Original Article

Gradual and Step-wise Halophilization Enables *Escherichia coli* ATCC 8739 to Adapt to 11% NaCl

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Abstract:
Introduction: *Escherichia coli* (*E. coli*) is a non-halophilic microbe and is used to indicate faecal contamination. Salt (sodium chloride, NaCl) is a common food additive and is used in preservatives to counter microbial growth. Previous studies had shown that pathogenic *E. coli* has a higher salt tolerance than non-pathogenic *E. coli*. The effect of how *E. coli* interacts with the salt present in the human diet is under-studied. Thus, it is important to investigate this relationship.
Methods: In this study, we observed the genetic changes and growth kinetics of *E. coli* ATCC 8739 under 3% - 11% NaCl over 80 passages. Growth kinetics was estimated by generation time, cell density and minimum inhibitory concentration (MIC) of NaCl.
Results: Our results suggested that *E. coli* was able to adapt from 1% NaCl to 11% NaCl with an increment of 1% NaCl per month. Our MIC results suggested that *E. coli* was able to grow at NaCl concentration of more than 7.5% based on the Area under Curve (AUC) from 5% at passage 44 (cultured in 5% NaCl) to 13% at passage 72 (cultured at 7% NaCl).
Conclusion: We conclude that *E. coli* ATCC 8739 can be adapted to grow in 11% NaCl by incremental adaptation.

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1. Introduction

Escherichia coli is a textbook example of non-halophilic bacteria and is an indicator organism for faecal contamination of water as *E. coli* is a more consistent predictor of gastrointestinal illness than other bacterial indicators in water (1). This corroborates Burton et al. (2) whom suggested that *E. coli* was a suitable predictor of *Salmonella enterica serovar newport* in various freshwater sediments and has been observed to survive as long as or longer than *Salmonella spp.*; thus, fulfilling its requirement as an indicator for pathogenic bacteria. In addition, the survival of *E. coli* is independent of the amount of organic matter (2). This suggested that *E. coli* is a suitable indicator as it can survive in media of different nutritional richness.

Doudoroff (3) demonstrated that the viable count of *E. coli* previously cultured in ordinary fresh water media then transferred to saline nutrient solutions remained at a constant up to 7% NaCl concentration. The viable count dropped progressively with further increase in concentration suggesting that *E. coli* is non-halophilic and 7% NaCl is bacteriostatic. It was observed that concentration of yeast autolysate, aeration and physiological conditions affected *E. coli*'s overall salt tolerance at high concentration of NaCl (3). The relation of viable count to NaCl

concentration (3) corroborated the findings of Vaas (4) for *Bacillus megatherium* where the growth of *E. coli* in a saline environment is greatest at the early stationary phase and lowest in the logarithmic phase (3).

Previous studies (5-8) on *E. coli*'s adaptation and evolution were carried out using antibiotics and drugs. Recently, a study in which pigs treated with ampicillin, a common antibiotic, demonstrated a significant increase in the occurrence of ampicillin-resistant *E. coli* from 6% to more than 90% after a course of 7 days (9) suggesting incomplete intestinal absorption as the evolutionary pressure for intestinal bacteria such as *E. coli* towards antibiotic resistance (10-11). However, common food additive such as salt, which is used to preserve food and inhibit microorganisms, is less understood in terms of *E. coli*'s adaptive mechanism although recent studies (12-13) had suggested that *E. coli* is able to adapt to food additives over extended culture. This suggests that *E. coli* may be able to adapt to higher concentrations of food additives but this has yet to be studied. The adaptation of *E. coli*salt may suggest similar resistance to other preservatives and its ability to grow in saline environment may be underestimated. In addition, number of stress adaptation studies had demonstrated that the growth phases may have an impact on *E. coli* adaptation. Nair and Finkel (14) suggested that a non-specific DNA binding protein, dps, may confer multiple stress tolerance at stationary phase, which concur with Jolivet-Gougeon et al. (15).

We define halophilization as the adaptation of the organism to the inhibitory effects of salt (sodium chloride; NaCl) by introducing increasing concentrations of salt into the growth environment. The adaptation can either be gradual which requires more than one genetic or epigenetic change or instantaneous whereby only one genetic or epigenetic change is required. Increasing salt concentration can cause a variety of stresses (16) such as osmotic and ionic stress. Organisms that grow in a salty environment experience osmotic stress cause by the influx of salt (reduced a_w) and efflux of water molecule out of the cells. This is important to water monitoring as osmotic stress is closely related to water activity (a_w), defined as the amount of water that is available for growth and reproduction of an organism (17) and is inversely proportional to the solute concentration at the same temperature. Pure water has an a_w of one.

