Experimental evolution of halotolerance in *Escherichia coli*

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

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2011

2007-2011

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Date of defence: 18.08.2011

Acknowledgments

First of all, I would like to praise and thank my gracious and great God **“ALLAH”** who enables me to carry out this work.

The completion of this study leaves me indebted to many people. My first indebtedness goes to **Professor Dr. Rupert Mutzel** who gave me the opportunity to work “under his roof”. I would like to express my deepest thanks for his kind supervision, continuous encouragement and valuable comments, in addition to improving the writing and presentation of my thesis.

Further, I would like to thank **Professor Dr. Kürsad Turgay** for evaluating my thesis.

All the best feelings and deepest thanks for all the members of Mutzel's work group;

Dr. Malgorzata Czarna and **Dr. Sascha Thewes** shared their experience in biochemistry, microbiology and molecular biology with me, were always enthusiastic about my project and its progress, contributed many helpful suggestions, and critically read the thesis. Dr. Czarna also introduced me into the mysteries of "2 D technology". Many thanks, Gosia and Sascha!

I would also like to thank **Angela Lahrz** and **Sabine Sieboth-Gutschmidt** for their constant technical support, valuable practical advices and help with continuous cultures.

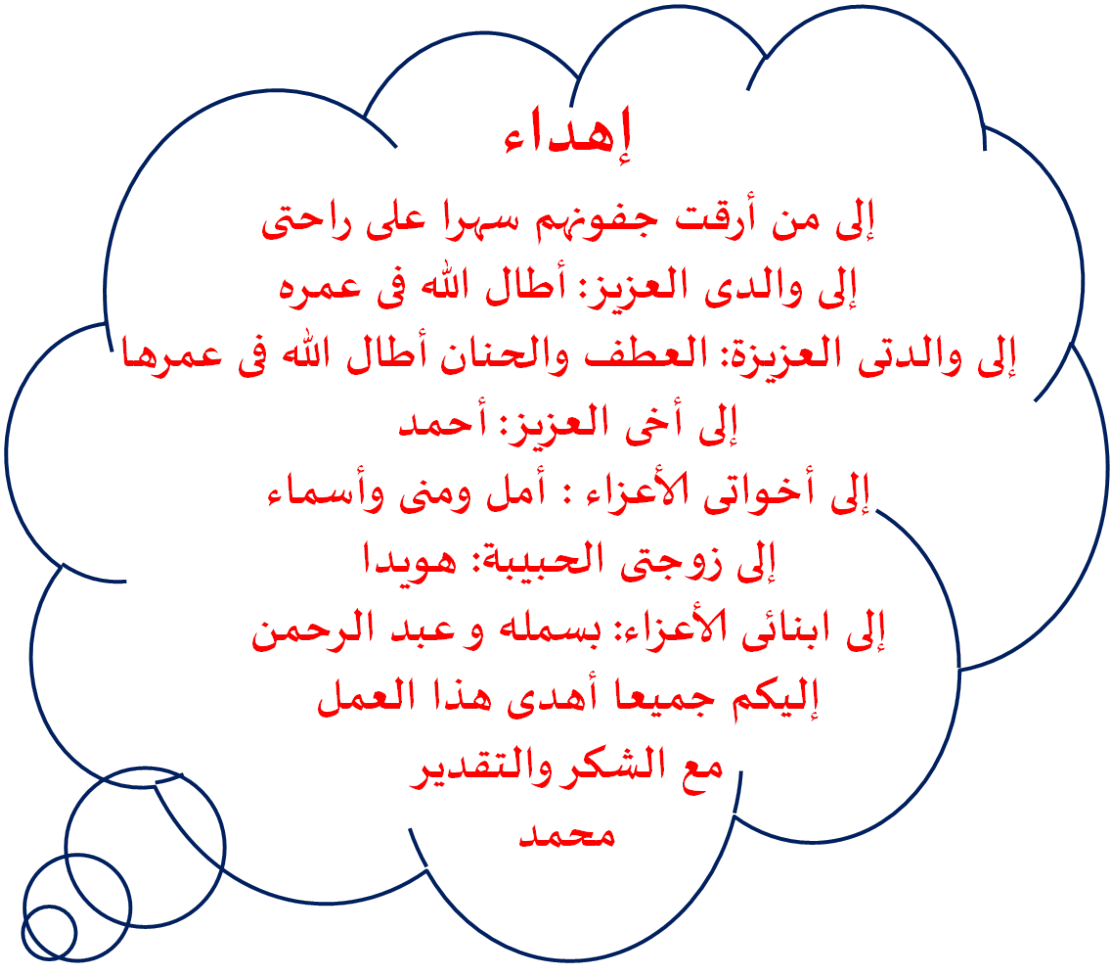
Several students, whose bachelor theses I accompanied, also contributed to the work presented here: **Elena Ulbricht** and **Stefanie Wolter** on trehalose and amino acid analyses; **Anna-Leonie Wolfrum** on measuring generation times and salt tolerance analysis of some strains. **Lilian Löwenau, Susanne Vettermann, Robert Hauffe** and **Florian Scholz** communicated results of ongoing experiments which helped to make some points of the story clearer.

Quantification of amino acids by HPLC would not have been so straightforward without the help of **Kathleen Dahncke** who postponed her own experiments to get the HPLC running for my samples.

Special thanks for **Eberhard Klauck** for the kind gift of anti-RpoS.

Great thanks to the **Egyptian Government** for the financial support offered to me to undertake this study in Germany.

Dedication



I would like to dedicate this thesis to the dearest people to my heart.

To my family whose cooperation and understanding, at all stages of this study and against all odds, have been simply overwhelming

With my thanks and appreciation

Mohammed

Summary

This thesis describes experiments to evolve a mesophilic bacterium, *Escherichia coli*, towards halophily. The applied technology consisted in the continuous proliferation of vast populations of cells in suspension in the "Genemat" format under self-adjusting, limiting NaCl concentrations in the growth medium. This was achieved by applying a conditional pulse feed regime where the proliferating suspension culture is periodically diluted with a "permissive" medium of an osmolarity that is compatible with growth of the cells if cell density falls below a pre-set value, and with a pulse of "nonpermissive" medium of high osmolarity when cell density is superior to this threshold. This regime favors genetic variants with elevated osmotolerance, which rapidly replace their less-adapted progenitors.

Two parallel cultures were run over approximately 8800 and 6700 generations, respectively. The first culture consisted of the prototypic *E. coli* strain MG1655, the second of a Δ*def/fmt/mutD* variant (ε2124) which is distinct from other eubacteria in its mechanism of initiation of translation and, in addition, shows a mutator phenotype.

Both experiments yielded offspring with greatly enhanced levels of halotolerance, MG1655 evolvants growing at steady state concentrations of 1.3 M NaCl with generation times of about 4 hours, ε2124 evolvants supporting steady state concentrations of about 1.2 M NaCl. The former can grow in salinities of up to 2.0 M, the latter up to 1.6 M. They tolerate similar concentrations of KCl and K_2SO_4, as well as the uncharged, osmotically active sugar, sorbitol. Evolved derivatives are impaired in growth at low salinities but can still be adapted to grow in the absence of extracellular salt. Whereas adaptation proceeded smoothly for MG1655 cells, the ε2124 culture showed massive and sustained oscillations in the consumption of nonpermissive medium, an effect that could be due to its high genetic variability.

