC*, *glnE*, *nuoG548* and *nuoG10*. The assays were performed in accordance with the recommended thermal cycling conditions in 96-well plates on the qPCR instrument QuantStudio 3. According to the manufacturer's instruction, the genotyping reactions were carried out in a total volume of 5 μL. The isolated mutant with the *rpsL*, *dsbC*, *glnE*, *nuoG548* and *nuoG10* mutant alleles confirmed by the SNP genotyping assay and the wild type were used as controls. To quantify the fraction of each pair of strains, we also set different fractions of the standard genomic DNA of the first strain to the standard genomic DNA of the second strain at: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%. Three biological replicates were performed. Thermo Fisher Cloud "Genotyping" application was used to generate allele calls.

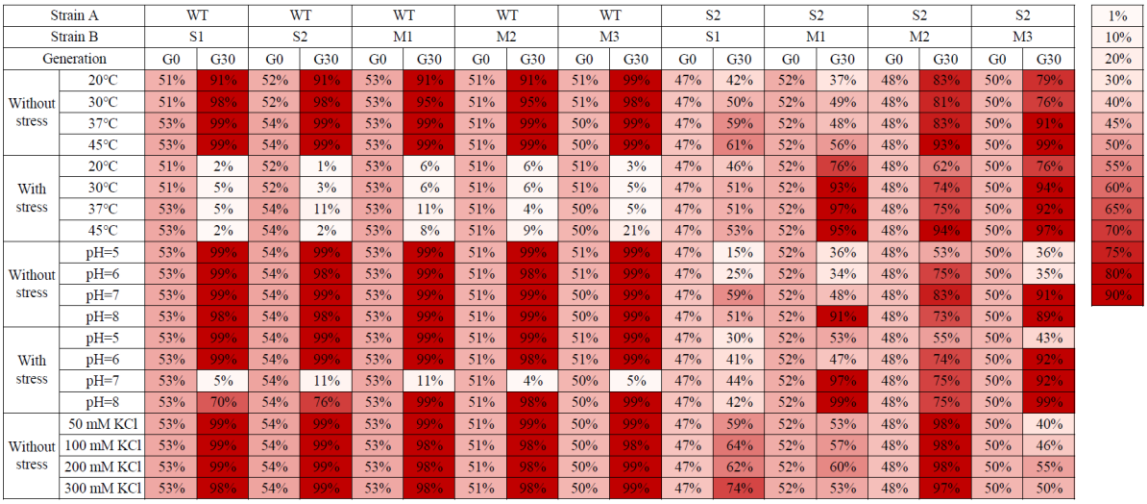
Statistical analyses. All statistical analyses were performed by R version 4.0.2. One-way ANOVA was performed for statistical analysis. Comparisons were performed by LSD tests (p<0.05).

RESULTS AND DISCUSSION

Wild-type outcompeted resistant mutants under conditions without selection stress. We conducted a competition test between the WT and five antibiotic-resistant mutants under different environmental conditions, including temperature, pH, and salinity. The initial fractions of WT/S1, WT/S2, WT/M1, WT/M3 and WT/M2 were around 50%. The results showed that the fractions of each mutant to WT mostly became 1% or 2% after the 30-genefractionn competition test. This indicated that the WT outcompeted the selected resistant mutants under the tested environmental conditions when there was no Streptomycin and pesticide selection stress. Mutations may have fitness costs and cause defects to the cell during the growth.[13] We monitored the growth of the wild-type and the selected mutant strains of *E. coli* K-12. Table 2 shows the growth parameters for the tested strains at four temperatures (20 ºC, 30 ºC, 37 ºC, 45 ºC), at four pH (5, 6, 7, 8), at different concentrations of KCl (50 mM, 100 mM, 200 mM, 300 mM) and under combined conditions with selection stress. At most tested conditions, WT reached a higher optical density after 24h incubation than the resistant mutants, which resulted in the WT outcompeting resistant mutants. However, at some conditions, the optical density after 24-h incubation of WT was lower than resistant mutants. But we observed from the growth curve that under these conditions, WT had the largest

maximum growth rate or had a relatively shorter lag phase. The rapid growth rate and short lag phase quickly established the growth advantage of WT, allowing WT to make full use of nutrients in the medium, thus inhibiting other resistant mutants.[14]

Figure 1. Growth competition between WT, S1, S2, M1, M2, and M3 under different conditions. The percentage represents the fraction of strain A in the total of strain A and strain B.