The growth of *E. coli* is optimal when it has a higher a_w which normally requires an a_w of at least 0.98 which corresponds to about 5% NaCl (18) at physiological temperature (19). This corresponds to Presser et al. (20) which demonstrates the inhibitory effect of combinations of water activity and pH varied with temperature for the growth of *E. coli*. *E. coli* demonstrated prolonged lag phase when exposed to NaCl and decrease in growth rate in increasing NaCl concentration (21) resulting in the efflux of water (16). However, *E. coli* 0157:H7 strain had been shown to be tolerant to low pH and low water activity (22). It has been suggested that *E. coli* copes with the high concentration of NaCl by accumulating organic osmolytes (23-24) such as glycine, betaine, glycerol and trehalose. It has also been suggested that high levels of betaine in *E. coli* enables adaptation to heat stress (24). Hence, it may be plausible that salt tolerance may result in increased tolerance to other chemical stresses.

A study (25) comparing the halotolerance of *E. coli* to a halophilic bacterium, *Vibrio spp.*, using carbonyl cyanide m-chlorophenylhydrazone (CCCP) and NaCl suggested that the growth-inhibitory effect of CCCP was less on *E. coli* compared to *Vibrio spp*. However, the growth-inhibition of NaCl was more pronounced for *E. coli* but stable for *Vibrio spp*. when concentration of sodium chloride increases. This suggests that the minimum inhibitory concentration (MIC) of *E. coli* is lower as compared to *Vibrio spp.* when grown on NaCl and the resistance of *E. coli* was dependent on the medium. Another study (26) demonstrated that *E. coli* O157:H7were cultured in brain heart infusion (BHI) experienced more morphological damage and alteration with increasing NaCl concentration compared to *Staphylococcus aureus* in 0%, 5% and 10% NaCl. This agrees with Jay (27) suggesting that *E. coli* O157:H7 tolerates up to 8% NaCl in culture media and up to 20% for certain strains of *S. aureus*.

Taken together, these studies (5, 18, 27) suggested that different strains of *E. coli* have different salt tolerance with the pathogenic *E. coli* O157:H7 strain having the highest salt tolerance. However, the reason as to why pathogenic *E. coli* are more salt tolerant to its non-pathogenic counterparts is not obvious although it may be hypothesized that constant low level exposure to chemicals, such as antibiotics and preservatives, during food preparation and medical treatment may provide a selective pressure towards chemical tolerance.

This study aims to observe gradual adaptation of *E. coli* ATCC 8739, a fully sequenced strain, cultured in NaCl supplemented medium up to 8% NaCl over 80 passages. Our results suggest that *E. coli* adapted to 1% increase in NaCl every month with a successful adaptation to 11% NaCl.

2. Materials and Methods

2.1. Culturing

The Culture Experiment was carried out with an initial inoculum of 9.7×10^6 *E. coli* ATCC 8739 cells from Passage 70 of Lee et al. (13) as the first passage in each of the 4 replicates of 10ml of 1X nutrient broth with a fixed concentration of NaCl and cultured in tightly-capped 15ml conical tubes. Subculture was performed using 1% of the previous culture on every odd day except Sunday (3 subculturing per week). NaCl concentration for the passages

were as follows: Passage 1 to 15 at 3% NaCl, Passage 16 to 31 at 4% NaCl, Passage 32 to 39 at 4.5% NaCl, Passage 40 to 50 at 5% NaCl, Passage 51 to 62 at 6% NaCl, Passage 63 to 74 at 7% NaCl, and Passage 75 to 80 at 8% NaCl.

2.2. Contamination Monitoring

The most likely contaminant is *Staphylococcus aureus*, a Gram positive, salt tolerant commensal on human skin. The cultures were monitored routinely for contamination using Gram staining and DNA fingerprinting. The DNA fingerprinting by PCR/restriction fragment length polymorphism was performed using the procedure in a previous similar adaptation study (13) where each of the 3 primers (Primer 5, CgCgCTggC; Primer 6, gCTggCggC and Primer 7, CAggCggCg) were used as both forward and reverse primers. The PCR product was digested with 1 unit of TaqI restriction endonuclease for 16 hours at 65^{o}C before analysis on 2% (w/v) agarose gel with 1X GelRed.