Whereas both populations of evolving cells relied on synthesis of the disaccharide trehalose as an intracellular osmoprotectant during the initial stages of the experiments, both cultures switched later this strategy to massive accumulation of the amino acid proline which can accumulated to intracellular concentrations of up to 500 mM during growth in high salt medium. Both derivatives may chronically enrich K^+ ions in their cytosol since both showed enhanced sensitivity for the presence of KI, and MG1655 derivatives could not grow at salt concentrations of more than 1 M NaCl when K^+ was omitted from the growth medium.

The general stress response of evolved cells was compared with that of wild type MG1655 cells by analyzing the expression of the stress sigma factor, RpoS, on Western Blots of cellular proteins isolated during growth at various salt concentrations. From these analyses it can be concluded that the stress answer sets in at higher salinities in the evolved derivatives.

Changes in the outer membrane and total proteomes were revealed by SDS-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis.

Further work should aim in doing analysis of the adaptive changes during the evolution process in molecular details by determination of the complete genome sequences as well as further functional, physiological, transcriptomic, and proteomic analysis, and both experiments should be continued.

Zusammenfassung

Diese Arbeit beschreibt Experimente zur Evolution des mesophilen Bakteriums *Escherichia coli* zur Halophilie. Dazu wurde die Technologie der kontinuierlichen Kultur großer Populationen von Zellen in Suspension unter automatisch eingestellten, gerade noch mit dem Wachstum der bestangepassten Varianten vereinbaren Salzkonzentrationen im "Genemat"-Format angewendet. Die Zellen wurden in einem *conditional pulse feed* Regime kultiviert. Dabei wird das Kulturgefäß mit Pulsen eines "permissiven" Mediums (mit einer Salzkonzentration, die mit dem Wachstum der Zellen vereinbar ist) verdünnt, wenn die Zelldichte unter einer vorgegebenen Schwelle liegt. Übersteigt die Zelldichte diese Schwelle, so wird stattdessen mit einem Puls von "nichtpermissivem" Medium (mit wachstumslimitierender Salzkonzentration) verdünnt. Unter diesen Bedingungen genießen genetische Varianten mit erhöhter Salztoleranz einen selektiven Vorteil gegenüber ihren weniger salztoleranten Vorläufern und verdrängen diese rasch aus der Kultur.

Es wurden zwei parallele Experimente über etwa 8800 Generationen und etwa 6700 Generationen durchgeführt. Für das erste Experiment wurde der prototypische *E. coli* Stamm MG1655 eingesetzt, für das zweite ein Abkömmling davon, ε2124, der sich durch eine Deletion des *def/fmt* Operons auszeichnet und sich daher im Mechanismus der Initiation der Proteinsynthese von anderen Eubakterien unterscheidet und der zudem auf Grund einer Mutation des *mutD* Gens einen Mutatorphänotyp zeigt.

Beide Experimente resultierten in der Selektion von Evolvaten, die gegenüber dem Ausgangstyp stark erhöhte Salzresistenz aufweisen, wobei MG1655 Derivate mit Generationszeiten von etwa 4 Stunden kontinuierlich bei 1,3 M NaCl wachsen können, ε2124 Derivate bei etwa 1,2 M NaCl. MG1655 Derivate zeigen Wachstum noch bei 2 M NaCl, ε2124 Derivate bis etwa 1,6 M. Ähnliche Konzentrationen anderer Salze wie KCl und K_2SO_4 werden ebenso toleriert wie hohe Konzentrationen des Zuckers Sorbitol. Die evolvierten Derivate wachsen schlecht in Abwesenheit von Salz, können aber noch an Medien mit geringen Salzkonzentrationen adaptieren. Während die Adaptation im Fall von MG1655 recht gleichmäßig voranschritt, zeigte die ε2124 Kultur starke, anhaltende Schwankungen im Verbrauch an nichtpermissivem Medium. Dies könnte im Mutator-Phänotyp dieser Linie begründet sein.

Während früher Stadien der Evolutionsexperimente akkumulierten Zellen beider Linien intrazellulär das Disaccharid Trehalose als osmoprotektive Substanz. Beide Linien wechselten dann auf die massive intrazelluläre Akkumulation der osmoprotektiven Aminosäure Prolin, die in den Zellen bei hoher externer Salinität Konzentrationen von bis zu 500 mM erreichen konnte. Eine erhöhte Sensitivität gegenüber dem toxischen KI und eine starke Verringerung der Salztoleranz in Abwesenheit von Kalium im Medium könnten darauf hinweisen, dass die evolvierten Derivate intrazellulär Kaliumionen anreichern.

Die Stressantwort evolvierter Zellen wurde mit der von Wildtyp Zellen anhand der Expression des "Stress-Sigmafaktors" RpoS während des Wachstums in unterschiedlichen Salinitäten verglichen. Aus den erhaltenen Ergebnissen kann geschlossen werden, dass die Stressantwort in den evolvierten Stämmen bei höherer externer Salzkonzentration induziert wird als im Wildtyp.

Analysen der Proteinzusammensetzung von Präparationen der äußeren Membran und von Gesamt-Zellextrakten durch 1- und 2-dimensionale Gelektrophoresen zeigen Änderungen im Proteom.

Neben der Fortsetzung der Evolutionsexperimente sollten in künftigen Arbeiten molekulare Details des Adaptationsprozesses anhand von Gesamtsequenzen der evolvierten Genome ermittelt werden, und weitere funktionelle und physiologische Studien sowie Transkriptom- und Proteomanalysen sollten durchgeführt werden, um Ablauf und Resultat der Evolution zu beschreiben.

Abbreviations

APS	Ammoniumperoxodisulfate
bp	Base pair(s)
DMSO	Dimethylsulfoxid
IEF	Isoelectric focussing
MQ	Salt-free, sterile, RNase-free water (MilliPore)
NTA	Nitrilotriacetic acid
OD_{600nm}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid

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1. Introduction

1.1 Halotolerance and halophily

Microbial life exists over the whole range of salinities from freshwater and marine biotopes to hypersaline environments with NaCl concentrations at saturation. Halophilic and halotolerant microorganisms are found in all three domains of life, *Archaea*, *Bacteria*, and *Eucarya* (Oren, 1999). While halotolerant organisms can be found in all three domains of life, halophily was long considered to be restricted to the Archaea, but more recently, a group of eubacteria has been shown to be extremely halophilic in the strict sense (Oren and Mana, 2002; Oren, 2008). Microorganisms, which can only live in extreme hypersaline environments, are designated halophiles, while those capable of growth in the absence of salt, but can thrive in varying concentrations, are considered to be halotolerant. Nevertheless, the concepts of halophilic and halotolerant organisms, as well as their response to salt, vary depending on the used criteria (Ventosa and Nieto, 1995).

Kushner (1985) identified several categories of microorganisms according to their salt concentration optimal for growth. He classified them as follows: non-halophiles grow best in media containing < 0.2 M NaCl (some of which, the halotolerant, can tolerate higher concentrations), slight halophiles (marine bacteria) grow best at 0.2 to 0.5 M NaCl, moderate halophiles grow best at 0.5 to 2.5 M NaCl, and extreme halophiles show optimal growth in media containing 2.5 to 5.2 M (saturated) NaCl.

Biological membranes are permeable to water and cells cannot maintain the water activity of their cytoplasm higher than that of the surrounding medium, because this would lead to a rapid loss of water to the environment (Brown, 1990). Therefore, the microorganisms, which live in high salt environments, are expected to keep their cytoplasm at least isoosmotic with the extracellular environment; to establish turgor pressure, the cytoplasm should be slightly hyperosmotic.