Strain A		WT		WT		WT		WT		WT		S2		S2		S2		S2	
Strain B		S1		S2		M1		M2		M3		S1		M1		M2		M3	
Generation		G0	G30	G0	G30	G0	G30	G0	G30	G0	G30	G0	G30	G0	G30	G0	G30	G0	G30
Without stress	20°C	51%	91%	52%	91%	53%	91%	51%	91%	51%	99%	47%	42%	52%	37%	48%	83%	50%	79%
	30°C	51%	98%	52%	98%	53%	95%	51%	95%	51%	98%	47%	50%	52%	49%	48%	81%	50%	76%
	37°C	53%	99%	54%	99%	53%	99%	51%	99%	50%	99%	47%	59%	52%	48%	48%	83%	50%	91%
	45°C	53%	99%	54%	99%	53%	99%	51%	99%	50%	99%	47%	61%	52%	56%	48%	93%	50%	99%
With stress	20°C	51%	2%	52%	1%	53%	6%	51%	6%	51%	3%	47%	46%	52%	76%	48%	62%	50%	76%
	30°C	51%	5%	52%	3%	53%	6%	51%	6%	51%	5%	47%	51%	52%	93%	48%	74%	50%	94%
	37°C	53%	5%	54%	11%	53%	11%	51%	4%	50%	5%	47%	51%	52%	97%	48%	75%	50%	92%
	45°C	53%	2%	54%	2%	53%	8%	51%	9%	50%	21%	47%	53%	52%	95%	48%	94%	50%	97%
Without stress	pH=5	53%	99%	54%	99%	53%	99%	51%	99%	51%	99%	47%	15%	52%	36%	48%	53%	50%	36%
	pH=6	53%	99%	54%	98%	53%	99%	51%	98%	51%	99%	47%	25%	52%	34%	48%	75%	50%	35%
	pH=7	53%	99%	54%	99%	53%	99%	51%	99%	50%	99%	47%	59%	52%	48%	48%	83%	50%	91%
	pH=8	53%	98%	54%	98%	53%	99%	51%	99%	50%	99%	47%	51%	52%	91%	48%	73%	50%	89%
With stress	pH=5	53%	99%	54%	99%	53%	99%	51%	99%	51%	99%	47%	30%	52%	53%	48%	55%	50%	43%
	pH=6	53%	99%	54%	99%	53%	99%	51%	98%	51%	99%	47%	41%	52%	47%	48%	74%	50%	92%
	pH=7	53%	5%	54%	11%	53%	11%	51%	4%	50%	5%	47%	44%	52%	97%	48%	75%	50%	92%
	pH=8	53%	70%	54%	76%	53%	99%	51%	98%	50%	99%	47%	42%	52%	99%	48%	75%	50%	99%
Without stress	50 mM KCl	53%	99%	54%	99%	53%	99%	51%	99%	50%	99%	47%	59%	52%	53%	48%	98%	50%	40%
	100 mM KCl	53%	99%	54%	99%	53%	98%	51%	98%	50%	98%	47%	64%	52%	57%	48%	98%	50%	46%
	200 mM KCl	53%	99%	54%	99%	53%	98%	51%	98%	50%	99%	47%	62%	52%	60%	48%	98%	50%	55%
	300 mM KCl	53%	98%	54%	99%	53%	98%	51%	98%	50%	99%	47%	74%	52%	53%	48%	97%	50%	50%

Influence of pH on competition between antibiotic resistant mutants. Acidity and alkalinity of the environment also significantly affected the growth kinetics of the tested strains. Baka et.al15 observed that the lowest pH yielding growth of *E.coli* K-12 was 4.5, the highest pH value for which growth was observed was 8.5, and the optimal pH for *E. coli* K-12 is around 7-7.5. The change of pH caused the lag phase to become longer and the maximum growth rate to become slower. Acidic conditions had a more obvious inhibitory effect than alkaline conditions. We competed the strongly resistant mutant S2 with another strongly resistant mutant S1 and three mildly resistant mutants M1, M2, and

M3 under four different pH. The competition results under different pH were shown in Figure 1 and Figure 2A. S2 had a growth advantage and became outcompeting in neutral and alkaline conditions. However, as the pH decreasing, S2 gradually lost its growth advantage. When competing with S1 in pH=7, S2 slightly outcompeted S1 with the fraction of S2 increasing to 59% after the competition. But when the pH decreased to pH=5 and pH=6, S2 lost its growth advantage and S1 outcompeted S2 significantly. As we can observed from the growth kinetic parameters (Figure 1) and growth curves (SI), S1 had a significant shorter lag phase ($p<0.05$) than S2 at pH=5 and reached to a higher optical density than S2 at pH=6. At pH=8, the growth of S1 and S2 were similar, which resulted in the competition fraction of around 50%. When incubating under acidic conditions, M1 and M3 outcompeted S2 significantly. The growth kinetics showing that M1 and M3 reached to higher cell densities than S2 also supported this conclusion (Figure 1). When incubated at pH=8, M1 and M3 lost growth advantages and S2 became outcompeted. The growth parameters also shown that S2 had higher optical density than M1 and M3. However, although the growth curves showed that M2 had higher or same cell densities than S2, the competition results were that S2 outcompeted M2 at all four different pH. Therefore, S2 was more susceptible to acidic conditions and was inhibited in the process of competing with other mutants for growth, thus losing its growth advantage.