2.3. Data Analysis

The cell density of each passage at the day of subculture and 5 and 7 day post-subculture was estimated from the OD600 readings using cell size correction suggested by Sezonov et al. (28) that the size of the cells remains constant up to OD600 0.3, which is equivalent to 5 x 10^7 cells per millilitre. After OD600 0.3, cells size decreases and the correlation between the OD600 and the cell density changes is estimated by the following equation: cell density = 52137400 * ln(OD600 Reading) + 118718650, as described in Lee et al. (13). To estimate generation time, 10 µl of *E. coli* culture from each replicate (A, B, C, D) was inoculated into 1ml of 1X nutrient broth and OD600 readings taken at intervals of up to 360 minutes was used to analyze the generation time of each sample tubes. This was conducted at every 3rd passage using the correction (28) before taking the geometric mean from the 3rd hour post-inoculation.

2.4. Minimum Inhibitory Concentration

1% of *E. coli* culture from each replicates (A, B, C, D) were inoculated into 1ml of 1X nutrient broth supplemented with 0% (w/v) NaCl, 1% (w/v) NaCl, 3% (w/v) NaCl, 5% (w/v) NaCl, 7% (w/v) NaCl, 9% (w/v) NaCl and 11% (w/v) NaCl of different salt concentration. This was incubated for 21-23 hours at 37^{o}C before taking OD600 readings. The readings were fitted to the following 4th order equation: $OD600 = M_4(\%NaCl)^4 + M_3(\%NaCl)^3 + M_2(\%NaCl)^2 + M_1(\%NaCl)^1 + M_0$.

3. Results

3.1. *E. coli* adapts to 1% increase in NaCl in about one month.

Our results suggest that *E. coli* were able to grow at 11% NaCl for all four replicates after about passage 64 (Table 1, Figure 1) as there was no measured growth at 11% NaCl before passage 64. The OD600 readings at 11% NaCl increased consistently after passage 64.

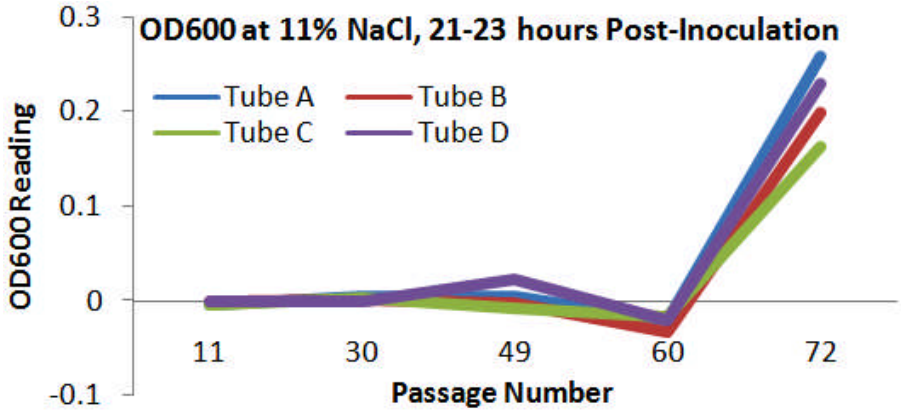

Figure 1. OD600 at 11% NaCl, 21 to 23 hours post-inoculation. This graph illustrates the OD600 reading in 11% NaCl of the MIC experiment demonstrating an increased OD reading post-passage 60, suggesting that the cells are able to divide at 11% NaCl

Table 1. OD600 tabulation for minimum inhibitory concentration estimation. Representative data from each salt concentration is shown.