In the case of organisms living in highly concentrated salt solutions extracellular ions compete with a cell's intracellular macromolecules for water. Two fundamentally different molecular strategies exist within the microbial world that enables microorganisms to cope with this environmental constraint, halophily and

halotolerance. Halophily is the genetic adaptation to a high salt environment which becomes manifest in the chemical composition and structures of cellular macromolecules and in the intracellular accumulation of monovalent cations (the “salt-in” strategy). Halotolerance is a regulatory response, which consists in either de novo synthesis or uptake from the medium of compatible solutes which bind water (Galinski, 1993) (the “compatible-solutes” strategy).

Halophilic organisms accumulate cations (in most cases K^+) in their cytoplasm, equilibrating their intracellular osmolarity with the extracellular (Oren, 1986). Extremely halophilic organisms are dependent on high extracellular salt and cannot live in diluted milieus.

In cells using the salt-in strategy for osmotic adaptation, all enzymes and structural cell components have to be adapted to the presence of high salt concentrations to ensure proper functioning of the intracellular enzymatic machinery. Halophilic proteins show unique molecular adaptations, a strikingly large excess of acidic amino acids and reduced amounts of hydrophobic amino acids (which even becomes manifest in the overall amino acid composition of the proteome (Gandbhir et al., 1995), allowing them to stay in solution due to hydration of acidic residues, charge shielding by electrostatic interactions, and the reduction of hydrophobic interactions which could cause a collapse of the structure (Lanyi, 1974; Zaccai and Eisenberg, 1990).

There is no need for specially adapted proteins in the cells, which use the compatible-solute strategy (Oren, 1999). Compatible solutes were defined as solutes that, at high concentrations, allow enzymes to function efficiently (Brown, 1990). Sugars and sugar derivatives (trehalose, sucrose, glucosylglycerol), polyols such as glycerol and arabitol, amino acids and their derivatives, and quaternary amines such as glycine betaine were detected in halophilic and halotolerant microorganisms as compatible solutes (Roberts, 2005).

Halomonas elongata (an extremely halotolerant member of the proteobacterial *Vibrio* group) shares properties of both halotolerant and halophilic microorganisms, massively overproducing the compatible solute ectoine in a high-salt medium but also displaying biased composition of its total cellular protein (Galinski, 1993; Gandbhir et al., 1995). This may be used as a hint to designing possible adaptive scenarios for the evolution of halophily. As the *Halomonaceae* are phylogenetically closely related to the main branch of mesophilic proteobacteria (Ventosa et al.,

1998), remodeling of a proteobacterium's macromolecules in a stringently selective, high-salt environment might be a rapid process, opening the possibility to reproduce and extend genetic adaptation to live in a hypersaline milieu under controlled laboratory conditions. Interestingly, a comparison of the rates of evolution of halophilic and freshwater micro-crustaceans demonstrated accelerated molecular evolution in halophilic lineages which was suggested to be due to the effects of salt on DNA-protein interactions (Hebert et al., 2002). Moreover, it was demonstrated that among haloarchaeal species intergenomic recombination even between highly conserved rRNA operons is a frequent event (Boucher et al., 2004).

1.2 Experimental evolution

In studies of experimental evolution, hypotheses and theories of evolution are tested by conducting controlled experiments. Evolution can be carried out in the laboratory by adapting a population to new environmental conditions through the action of variation (mutation) and natural selection. With the help of molecular tools, it is possible to determine and to identify the mutations that selection acts on, and to find out how exactly these mutations affect the biological fitness of the organism under selection.

In 1859 Darwin published *On the origin of species by means of natural selection* (Darwin, 1859). His biological *opus magnum* established the theory of evolution and, most important, defined the role of natural selection in determining its course. Darwin seeks to explain the adaptations of organisms, their complexity, and diversity as the result of natural processes (Ayala, 2009).

The use of microbial experimental systems is not new. In the fact, such experiments were carried out as early as 1887 by W. D. Dallinger who conducted long-term evolutionary experiments with protists and observed that specialization in one environment can yield a sort of adaptation (Jessup and Forde, 2008). Experimental evolution trials between 1880 and 1980 were practiced by a variety of evolutionary biologists, including Theodosius Dobzhansky whom we owe the immortal words "nothing in Biology makes sense except in the light of evolution" (Dobzhansky, 1964). But in this period much of the work lacked extensive replication and was carried out only for relatively short periods of time. In the 1980s, successful experiments with

extensive parallel replication of evolving lineages as well as large numbers of generations under selection appeared. One of the first in a new wave of experiments using this strategy was the laboratory "evolutionary radiation" of *Drosophila melanogaster* populations that Michael R. Rose started in 1980 (Rose, 1984).

In 1988, Richard Lenski, applying the technique of serial subculture (see below) started a long-term evolution experiment with the bacterium *Escherichia coli*. The experiment continues to this day, and is by now probably the longest controlled evolution experiment ever undertaken. Since the inception of the experiment, the bacteria have grown for 50000 generations (Lenski, 2011).

1.2.1 Continuous culture and the "sticker problem"

Basically, a device for continuous cultivation operates by renewing a liquid culture of constant volume with nutrient medium inflow, such that organisms must counteract dilution by growing at least at an equal rate.

Before 1944 continuous culture devices were designed primarily to give a continuous supply of microorganisms without routine subculturing. Much of this early work was industrial and was limited to increasing the efficiency of utilization of fermentable sugars. Reviews by Novick (1955) and James (1961) summarize the scientific attempts of this early period. Later, many investigators used exponential phase cultures to study and compare the growth of different microorganisms. The first successful prototype of the turbidostat appears to have been that of Myers and Clark (1944). In 1950 the technology for experimental evolution of organisms by long-term continuous cultivation of huge populations under strictly controlled, selective conditions was established around Monod's "bactogene" or the equivalent Novick and Szilard's "chemostat" (Monod, 1950; Novick and Szilard, 1950). Mutants proliferating at higher growth rates are selected during prolonged operation of these devices (Kubitschek, 1970).

In continuous cultures, control of growth can be either external or internal (Fig.1). In chemostats growth is controlled externally by limiting the supply of a critical growth factor, and the cells grow at a rate equal to a constant dilution rate. In turbidostats, growth is internally controlled and a photodetector/actuator-couple (the actuator

being a valve or pump) is used to maintain constant turbidity of the culture. Cells grow at or near their maximum rate in the nutrient medium that is provided.

Historically, continuous culture was initially welcomed with intense interest. It has for a long time been, and still is, a valuable instrument for physiological investigations. In spite of its solid theoretical foundations (Kubitschek, 1970), the technology has been progressively abandoned as a tool in experimental evolution. That is because conventional continuous culture suffers a major drawback, namely rapid selection of resident, adaptively static, biofilm-forming variants.