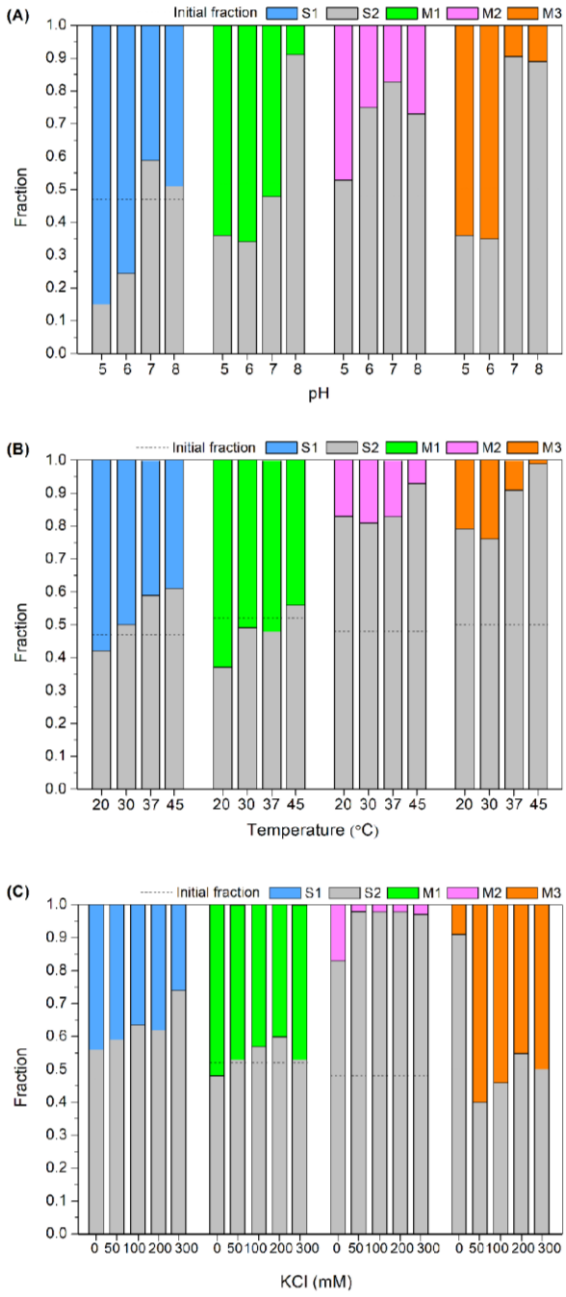


Figure 2. Fractions of growth competition between S1, S2, M1, M2, and M3 after 30 generations when incubated without selection pressure under different pH (A), temperatures (B), and KCl (C). Dash lines indicated the initial fraction of each pair of strains at G0.

Table 2. Growth kinetics of E.coli K-12 strains at different conditions without selection stress

	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD
Conditions	**pH=5**			**pH=6**			**pH=7**			**pH=8**		
WT	1.07^{ab}	1.59^{a}	3.33^{c}	1.47^{a}	6.19^{a}	1.53^{c}	1.33^{bc}	3.35^{a}	1.16^{b}	1.16^{a}	2.69^{a}	2.56^{a}
S1	0.75^{c}	1.09^{c}	6.10^{b}	0.95^{bc}	2.10^{c}	3.86^{a}	1.49^{a}	3.08^{b}	1.46^{a}	0.74^{bc}	1.34^{c}	1.57^{b}
S2	0.76^{c}	1.08^{c}	7.96^{a}	0.81^{c}	1.70^{c}	2.13^{b}	1.41^{ab}	3.21^{ab}	1.45^{a}	0.81^{bc}	1.64^{bc}	1.17^{b}
M1	0.95^{b}	1.65^{a}	2.13^{e}	1.29^{ab}	1.92^{c}	1.75^{c}	1.04^{d}	3.26^{ab}	1.43^{a}	0.83^{bc}	1.89^{b}	1.46^{b}
M2	1.12^{a}	1.40^{a}	2.02^{e}	1.29^{ab}	3.90^{b}	1.56^{c}	1.33^{bc}	2.44^{d}	1.00^{b}	0.85^{b}	1.79^{bc}	1.34^{b}
M3	0.96^{ab}	1.31^{a}	2.92^{d}	1.21^{ab}	5.4^{ab}	2.05^{b}	1.29^{c}	2.79^{c}	1.16^{b}	0.72^{c}	1.84^{b}	1.40^{b}
Conditions	**20°C**			**30°C**			**37°C**			**45°C**		
WT	1.50^{a}	1.72^{b}	1.78^{d}	1.51^{b}	2.26^{a}	1.70^{c}	1.33^{bc}	3.35^{a}	1.16^{b}	1.00^{a}	2.23^{b}	0.61^{c}
S1	1.50^{a}	1.78^{b}	2.44^{ab}	1.60^{a}	2.08^{ab}	2.79^{a}	1.49^{a}	3.08^{b}	1.46^{a}	0.79^{b}	2.01^{c}	0.93^{a}
S2	1.37^{b}	1.77^{b}	2.47^{ab}	1.59^{a}	1.95^{bc}	2.27^{ab}	1.41^{ab}	3.21^{ab}	1.45^{a}	0.78^{bc}	2.06^{c}	0.94^{a}
M1	1.54^{a}	2.01^{a}	2.06^{cd}	1.56^{ab}	2.11^{ab}	1.68^{c}	1.04^{d}	3.26^{ab}	1.43^{a}	0.75^{bcd}	2.46^{a}	0.64^{bc}
M2	1.37^{b}	1.76^{b}	2.36^{bc}	1.33^{c}	2.16^{ab}	1.76^{bc}	1.33^{bc}	2.44^{d}	1.00^{b}	0.64^{d}	2.26^{b}	0.77^{b}
M3	1.30^{b}	1.80^{b}	2.69^{a}	1.25^{d}	1.78^{c}	1.82^{bc}	1.29^{c}	2.79^{c}	1.16^{b}	0.67^{cd}	2.24^{b}	0.77^{b}
Conditions	**KCl 0 mM**			**KCl 100 mM**			**KCl 300 mM**					
WT	1.33^{bc}	3.35^{a}	1.16^{b}	1.43^{a}	3.11^{a}	0.91^{b}	1.32^{a}	3.52^{a}	1.79^{a}			
S1	1.49^{a}	3.08^{b}	1.46^{a}	1.33^{ab}	2.73^{bc}	1.04^{ab}	1.21^{b}	2.63^{c}	1.70^{a}			
S2	1.41^{ab}	3.21^{ab}	1.45^{a}	1.35^{ab}	2.93^{ab}	1.07^{ab}	1.20^{b}	2.60^{cd}	1.71^{a}			
M1	1.04^{d}	3.26^{ab}	1.43^{a}	1.31^{b}	3.14^{a}	1.33^{a}	1.18^{b}	3.17^{b}	1.71^{a}			
M2	1.33^{bc}	2.44^{d}	1.00^{b}	1.19^{c}	2.69^{bc}	0.73^{b}	1.08^{c}	2.35^{de}	0.82^{b}			
M3	1.29^{c}	2.79^{c}	1.16^{b}	0.99^{d}	2.47^{c}	0.82^{b}	0.93^{d}	2.17^{e}	1.01^{b}			

OD24h=optical density after 24h incubation; Vmax=maximum growth rate; LPD=lag phase duration. Values with different letters are significantly different ($p<0.05$).