Passage ([NaCl]% in media)	Replicate	[NaCl]% for Minimum Inhibitory Concentration						
		0%	1%	3%	5%	7%	9%	11%
11 (3%)	A	0.684	0.672	0.866	0.464	0.118	0.006	-0.004
	B	0.616	0.613	0.863	0.355	0.075	0.006	-0.001
	C	0.637	0.657	0.706	0.376	0.089	-0.003	-0.004
	D	0.686	0.719	0.870	0.370	0.090	0.008	-0.001
30 (4%)	A	0.644	0.703	0.793	0.362	0.139	0.006	0.005
	B	0.678	0.658	0.744	0.381	0.085	0.021	0.002
	C	0.659	0.627	0.757	0.425	0.005	0.010	0.003
	D	0.670	0.639	0.784	0.363	0.035	0.007	-0.001
49 (5%)	A	0.705	0.680	0.840	0.696	0.104	0.012	0.006
	B	0.761	0.720	0.927	0.757	0.123	0.030	-0.003
	C	0.919	0.705	1.354	0.792	0.158	0.064	-0.007
	D	0.726	0.767	1.060	0.582	0.115	0.002	0.023
61 (6%)	A	0.471	0.515	0.771	0.433	0.049	-0.022	-0.020
	B	0.432	0.446	0.732	0.461	0.078	-0.018	-0.033
	C	0.54	0.561	0.628	0.444	0.085	-0.021	-0.016
	D	0.985	0.569	0.760	0.431	0.124	-0.009	-0.020
72 (7%)	A	0.549	0.746	0.610	0.401	0.418	0.289	0.259
	B	0.804	0.742	0.791	0.501	0.301	0.288	0.200
	C	0.55	1.013	0.984	0.407	0.309	0.241	0.163
	D	0.873	0.790	0.689	0.387	0.340	0.286	0.230

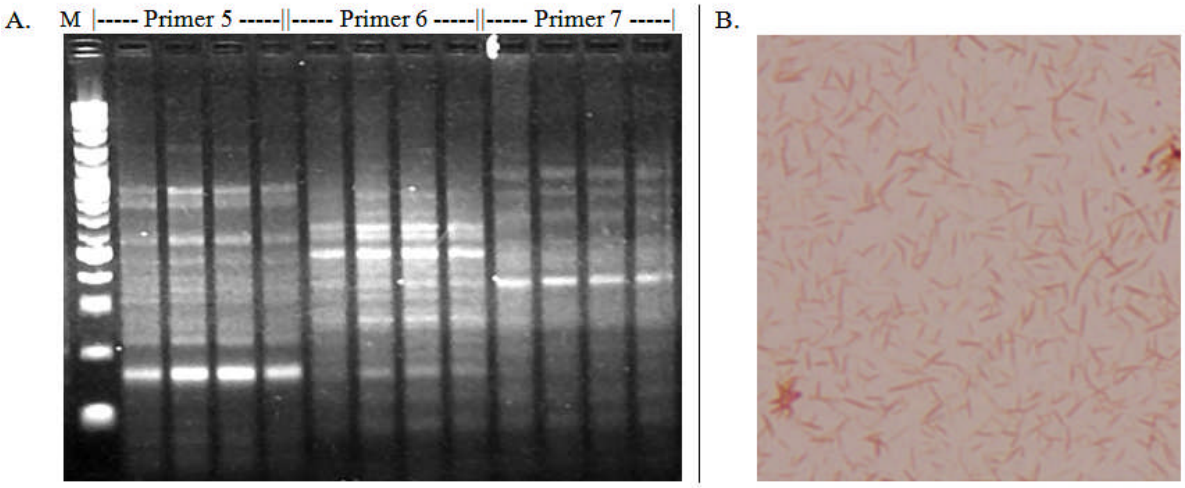

Figure 2. PCR-RFLP and Gram staining of Passage 72.
(A) Genomic DNA extraction and PCR using Lee et al. (2012) and 16-hour TaqI restriction endonuclease digestion showed similar profile in all 4 replicates.
(B) Gram staining showed predominately Gram negative cultures. These suggest that the culture were uncontaminated by *Staphylococcus aureus* (a common Gram positive salt tolerant bacterium)

This suggests that *E. coli* is able to grow at 11% NaCl even though they had been adapted to grow at 7-8% NaCl from passage 63. In addition, Gram staining and PCR-RFLP at Passage 72 (Figure 2) showed that the cultures are Gram negative and the PCR-RFLP profiles of all 4 replicates to be similar. This suggested that the cultures had not been contaminated with *S.aureus*, which is Gram positive and the most likely contaminant as *S. aureus* is a salt tolerant commensal found on human skin. The Area under Curve (AUC) has increased for all four replicates over 80 passages (Figure 3) when NaCl concentration is more than 7.5%. The rate of increase in ascending order is as follows: Tube A (0.003% per passage), Tube D (0.0049% per passage), Tube C (0.0094% per passage) and Tube B (0.0144% per passage).