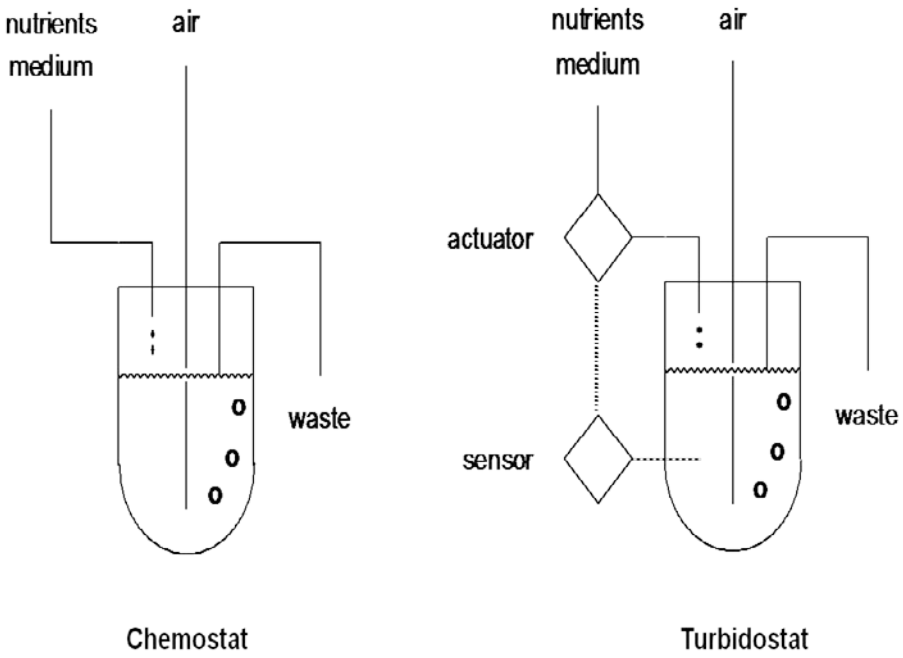

Figure 1: Schematic representation of chemostat and turbidostat. Drawing (modified) courtesy of Dr. P. Marlière.

Physical adhesion to niches is certainly one of the most effective adaptive strategies that populations of living organisms pursue in their struggle for life. For the biotechnological task of experimental evolution of microbial cells in continuous culture, surface adhesion has been recognized a primary obstacle (Chao and Ramsdell, 1985) because resident populations are not constrained to adaptively compete with their pelagic counterparts under a regime of continuous dilution. In principle, this can be avoided by serial subculture of cells in suspension (Lenski and Travisano, 1994), a technique where cells in suspension are frequently transferred

into fresh culture vessels (i.e., surfaces that could be colonized are periodically discarded), creating a selective disadvantage for static variants.

The most extensive experiments of microbial adaptation published so far by the Lenski group (50000 generations of *E. coli;* Lenski, 2011) were therefore conducted by tedious, manual, serial sub-culturing in common glassware, and not by automated propagation in continuous culture equipment. At an industrial scale, serial subculture technology has not been systematically exploited because it is laborious and requires absolute sterility during transfers. Moreover, the technology has major conceptional drawbacks. Cells are exponentially growing only during a very limited fraction of the cultivation process because every fresh inoculum will undergo initial lag and final stationary phases, respectively, and natural selection will not exclusively favor faster-growing variants but also variants with shorter lag phase or later entry into stationary phase. Most important, however, serial subculturing forces the evolving population at each transfer through a narrow bottleneck of small numbers of individuals since only a small part of the population is preserved. Indeed, when the statistical chance for a beneficial mutation to arise is highest (during the last generation before transfer), the size of the subpopulation, which carries this mutation and can be transferred to the next cycle is minimal (Wahl and Gerrish, 2001). Also, frequent passage through small population sizes may lead to fixation of deleterious mutations by genetic drift (Silander et al., 2007).

1.2.2 Genemat technology

The technological solution for these drawbacks is an automated fluidics device ("Genemat") with two growth chambers undergoing transient phases of sterilization (Marlière and Mutzel, 2004). Its function ensures that (i) a population of cells in suspension is kept under permanent proliferation over long periods of time and (ii) resident cells in any part of the device are frequently and effectively destroyed. Fully defined and strictly controlled chemostat or turbidostat conditions can be imposed on evolving populations.

In addition to its potential for directed evolution of cell lines of industrial interest, this technology opens avenues for full mathematical analysis and modeling of evolutionary processes and the underlying molecular mechanisms since the properties of evolving populations can be readily extracted from their growth

characteristics, which in turn are strictly correlated to cell densities and dilution rates (Kubitschek, 1970).

1.3 Experimental evolution of halophily

Halophilic microorganisms and the proteins encoded in their genomes are highly adapted to function in a milieu of up to 5 M inorganic salt. They offer a valuable source of catalysts for biotechnical applications. Common physiological properties of halophilic microorganisms and structural and compositional features of halophilic proteins have been elucidated over the past decades (see above); however, the adaptive mechanisms which allow mesophilic organisms to invade and explore high salt environments are still obscure. In this study we set out to experimentally evolve the mesophilic bacterium *Escherichia coli* to halophily by long-term proliferation of the organism under strictly controlled, highly challenging laboratory conditions.

Species such as *E. coli* dispose on a limited inventory of regulatory answers ranging from intracellular enrichment of compatible solutes such as glycine betaine or trehalose, to induction of general stress responses (Wood et al., 2001). With increasing osmolarity this limited regulatory response will no longer allow for rapid growth, allowing better adapted genetic variants to displace the original genotype. A first adaptive response could, for example, consist in the deregulation or overexpression of an enzymatic pathway for compatible solute synthesis, leading to massive intracellular accumulation of osmolytes. Even such a strain would, however, eventually come to an upper limit of osmolarity where growth is inhibited, not only because water will leak from the cell, but also because those proteins which are in contact with the extracellular medium (outer membrane proteins, periplasmic proteins, and the outer face of inner transmembrane proteins) will lose their activity. At this point the only escape from the challenge consists in structural alterations of these proteins such that they remain active in high salt. Finally, at an even more stringent level of selection the evolving bacteria will no longer be able to retain water in their cytoplasm, entailing selective pressure to adapt the intracellular ionic milieu and the structures of intracellular macromolecules to maximal retention of water.

Two pilot experiments were carried out previously in this laboratory to genetically adapt the gram-negative bacteria *Escherichia coli* to highly challenging osmotic conditions. After about 1250 generations of *E. coli* ε2124 (M. von Scheibner, Diploma thesis, 2006) and 1745 generations of *E. coli* MG1655 (L. Corvers, Diploma thesis,

2007) under constant selection, *E. coli* derivatives were isolated that grow in the presence of 1 M NaCl. In high salt medium these derivatives accumulate the osmoprotective solute, trehalose.

1.4 The *Escherichia coli* model

The wealth of genetic and biochemical knowledge available for *E. coli* makes it an obvious choice as a model organism for experimental evolution.

In this study we used two *E. coli* K-12 strains, MG1655 and ε2124. MG1655 strain is a wild type of *E. coli* and ε2124 strain is derived from strain β2124 (an MG1655 Δ*def/fmt/mutD* derivative), which is devoid of the genes for Met-tRNAi transformylase and polypeptide deformylase. The products of these genes are present in all eubacteria and their mitochondrial and plastid descendants, but are absent in archaea and eukaryotes. The β2124 lineage is distinct from all known eubacteria in being devoid of N-formylation and deformylation activities, resembling eukaryotes and archaea in this trait, and thus representing a unique form of life (Marlière et al., 2005). This strain can be considered the ancestor to a whole lineage of artificially evolved bacteria which are devoid of N-formylated peptides and therefore ideal hosts, e.g. for the production of proteins for therapeutical use in mammals. Moreover, these strains carry an unambiguous "genetic watermark" (the absence of the ubiquitous eubacterial *fmt/def* genes).

1.5 Biotechnological applications for experimentally evolved halotolerant and halophilic bacteria

Lineages of experimentally evolved halotolerant/halophilic bacteria and their products have an evident potential for every biotechnical application where high salt conditions are required or desired.