Influence of temperature on competition between antibiotic resistant mutants. Temperature was considered as one of most important factors of inhibiting antibiotic resistant bacteria.[16] Duffy[17] found that the temperature had a significant effect on the growth kinetics of *E. coli* with a lower growth temperature significantly lengthening the lag phase and slowing the growth rate from that observed at 37 ºC. When we lowered the growth temperature to 20 ºC and 30 ºC, or increased the temperature to 45 ºC, the lag

phase of all the strains became longer and the maximum growth rate slower than at 37 ºC. When we increased the temperature to 45 ºC, all the strains were greatly inhibited and reached to the lowest OD_{24h}. Therefore, higher temperature seems to favor for the elimination of resistant mutants.[6]

The fractions of each pair of mutant strains after 30 generations were showed in Figure 1. For the competition between two strongly resistant mutants S1 and S2, there was an increasing trend of the fraction of S2 to S1 with the increase of temperature, and S1 outcompeted S2 at 20 ºC, 30 ºC and 37 ºC (Figure 2B). When we increased the temperature to 45 ºC, S2 became outcompeting but the growth advantage was not obvious. The competition between S2 and the mildly resistant mutant M1 had a similar increasing trend with the fraction increasing from 37% at 20 ºC to 56% at 45 ºC. For the other two mildly resistant mutant M2 and M3, S2 outcompeted at all tested temperatures, and the growth advantages of S2 over M2 and M3 were evident.

Influence of salinity on competition between strongly resistant mutants and mildly resistant mutants. The effects of different concentrations of KCl were diverse on the mutants used in this experiment. As the concentration of KCl increased, S2 gradually showed more growth advantages over S1, with the fraction of S2 to S1 risen from 59% to 74%. This indicated that high concentration of KCl was more suitable for S2 but inhibited the growth of S1. The competitions between M1 and S2 were roughly similar to that of S1 and S2. As for M2, the fraction of S2 to M2 increased from 83% to nearly 100% and it did not matter how much KCl we added. The OD_{24h} of M2 were

significantly smaller relative to the condition without KCl. The growth of M2 was significantly inhibited by the addition of KCl compared with the no-KCl condition. It was noted that the growth advantage of M3 over S2 was greatly promoted when incubated under conditions with KCl. There were astounding 36%-51% decrease. However, the growth curves showed that the OD_{24h} and V_{max} of M3 were smaller than that of S2. But we noted that the LPD of M3 was significantly shorter than that of S2, from which we could speculate that the shorter LPD made it more advantageous for the growth of M3 at the beginning of the competition. However, although the addition of KCl helped M3 to establish the growth advantages over S2, M3 did not outcompete S2 at higher KCl concentrations, and the results were that these two mutants grew roughly equally well.

Adding selection stress changed the competition results under certain conditions. When we combined (1/5 Strep, 100 P) selection stress with pH, the competition results changed greatly that WT was outcompeted by the antibiotic resistant mutants at pH=7 (Figure 1). The growth kinetics showed that the growth of WT at pH=7 had been significantly inhibited (Table 3). The V_{max} became slower and the stationary phase was shorter that the cells soon came to the death phase. However, the selection stress did not have significant effect at pH=5, pH=6 and pH=8. The change of pH, no matter increasing or decreasing, reversed the effect of antibiotic and pesticide on WT, and WT became the dominant strain in the culture again. When we compare S1 and S2 under different pH conditions with selection stress, we found that the fractions of S2 did not change much and were roughly around 40%. When competed with mildly resistant mutants, S2 had absolute growth advantages in neutral and alkaline conditions. The growth advantages

were also reflected in the growth kinetics that S2 had slightly higher OD_{24h} and V_{max} than the other three mildly resistant mutants at pH=7 and pH=8.

As for the combination of selection stress and temperature, the competition results showed that all the antibiotic resistant mutants outcompeted WT at the four tested temperatures (Figure 1). As expected, the combination had a significant effect on the growth kinetics of WT with selection stress, significantly slowing the V_{max} and reducing the OD_{24h} (Table 3). The mildly resistant mutants were also inhibited by the selection stress, but the effects were not as evident as WT. The V_{max} of strongly resistant mutants increased, and the LPD decreased after adding selection stress. S2 outcompeted the other four mutants and the growth advantages became more obvious as the temperature increased (Figure 3B).

When we combined selection stress with salinity, both S1 and S2 outcompeted WT under conditions with stress. But as the concentration of KCl in the medium increasing, S1 and S2 mutants gradually lost their growth advantages over WT, as the fractions of WT to the two strongly resistant mutants increased around 40%. As for competition between S1 and S2, the addition of selection to the salinity would benefit the growth of S1 compared to S2, but the growth of two strongly resistant mutants under such conditions were similar, with the final fraction remained close to the initial fraction.