MIC experiments were conducted every two weeks to evaluate the adaptability of *E. coli*. The 1/2ODMax is used as a gauge to determine the maximum cell density after 21 to 23 hours post inoculation (Figure 1). The increase in 1/2ODMax is possibly due to the fast growers proliferating faster than the slower growers. This would also result in both an increase in 1/2ODMax and AUC.

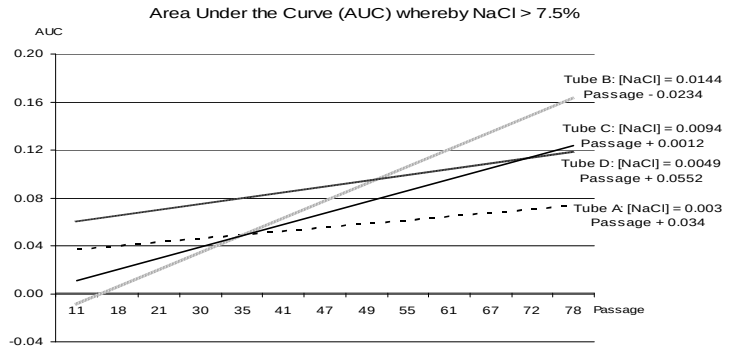

Figure 3. Area under the curve whereby the concentration of NaCl is more than 7.5%. Increasing AUC suggest that the cells are able to grow at higher salt concentrations with increasing passage counts.

3.2. Rapid Increase of *E. coli* population in 4% NaCl concentration

Our results suggest the number of generations is generally constant from passage 1 – 43, until it experienced a rise in number of generation from passage 16 – 21. From passage 43 onwards, the number of generations is more inconsistent compared to earlier passages (Figure 4). The coefficient of variations increased from P1 – P15 (3% NaCl) to P16 – P31 (4%NaCl). It decreased in P32 – P39 (4.5%) before increasing again in P40 – P50 (5% NaCl). From P51 onwards, it starts to decrease when NaCl reaches 6% and above (Figure 4).

Our results showed a visible spike in the number of generations can be seen over passage 19 which is at 4% NaCl (Figure 4). The large spike indicates that there is a rapid increase in the number of generations which could be the fast growers of *E. coli* that adapt well to the 4% NaCl and leading to proliferation. This supports the weeding point as observed in the mean DI values and Coefficients of Variation (CV). The differences in spike for the samples (ranging from 7.6 in Tube A and 12.1 in Tube B) might be a random variation in the samples. Nevertheless, the increase in number of generations was observed in all 4 samples. The adaptability of the *E. coli* to the NaCl can be known and predicted by looking at the CV. A decrease in CV would indicate a more stable growth of *E. coli*.

3.3. Generation Time Increases as Salt Concentration Increases

All four replicates have shown an increasing rate of generation time (Figure 5). The rate of increase in ascending order is as follows: Tube C (0.427 minutes per passage), Tube A (0.6777 minutes per passage), Tube B (0.7578 minutes per passage) and Tube D (1.1781 minutes per passage). The average number of generations between each passage was 6.93, with a standard deviation of 0.66 generations. The average OD600 readings at Day 5 and 7 were 0.467 (with a standard deviation of 0.096) and 0.482 (with a standard deviation of 0.140) respectively. Two factor analysis of variance (ANOVA) on the generation time of different concentration of NaCl (treatment) and tube (A, B, C & D) demonstrated that only treatment is significant ($F = 3.176$, p-value = 0.01) and there is no interaction between the treatments and replicates ($F = 0.936$, p-value = 0.539).