Salt-resistant enzymes were produced from several halotolerant bacteria, such as the amylase produced by a *Bacillus sp.,* which is stable at 5 M NaCl and could be used in the treatment of effluents containing starchy or cellulosic residues (Khire and Pant 1992).

Compatible solutes (in particular amino acids and their derivatives such as ectoine) have a proven commercial value for industrial, medical and cosmetical use and can be easily extracted from halotolerant producers (Ventosa and Nieto, 1995). These compatible solutes may be applied as stabilizers of enzymes and whole cells (Galinski and Tindall, 1992). Thus, hydroxyectoine protects lactate dehydrogenase and other enzymes against high and low temperatures, salt and desiccation (Galinski and Lippert, 1991). Industrial use of some of these compatible solutes, which are easily produced by biotechnological processes, is a very promising field.

As a result of industrial activities, ecosystems are often subjected to heavy metal pollution and chemical industries pay increasing attention to biotransformations, which can greatly reduce production costs and pollution of the environment. Substrates for these enzymatic transformations may stem from upstream chemical processes that require the presence of high salt. Halophilic biocatalysts will reduce the costs and efforts to make biotransformation in such reaction media compatible with proper function and high activity of the catalyst. Similarly, bioremediation of pollutants in high salt environments would be facilitated by the use of halophilic microorganisms or halophilic enzymes encoded by them.

In the case of large-scale industrial biotransformation processes, enzyme extracts, substrates, products, and the ionic composition of the reaction medium often provide ideal growth conditions for contaminating microorganisms that enter the reactor with the freshwater supply. Such contaminants can drastically reduce the yield of the process due to metabolism of substrates and products, and the degradation of the enzyme extract. In many cases, this could be easily circumvented by the use of a catalyst that functions in high-salt medium, which inhibits growth of freshwater microorganisms, economizing frequent refeeding of enzyme extract or expensive treatment of the freshwater supply.

Production of bio-fuel is one of the most important processes, which could be achieved in the future by contribution of halophiles. Bio-fuel products such as ethanol and hydrogen could be produced from lignocellulosic biomass by fermenting processes after removal of lignin components. The required alkaline pre-treatment (to remove lignin) and subsequent partial neutralization will create an environment for halophilic or haloalkaliphilic fermentative bacteria in the cellulose-converting process (Ma et al., 2010).

Another process in which moderate halophiles could contribute is removal of phosphate from saline environments in a cheaper alternative to chemical approaches

(Ramos-Cormenzana, 1989). Increasing irrigation has led to 30% to 50% of agricultural areas being affected by salinity. The potential use of moderate halophiles in the recovery of saline soils is therefore gaining importance.

It is thus possible that in the future, halophilic microorganisms that are readily accessible to genetic engineering would be ideal hosts for the in vivo adaptation of valuable but salt-labile macromolecules to high salt stability. Recombinant enzymes from mesophilic donor organisms evolved to halophily would greatly broaden the spectrum of all of the applications mentioned above.

1.6 Aim of the work

Two major goals were pursued during the research presented in this thesis:

I. Experimentally adaptation of a mesophilic model organism, *Escherichia coli*, to osmotic environments in which its progenitor cannot grow, and experimentally evolving the cellular macromolecules of the bacterium to function in highly concentrated salt solutions.

II. Analysis of the adaptive kinetics which allows mesophilic organisms to invade and explore high salt environment, as well as the analysis of the adaptive changes that the evolving organism undergoes during adaptation to the extreme environment.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were purchased from AppliChem, Bio Rad, Fluka, GE-Healthcare, Merck, Riedel-de Haen, Roche, Roth, Sigma Aldrich and VWR.

2.1.2 Instruments

Instrument	**Manufacturer**
Autoclave	Zirbus Technology
Centrifuges:	
Model 4K10	Sigma
Model mini spin plus	Eppendorf
Sorvall	Dupont Instruments
Genetic machine	Home-made, AG Mutzel
HPLC machine Model 1200 SL with ZORBAX Eclipse Plus C18. 3.0 x 150, 3.5 µm column	Agilent Technologies,
IPGphor	Amersham Pharmacia Biotech
PCR-Sprint	Hybaid
Power Supply	BioRad
Protean II xi Cell	Bio Rad
Scanner	Fuji Film
Spectrophotometer	Hitachi, model U-1100
Thermoblock	Biometra
Trans Blot Semi Dry Transfer Cell	Bio Rad
UV transilluminator	Ray test Isotope Measurement GmbH
X-Ray film developer	Optimax

2.1.3 *Escherichia coli* strains

***Escherichia coli* strain**	**Relevant genotype**	**Source**	**Reference**
MG1655	*E.coli* Wild type	Laboratory collection AG Mutzel	Blattner et al., 1997
$MG1655_{abc}$ (abc, number of days under cultivation)		Evolved from MG1655 for around 8 months (Corvers, L. Diploma thesis, 2007) and further in this study	This work
$\varepsilon2124_{abc}$ (abc, number of days under cultivation)	derivative of strain β2124 (MG1655, Δ*def/fmt, mutD*) which is devoid of the genes encoding Met-tRNAi transformylase and polypeptide deformylase and carries a mutation in the *mutD* gene	β2124 from Marlière, P. via Mutzel, R.	Mazel et al., 1994
		Evolved from ε2124 for around 8 months (von Scheibner, M. Diploma thesis, 2006) and further in this study	This work

2.1.4 Media

Minimal (MS-man) liquid medium

MS-Mannitol medium was used as a liquid medium for cultivation of bacteria.

Composition of the medium:

Components	**Concentration in M**	**Amount in g per 1 l bidest. water**	**Amount in g per 5 l bidest. water**
Ammonium chloride	20 mM	0.53 g	2.65 g
Citric acid monohydrate	4 mM	0.84 g	4.2 g
D (-)-Mannitol	11 mM	2 g	10 g
di-potassium hydrogen phosphate	50 mM	8.71 g	43.55 g
Magnesium sulfate heptahydrate	1 mM	0.25 g	1.25 g
NTA-Mix (1000x)	1ml	1 ml	5 ml

MS-man medium supplemented with various concentrations of NaCl was used as specifically indicated. Defined NaCl concentrations were adjusted either by addition of solid NaCl before final volume adjustment or by mixing media containing different NaCl concentrations to reach the desired final NaCl concentration. MS-man medium containing other additions (e.g., KCl, sorbitol, etc.) was prepared in an analogous manner.

Composition of the NTA-Mix (1000x):

Component	Concentration in M	Amount in g per 1 l bidest. water
Boric acid	3×10^{-4}	0.02 g
Calcium chloride dihydrate	3×10^{-3}	0.45 g
Chromium chloride hexahydrate	3×10^{-4}	0.08 g
Cobalt chloride hexahydrate	3×10^{-4}	0.07 g
Copper chloride dihydrate	3×10^{-4}	0.05 g
Ferric chloride hexahydrate	2×10^{-3}	0.81 g
Manganese chloride tetrahydrate	10^{-3}	0.20 g
Nickel chloride hexahydrate	3×10^{-4}	0.07 g
Nitrilotriacetic acid (NTA)	10^{-2}	1.91 g
Sodium molybdate dihydrate	3×10^{-4}	0.07 g
Sodium selenite pentahydrate	3×10^{-4}	0.08 g
Zinc chloride	10^{-3}	0.04 g

Nitrilotriacetic acid was dissolved in 100 ml bidestilled water and the pH was adjusted to 6.5. Then, the rest of the components were added, completed up to 1 l, and the NTA-Mix was sterilized at 120 ºC for 20 min.