Table 3. Growth kinetics of E.coli K-12 strains at different conditions with selection stress**.**

	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD	OD_{24h}	V_{max}	LPD
Conditions	**pH=5**			**pH=6**			**pH=7**			**pH=8**		
WT	1.08^{a}	1.58^{a}	3.21^{c}	1.35^{b}	7.21^{a}	1.72^{bc}	0.76^{c}	2.79^{b}	0.97^{c}	1.06^{a}	2.63^{a}	2.89^{a}
S1	0.87^{b}	1.12^{cd}	6.04^{b}	0.74^{d}	1.45^{d}	1.88^{b}	1.42^{a}	3.05^{a}	1.48^{a}	0.87^{b}	2.09^{b}	2.39^{b}
S2	0.81^{bc}	1.00^{d}	7.72^{a}	1.04^{c}	1.70^{d}	1.77^{bc}	1.45^{a}	3.02^{a}	1.25^{ab}	0.81^{bc}	2.12^{b}	2.48^{b}
M1	0.75^{bc}	1.57^{a}	2.11^{e}	1.35^{b}	5.70^{b}	1.64^{c}	1.19^{b}	2.40^{c}	0.70^{d}	0.78^{bc}	1.92^{bc}	1.38^{c}
M2	0.65^{cd}	1.41^{b}	2.00^{e}	1.53^{a}	2.89^{c}	1.55^{c}	1.16^{b}	2.17^{d}	0.89^{cd}	0.77^{bc}	1.72^{c}	1.22^{c}
M3	0.52^{d}	1.2^{c}	2.86^{d}	1.23^{b}	4.95^{b}	2.14^{a}	1.11^{b}	2.46^{c}	1.23^{b}	0.73^{c}	1.80^{bc}	1.46^{c}
Conditions	**20℃**			**30℃**			**37℃**			**45℃**		
WT	0.56^{d}	1.36^{b}	1.46^{b}	0.89^{e}	2.58^{a}	2.41^{b}	0.76^{c}	2.79^{b}	0.97^{c}	0.56^{c}	2.25^{ab}	0.68^{b}
S1	1.47^{a}	1.90^{a}	2.71^{a}	1.50^{a}	2.34^{b}	2.85^{a}	1.42^{a}	3.05^{a}	1.48^{a}	0.74^{a}	2.07^{bc}	1.00^{a}
S2	1.44^{ab}	1.75^{a}	2.40^{a}	1.54^{a}	2.34^{b}	2.69^{a}	1.45^{a}	3.02^{a}	1.25^{ab}	0.73^{a}	2.04^{bc}	0.96^{a}
M1	1.30^{bc}	1.89^{a}	2.61^{a}	0.97^{d}	2.55^{a}	2.18^{bc}	1.19^{b}	2.40^{c}	0.70^{d}	0.64^{b}	2.34^{a}	0.64^{b}
M2	1.27^{c}	1.87^{a}	2.75^{a}	1.28^{b}	2.22^{b}	2.24^{b}	1.16^{b}	2.17^{d}	0.89^{cd}	0.56^{c}	1.98^{c}	0.68^{b}
M3	1.22^{c}	1.67^{ab}	2.75^{a}	1.20^{c}	1.93^{c}	1.98^{c}	1.11^{b}	2.46^{c}	1.23^{b}	0.59^{bc}	2.04^{bc}	0.72^{b}
Conditions	**KCl 0 mM**			**KCl 100 mM**			**KCl 300 mM**					
WT	0.76^{c}	2.79^{b}	0.97^{c}	0.75^{d}	2.81^{ab}	0.91^{c}	0.90^{cd}	2.50^{a}	1.39^{abc}			
S1	1.42^{a}	3.05^{a}	1.48^{a}	1.23^{ab}	2.70^{b}	1.32^{a}	1.36^{a}	2.75^{a}	2.26^{a}			
S2	1.45^{a}	3.02^{a}	1.25^{ab}	1.32^{a}	2.84^{ab}	1.07^{bc}	1.15^{abc}	2.47^{a}	1.80^{ab}			
M1	1.19^{b}	2.40^{c}	0.70^{d}	0.84^{d}	2.96^{a}	0.85^{c}	0.85^{d}	2.68^{a}	1.21^{bc}			
M2	1.16^{b}	2.17^{d}	0.89^{cd}	1.15^{b}	2.70^{b}	1.19^{ab}	1.07^{bcd}	2.23^{a}	0.87^{c}			
M3	1.11^{b}	2.46^{c}	1.23^{b}	0.98^{c}	2.66^{b}	1.02^{bc}	1.19^{ab}	2.20^{a}	1.11^{bc}			

OD_{24h}=optical density after 24h incubation; V_{max}=maximum growth rate; LPD=lag phase duration. Values with different letters are significantly different ($P<0.05$).