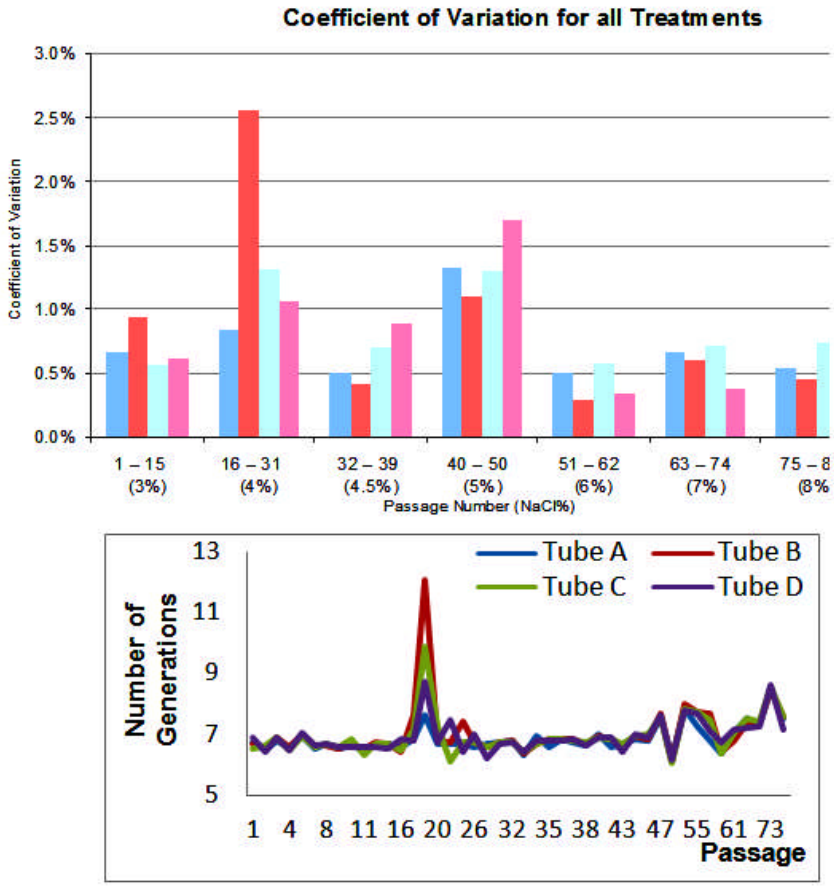

Figure 4. Coefficient of variation and 2-day generations for all four tubes over 80 passages. Coefficient of variation (CV) is calculated based on the density of cells at day 2 of culture. CV is defined as quotient of standard deviation and mean. Low CV suggests low variation of the cell density at day 2 of culture.

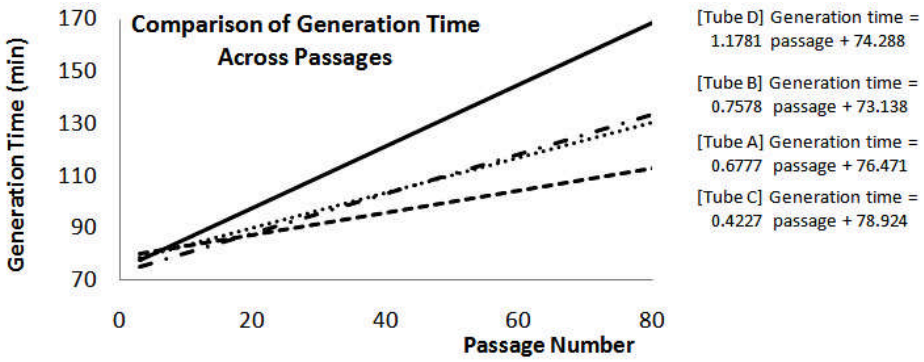

Figure 5. Generation time of four tubes: Tube A, Tube B, Tube C and Tube D across 80 passages. OD600 readings were taken from 2 hours post-inoculation to reduce the impact of varying length of lag phase across different passages and the generation time for each passage is calculated as the geometric mean of the estimated generation time between intervals of OD600 readings from 2 to 5 hours post-inoculation.

4. Discussions

A study by Adam et al. (29) had demonstrated that the increase in resistance can be improved by repetitive exposures to increasing concentration of antibiotics. Similarly, our MIC result showing that the 1/2ODMax had increased in 3 of the 4 replicates suggesting that repetitive exposures to increasing concentration of NaCl could result in increased NaCl tolerance. Perron et al. (30) demonstrated that the speed of adaptation of *E. coli* to various antibiotics varies according to rate of selection where the evolving population was dependent on the rate of change of antibiotic concentration, and changed over time. Our AUC results suggest that the cells' proportion which is able to grow at more than 7.5% NaCl as the number of passages increase. 7.5% NaCl is used as a benchmark for testing the adaptability of *E. coli* as there is no significant growth of *E. coli* at 7% NaCl (18). Cells that are able to adapt to the 7.5% NaCl have the highest adaptability within the whole population in which they will continue to proliferate resulting in the increase of AUC. Since there is an increment of about 1% NaCl every month during the course of 80 passages, these cells are able to handle the additional stress caused by the higher NaCl concentration. This suggests that the cultures diverge differently from their initial low salt environment and the range of NaCl concentrations at which *E. coli* adapts had shifted.