Minimal (MS-man) solid medium

Components of MS-man medium were dissolved in 500 ml bidestilled water, while 1.5-2 % agar was added to 500 ml bidestilled water and those two suspensions were separately sterilized at 120 ºC for 20 min. After sterilization and cooling to around 65 ºC they were mixed together and poured into plates.

LB medium

Components	Amount in g per 1 l bidest. water
Bacto-pepton	10 g
NaCl	5 g
Yeast extract	5 g

pH was adjusted to 7.3 and the medium was sterilized at 120 ºC for 20 min.

LB solid medium

1.5-2 % agar was added to the components of the above liquid medium and sterilized at 120 ºC for 20 min.

2.1.5 Buffer and sterilizing agent for the genetic machine

Potassium phosphate buffer

Components	Amount
Potassium dihydrogen phosphate	34.03 g
Bidest. water	5 l

pH was adjusted to 5 by adding of (50mM) of dipotassium hydrogen phosphate, then the buffer was sterilized at 120 ºC for 20 min.

Sterilizing agent (5 M sodium hydroxide)

Component	Amount
Sodium hydroxide	1000 g
Bidest. water	5 l

2.1.6 Reagents for PCR

Component	Manufacturer
2x PCR Master Mix	Fermentas
Oligonucleotides oFUM301=TGC AGA AAT TCA GCG TAT CG oFUM308=AAC GTG GTG ATA AGC CCT TG	BioTez (Berlin)

Standards for agarose gels

Component	Manufacturer
Gene Ruler 100 bp plus DNA-Ladder	Fermentas
Mass Ruler DNA-Ladder Mix	Fermentas
6x DNA loading dye	Fermentas

TAE (50x)

Component	Amount
Acetic acid	28.55 ml
EDTA	9.31 g
Tris	121 g
Bidest. water	to 1 l

2.1.7 Materials and solutions for detection of amino acids and trehalose by thin-layer chromatography

Trichloroacetic acid	15% v/v

TLC plates

Silica gel 60 F 254, layer thickness 0.2 mm	Merck (Darmstadt)

a) Trehalose detection

Standards

Trehalose	10 mM
Glucose	10 mM

Eluent

Butanol	250 ml
Ethanol	150 ml
Bidest. water	100 ml

Spray reagent

Methanol	80ml
Sulfuric acid	20 ml

b) Amino acids detection

Standards

Proline	10 mM
Glutamic acid	10 mM

Eluent

Chloroform	100 ml
Ethanol	64 ml
Acetic acid	20 ml
Bidest. water	16 ml

Spray reagent

Ninhydrin spray solution for TLC	Roth (Karlsruhe)

2.1.8 Materials and reagents for detection of amino acids by HPLC

Column

ZORBAX Eclipse Plus C18, 3.0 x 150, 3.5 µm	Agilent Technologies (Waldbronn)

Amino acids standard

Amino acids standard solution (AAS18_10ML)	Sigma-Aldrich (Steinheim)

Mobile phase A

di-Sodium hydrogen phosphate	10 mM	
Sodium borate	10 mM	pH 8.2
Sodium azide	500 µM	

Mobile phase B

Component	Volume ratio
Acetonitrile: methanol: water	45: 45: 10

Derivatization

OPA

o-phthalaldehyde	10 mg / ml	
3-mercaptopropionic acid	10 mg / ml	
Sodium borate buffer	400 mM	pH 10.2

FMOC

9-fluorenylmethyl chloroformate	2.5 mg / ml
Acetonitrile	99.8 %

Diluent injection

Mobile phase A	100 ml
Concentrated phosphoric acid	250 ml

2.1.9 Buffers and materials for Western-Blot

Anode I (pH 10.4)

Component	**Amount**
Tris-HCl	36.34 g
Methanol	200 ml
Bidest. water	to 1 l

Anode II (pH 10.4)

Component	**Amount**
Tris-HCl	2.3 g
Methanol	200 ml
Bidest. water	to 1 l

Cathode (pH 9.4)

Component	**Amount**
Tris-HCl	3.02 g
ε-Amino-n-caproic acid	5.2 g
Methanol	200 ml
Bidest. water	to 1 l

TBS (pH 7.4)

Component	**Amount**
Tris-HCl	1.212 g
Sodium chloride	8.766 g
Tween 20	500 µl
Bidest. water	to 1 l

ECL I

Solution A	0.5 M Luminol (3-aminophthalhydrazide) in DMSO
Solution B	0.18 M p-Cumaric acid in DMSO
Solution C	1 M Tris-HCl, pH 8.5
Bidest. water	450 ml

450 ml bidestilled water and 50 ml solution C were treated with a stream of nitrogen for 5 min. Then, 2.5 ml solution A and 1.11 ml solution B were added dropwise, and the solution was again treated with a stream of nitrogen for 30 min and stored in the dark at 4 °C.

ECL II

Hydrogen peroxide (H_2O_2) 30 %

Immediately before use 3 µl ECL II were added to 1 ml ECL I and applied on the nitrocellulose membrane.

Antibodies

Antibody	Dilution
Anti RpoS primary antibody (gift from E. Klauck, AG Hengge, FU Berlin)	1:5000 in Roti-Block
Goat peroxidase-conjugated anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, INC)	1:10000 in TBST + milk powder (Frema Reform) Finck (Herrenberg)

Film, membrane and Whatman paper

Kodak BioMax Light Film	Sigma Aldrich
Nitrocellulose Transfer membrane BA85	Schleier & Schuell BioScience GmbH
Whatman paper 1mm	Whatman

2.1.10 Buffers and staining kit for detection of outer membrane proteins on SDS-polyacrylamide gels

Buffers	Staining kit
0.9 % NaCl	Page Silver™ Silver Staining Kit (Fermentas)
50 mM Tris-HCl pH 8.5 + 2 mM EDTA	
2 mM Tris-HCl pH7.8	

2.1.11 Buffers and solutions for 1-dimensionsional SDS-polyacrylamide gel electrophoresis

2x gel sample buffer (2xGSB)

Component	Amount
0.1 % Bromophenol blue in Ethanol	15 ml
1x upper gel buffer	300 ml
Glycerol	150 ml
SDS	30 g
Bidest. water	to 1 l

2% β-mercaptoethanol was added before use.