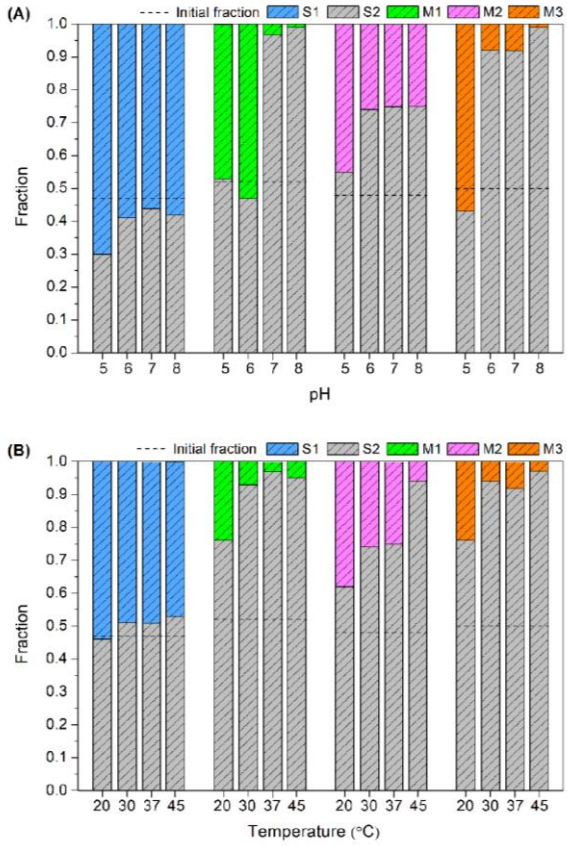


Figure 3. Fractions of growth competition between S1, S2, M1, M2, and M3 after 30 generations when incubated with (1/5 Strep, 100 P) selection stress under different pH (A) and temperatures (B). Dash lines indicated the initial fraction of each pair of strains at G0.

Environmental implications. In the absence of the selection stress, wild type had a completely dominant growth advantage. However, we cannot avoid antibiotics and pesticides in the natural environment, so we studied the combination of selection stress and environmental conditions. High temperature, high pH and high salt concentration were conducive to the growth of the strongly resistant mutant, while adding selection stress would make the growth advantage more obvious. However, low temperature and

acid conditions could weaken and inhibit the growth of the strongly resistant mutant but benefit the growth of mildly resistant mutants while they were competed. Therefore, to eliminate the strong antibiotic resistant bacteria, we should control the antibiotics in the environment and avoid high temperatures and high pH.

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SUPPORTING INFORMATION

Table S1 Environmental concentrations of selected pesticides

Name	**Classification**	**Conc. (µg/L)**	**Environmental samples and references**	**Purchase**
2,4-D	Herbicide	0.2	Urban run-off[1]	Sigma
Atrazine	Herbicide	0.5	Groundwater and surface water[2]	Sigma
Benomyl	Fungicide	0.2	Surface water[3]	Sigma
Carbaryl	Insecticide	4.8	Surface water[4]	Sigma
Carbofuran	Insecticide	0.38	Ground and surface water[5]	Sigma
Chlorpyrifos	Pesticide	0.4	Lake[6]	AK Scientific
Clotrimazole	Fungicide	0.1	Wastewater[7]	AK Scientific
DEET	Biocide	3	Wastewater influent[8]	Sigma
Diazinon	Insecticide	0.3	Wastewater[9]	Sigma
Diuron	Herbicide	1	Urban run-off[1]	AK Scientific
Fipronil	Insecticide	0.2	Urban surface water[10]	AK Scientific
Imazalil	Fungicide	0.4	River[11]	AK Scientific
Imidacloprid	Insecticide	0.4	Ground and surface water[5]	Sigma
Irgarol	Biocide	0.2	Coastal water[12]	Sigma
Linuron	Herbicide	2	Rivers[13]	Sigma
Mecoprop	Herbicide	2	Urban run-off[1]	Sigma
Metaldehyde	Pesticide	0.5	Surface water[14]	Sigma
Metolachlor	Herbicide	0.4	Wastewater[9]	Sigma
Propiconazole	Fungicide	1	Wastewater[15]	Sigma
Tebuconazole	Fungicide	0.5	Wastewater[16]	Sigma
Terbuthylazine	Herbicide	0.65	Ground and surface water[5]	AK Scientific
Terbutryn	Herbicide	0.5	Rivers[17]	Sigma
Thiabendazole	Fungicide	0.2	Wastewater influent[18]	Sigma
Total		**19.9**		