For Tube C, the 1/2ODMax varies inversely with its AUC. A reason could be that *E. coli* in Tube C are able to grow in a wider range of NaCl concentrations. For Tubes A, B and D, the 1/2ODMax varies directly with the AUC which suggests that the cells in Tubes A, B and D adapts at a higher salt concentration as compared to cells in Tube C. This suggests that Tube A, B and D had their salt tolerance shifted to adapt to a higher salt concentration whereas Tube C had its salt tolerance expanded in which it can grow at a wider range of salt concentration. This suggests that Tubes A, B and D had lost the low salt phenotype and unlikely to be able to grow as well in low salt environment compared with the original *E. coli* ATCC 8739 but Tube C seems to had acquired both high and low salt phenotypes. Our results suggest that a portion of cells (Tube C) are able to adapt to high salt diet but seems to retain preference to a low salt diet compared to others (Tubes A, B and D) which seems to adapt to a high salt phenotype. A recent study (31) had demonstrated that constant culture of *E. coli* in cellobiose resulted in higher efficiency in cellobiose use. As the MIC was carried out on entire populations, it may be plausible that the population in Tube C had evolved for both high and low salt preferences while the other populations (Tubes A, B and D) might have a larger population of high salt phenotype. However, the isolation of evolved cells with different salt tolerance and preference will require further study.

In Fantin et al. (32), the speed of commensal *E. coli* adapting to nalidixic acid, ciprofloxacin and levofloxacin was found to be 7 days. Oral ciprofloxacin of varying concentration was administered over 14 days on healthy individuals. This study suggests that the speed of adaptation of *E. coli* may be similar in adapting to NaCl. However, by considering factors such as the difference in microbicidal effects of NaCl and antibiotics along with the conditions of the experiment, the actual speed of *E. coli* adapting to NaCl could be different.

Our results suggest that the rate of 1% NaCl concentration increment per month from the previous concentration does not exhibit a bacteriostatic effect as the culture remains sustainable; thus, within the growth/no-growth boundary (20). This suggests that the rate of 1% increment of NaCl concentration per month is within the tolerability of *E. coli* ATCC 8739 and capable of adapting to this rate of salt concentration increment. However, our results do not suggest that 1% increment per month as the maximum as this will require further study to determine the increment at each salt concentration which demonstrates bacteriostatic effect.

Our results suggest that the CV at 4% is the highest which indicates that the growth of *E. coli* is the most unstable during 4% NaCl (0.83% to 2.55%). This could be that 'weeding' of the slow replicators of *E. coli* is occurring during 4% NaCl. However, this weeding range is not observed at above 5% NaCl as *E. coli* in nutrient broth is able to reproduce actively up to 5% with no significant growth at 7% NaCl (18). This suggests that weeding is likely to span between 4% and 5% NaCl. In Hrenovic and Ivankovic (18), *E. coli* exhibits an absence of bacterial die-off at 3.5% salt concentration. This is primarily attributed to the enriched nutrients available in the media which seemed to be osmotolerant to *E. coli*. This suggests that 1X nutrient broth media used is able to delay bacterial die-off and for the halophilization of *E. coli*.

An increase in generation time demonstrated that the treatment affects *E. coli* ability to adapt and divide. This is supported by Carlucci and Pramer (21) showing that bacteria that are exposed to NaCl typically exhibit a prolonged lag phase and decreasing growth rate in increasing NaCl concentration. Although Liu et al. (33) demonstrated that bacterial cells need time to adapt to the change in environmental conditions, this is not observed in our results as the salt concentration is increased over time. However, our results suggest that the *E. coli* cells are able to grow at every passage despite the increase in salt concentration. This further suggests that the cells can adapt to the rate of 1% NaCl concentration increment per month.

5. Conclusion

In summary, our findings showed that *E. coli* is able to adapt to high salt concentrations in a relatively short amount of time. The most important practical implication of our findings is the likelihood of adaptation to medical therapies, such as antibiotics treatment, within human intestines, as well as food preservation techniques.

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