4x lower gel buffer (pH8.8)

Component	Amount
Tris-HCl	182 g
SDS	4 g
Bidest. water	to 1 l

4x upper gel buffer (pH6.8)

Component	Amount
Tris-HCl	61 g
SDS	4 g
Bidest. water	to 1 l

SDS-polyacrylamide gel electrophoresis

Small gel (5 ml Separation gel, 2.5 ml Stacking gel)

Separation gel

Components for 2 gels	**12.5%**
4x Lower gel buffer (ml)	3.1
30% acrylamid/ bisacrylamid (ml) (Rotiphorese Gel 30)	5.2
MQ water (ml)	4.1
TEMED (µl)	10
10% APS (µl)	100

Stacking gel

Components for 2 gels	**Volume**
4x Upper gel buffer	1.25 ml
30% acrylamid/ bisacrylamid (Rotiphorese Gel 30)	0.5 ml
MQ water	3.2 ml
TEMED	5 µl
10% APS	30 µl

Large gel (35 ml Separation gel , 8 ml Stacking gel)

Separation gel

Components for 2 gels	**12.5%**
4x Lower gel buffer (ml)	18.6
30% acrylamid/bisacrylamid (Rotiphorese Gel 30) (ml)	31.2
MQ water (ml)	24.4
TEMED (µl)	54
10% APS (µl)	540

Stacking gel

Components for 2 gels	Volume
4x Upper gel buffer	5 ml
30% acrylamid/ bisacrylamid (Rotiphorese Gel 30)	2 ml
MQ water	12.8 ml
TEMED	20 µl
10% APS	120 µl

10 x running buffer

Component	Amount
Tris-HCl	30.3 g
Glycine	144 g
SDS	10 g
Bidest. water	to 1 l

Coomassie stain

Component	Volume
Acetic acid	100 ml
Methanol	300 ml
Bidest. water	600 ml

Standard for protein gel

Component	Manufacturer
Protein molecular weight marker	Fermentas

2.1.12 Buffers and solutions for two-dimensionsional SDS-polyacrylamide gel electrophoresis

Washing buffer

10mM Tris-HCl pH 8, 5 mM magnesium acetate

SA buffer

SA buffer contained 0.3% (wt/vol) SDS, 4.44 mg of Tris-HCl liter $^{-1}$, 2.56 mg of Tris base liter $^{-1}$, and 20 mM dithiothreitol (DTT) added freshly.

DNase I and RNase A

DNase I and RNase A were added at concentrations of 0.064 mg ml^{-1} and 0.09 mg ml^{-1}, respectively, together with 58mM $MgCl_2$.

Lysis buffer

Component	Amount per 10 ml	Final concentration
Urea	4.8 g	8M
Thiourea	1.52 g	2M
300mM Tris-HCl pH 8.5	1 ml	30 mM
ASB-14	0.2 g	2%
DTT	0.030 g	20mM
Antiprotease cocktail	a little amount (optionally)	

Urea and thiourea were dissolved by intensive shaking, then 0.5 g Resin (AG 501-X8 Resin, Bio Rad) was added, and the solution was filtrated through a Milipore filter (0.22 µm). 0.2 g ASB-14 was added and 2 ml aliquots were stored at -20 °C. 20mM DTT (0.006 g) and half of tablet of antiprotease cocktail (EDTA free, Roche) were added to aliquot 2 ml prior to use.

SDS equilibration buffer

Component	Amount	Final concentration
Urea	21.66 g	6M
Tris-HCl 1.5 M pH 8.8	2.02 g	50 mM
Glycerol (50 %)	36 ml	30 %
SDS	1.2 g	2 %
MQ water	to 60 ml	

Rehydration buffer (for IPG Strips 4-7, GE Healthcare)

Component	Amount per 25 ml	Final concentration
Urea	10.51 g	7M
Thiourea	3.8 g	2M
ASB-14	0.5 g	2 %
IPG buffer pH 4-7 (GE Healthcare)	150 µl	0.6 %
Destreak solution (GE Healthcare)	300 µl	
DTT		1%

Urea and thiourea were dissolved in around 20 ml MQ water by vigorous vortexing for 20 min at RT, then 5 g resin/100 ml was added and the suspension was vortexed for 1 h at RT, then the solution was filtrated through a 0.22 µm filter. ASB, IPG buffer and Destreak solution were added and the suspension was completed to 25 ml with MQ water. The buffer was stored aliquoted as 0.8 ml at -20 °C. Before using, DTT were added to final concentration 1%. For 18 cm dry strips the total volume of rehydration buffer including the protein sample was equal to 340 µl.

Agarose sealing solution

Component	Amount
1xSDS electrophoresis buffer	100 ml
Agarose	0.5 g
1% Bromophenol blue stock solution	200 µl

Bromophenol blue stock solution

Component	Amount
Bromophenol blue	100 mg
Tris-base	60 mg
MQ water	to 10 ml

SDS electrophoresis buffer (10x)

Component	Amount
Tris-base	60.5 g
Glycine	288.2 g
SDS	40 g
MQ water	to 2 l

2D gels (150 ml solution)

Components for 2 gels	**Amount**
1.5 M Tris pH 8.8	37.5 ml
30 % acrylamid/bisacrylamid (Rotiphorese Gel 30)	62.55 ml
MQ water	47.7 ml
10 % SDS (filtrated 0.22 µm)	1.5 ml
TEMED	0.075 ml
10% APS	0.8 ml

Kits

Kits	**Manufacturer**
2-D Clean-Up Kit	GE Healthcare
Immobiline™ Dry Strip gels, 18 cm, pH 4-7	GE Healthcare
RC/DC Protein Assay II Kit	BioRad
SYPRO® Ruby Protein Stain	BioRad

Gel fixing solution

Component	**Amount**
Acetic acid	100 ml
Methanol	400 ml
MQ water	to 1 l

Gel washing solution

Component	**Amount**
Acetic acid	70 ml
Methanol	100 ml
MQ water	to 1 l

2.2 Methods

2.2.1 Operation of the genetic machine

Continuous proliferation under osmotic selection

All evolved strains were cultivated aerobically in simple inorganic minimal media supplemented with a chemically stable, autoclavable carbon source (mannitol) at 30 °C. In the 26.5-ml reactors, which were used for continuous cultivation, cell densities were adjusted to approximately 1 x 10^9/ml, resulting in a total of ca. 2.5 x 10^{10} cells under constant selection (the mutation rate for *E. coli* is ca. 5 x 10^{-10} per base pair per generation; Drake, 1991).

Technically (Fig. 2 and 3), the continuous cultivation process is executed automatically as follows. Cultures are grown under the regime described above (for 6 h in case of MG1655 and for 12 h in case of ε2124) in one of the two reactors (the second reactor being empty and sterile). The culture is then transferred to the second reactor. The first reactor and all of the other inner surfaces of the device which were in contact with living cells are sterilized with an excess of concentrated (5 M) NaOH. After removing the sterilizing agent, all surfaces that were in contact with it are neutralized by rinsing with an excess of slightly acidic (pH 5.0) 50 mM K-phosphate buffer. After removal of the buffer solution, this part of the device is empty and sterile, ready to accommodate the proliferating cell suspension which was in the second reactor.

During normal operation of the device, the risk of contamination with undesired microorganisms is nil, however, any "open" manipulation necessary during inoculation, exchange of reservoirs for liquids or withdrawal of samples bears such a risk. This is controlled by (i) applying standardized procedures for autoclaving liquids and connecting reservoirs or devices for inoculation or sampling to the device (for example, all entries into and exits from recipients are protected by tandem sterile filters during and after autoclaving; connections between tubing are realized via isolated sectors delimited by stainless steel adapters which can be flame-sterilized) and (ii) by regularly checking the genetic identity of the evolved strains (every 200-

250 generations) by PCR amplification and gel electrophoretic analysis of unambiguous diagnostic genetic markers.