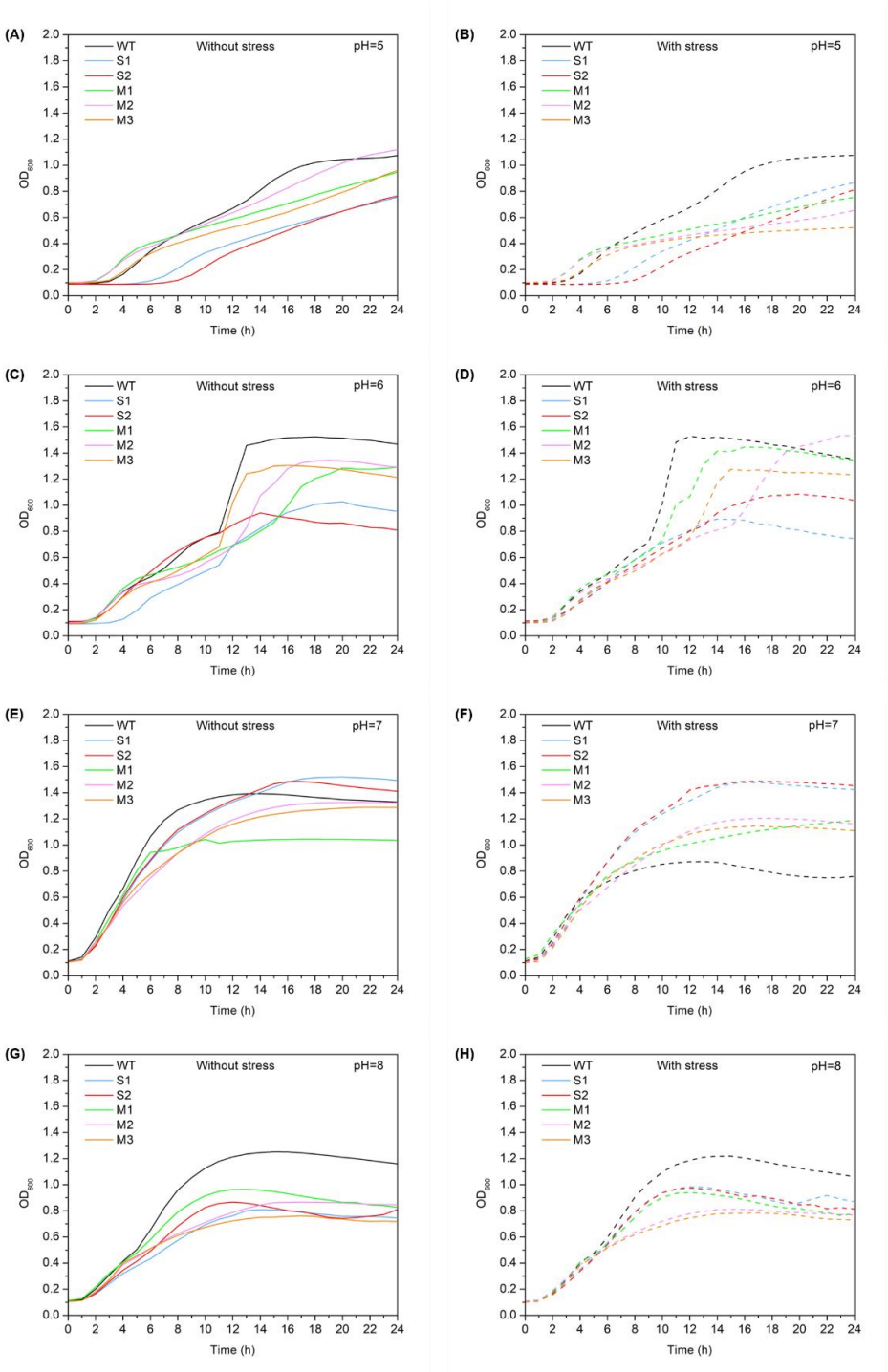

Figure S1. Growth curves of WT, S1, S2, M1, M2, and M3 at pH=5 (without stress (A), with stress (B)), pH=6 (without stress (C), with stress (D)), pH=7 (without stress (E), with stress (F)), and pH=8 (without stress (G), with stress (H)).

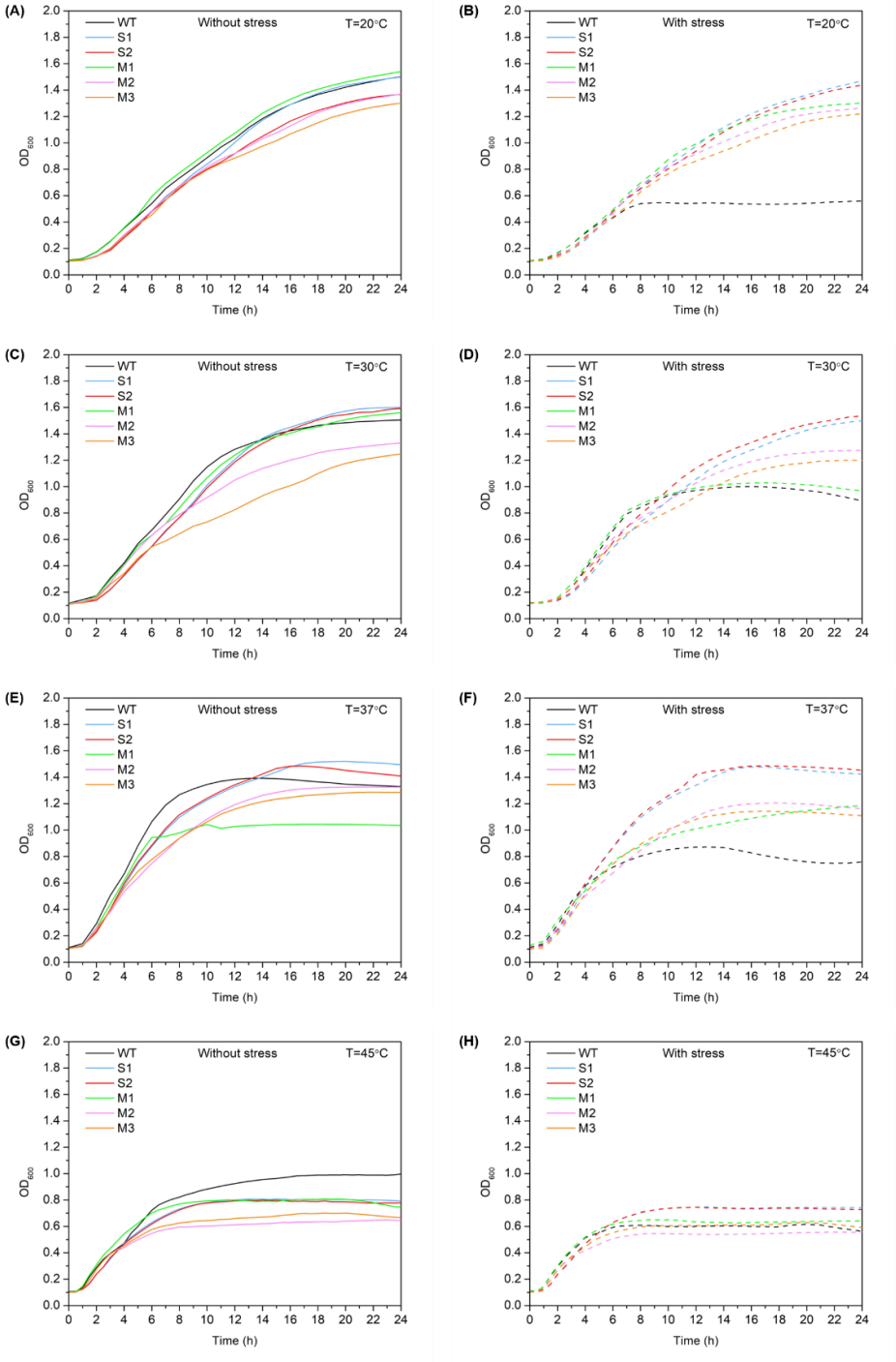

Figure S2. Growth curves of WT, S1, S2, M1, M2, and M3 at T=20 ºC (without stress (A), with stress (B)), T=30 ºC (without stress (C), with stress (D)), T=37 ºC (without stress (E), with stress (F)), and T=45 ºC (without stress (G), with stress (H)).

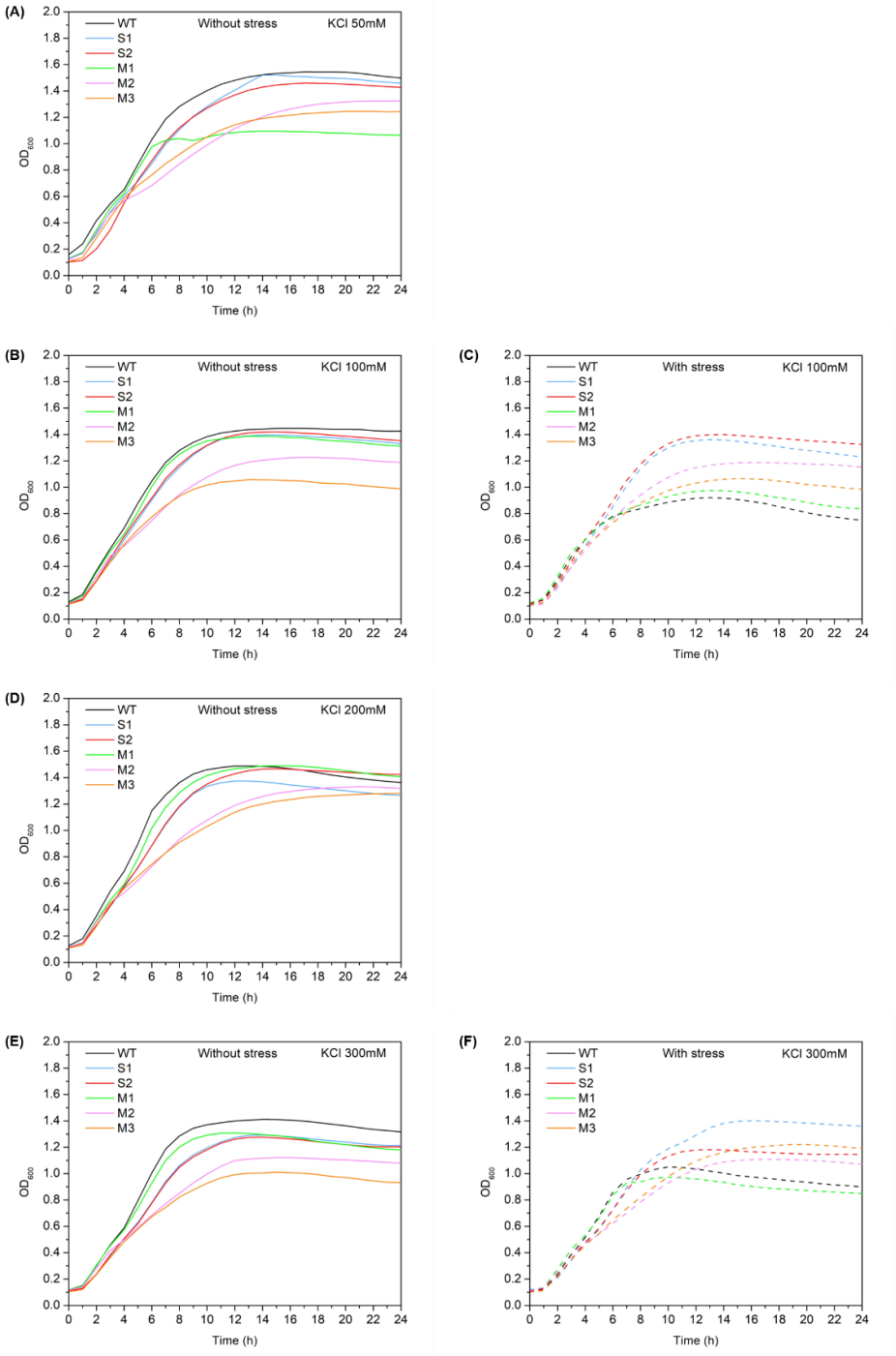

Figure S3. Growth curves of WT, S1, S2, M1, M2, and M3 at KCl=50 mM (without stress (A)), KCl=100 mM (without stress (B), with stress (C)), KCl=200 mM (without stress (D)), and KCl=300 mM (without stress (E), with stress (F)).

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