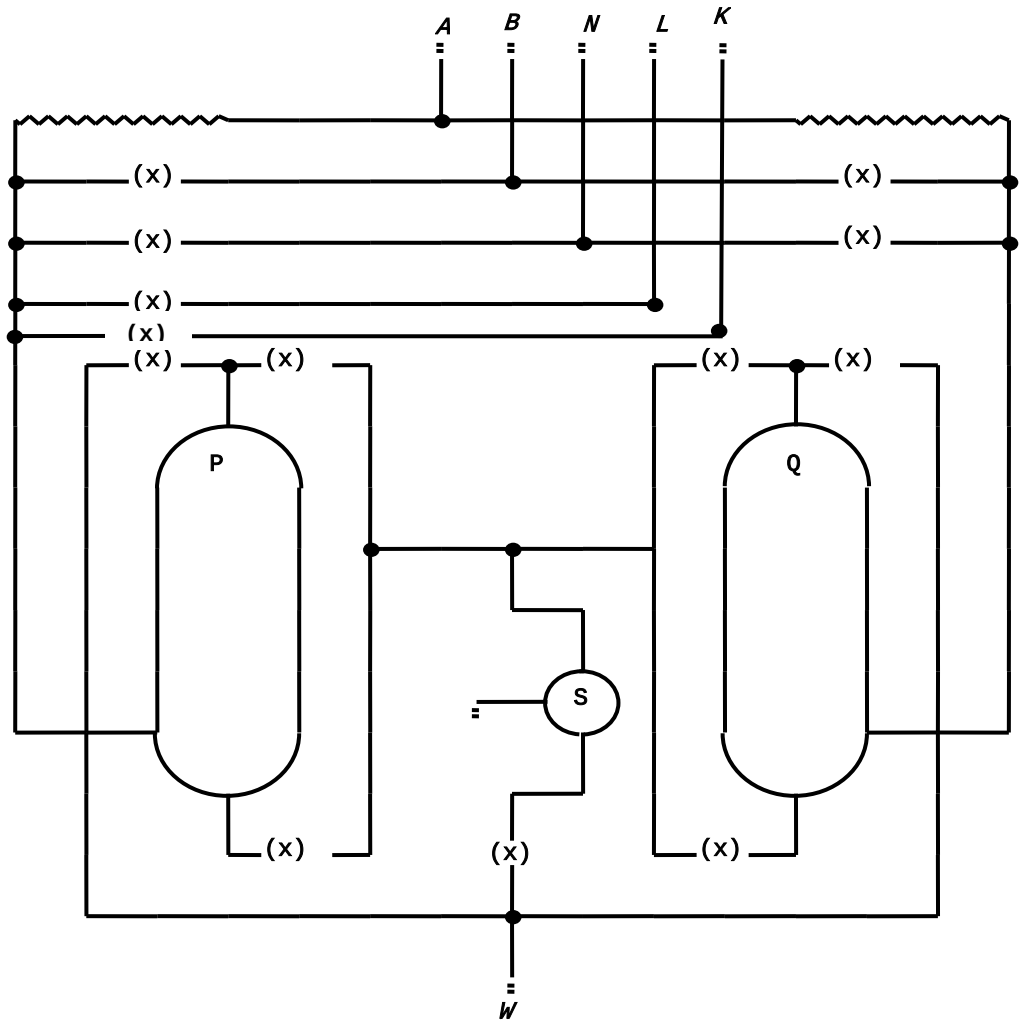

Figure 2: Schematic representation of the Genemat.

Connection are indicated by solid lines; P, Q, reactors; S, sampling port; (x) solenoid valves; A, gas supply; B, buffer supply; N, supply of sterilizing agent; L, permissive medium supply; K, nonpermissive medium supply; W, waste.

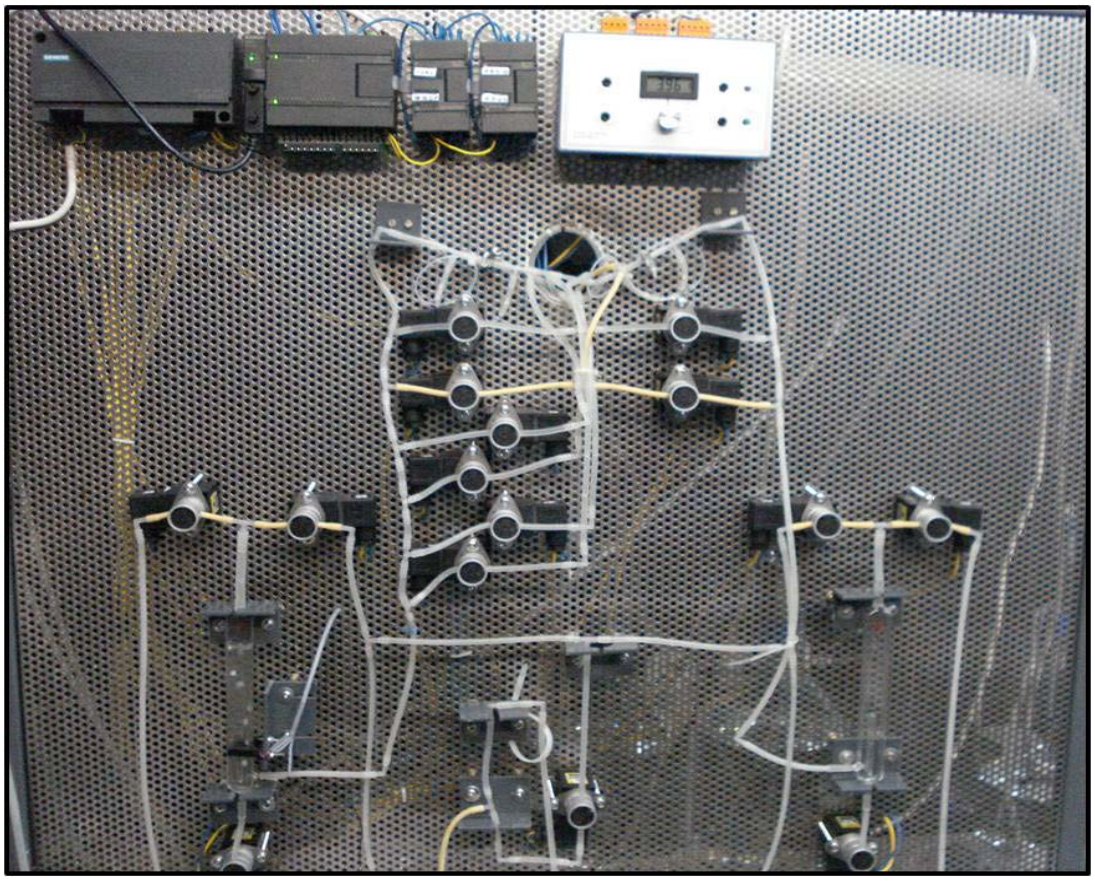

Figure 3: Setup of the Genemat.

The scenario of halophilic adaptation

For the purpose of experimental evolution to high salt tolerance and, ultimately, halophily, a fully automated, combined chemostat/turbidostat regime was applied (Fig. 4) which we name the "conditional pulse feed regime". Populations of cells grow in an excess of nutrients at a rate close to their maximal growth rate. The culture is periodically diluted with a defined volume of fresh, permissive medium at regular, defined intervals (chemostat regime). Permissive medium contains a salt concentration that is fully compatible with growth of the population. Whenever the cell density becomes superior to a prefixed threshold value (e.g., 10^9/ml; "turbidostat threshold"), a defined volume of nonpermissive medium is applied. Nonpermissive medium contains a salt concentration that is inhibitory to growth of the population. If growth of the population is still compatible with this challenge, cell density will again pass the threshold, and the culture will be diluted with another pulse of non permissive medium. If, however, growth slows down or is even halted due to the

osmotic challenge, repeated pulses of permissive medium will partially relieve the challenge, and growth can resume. This regime thus automatically adjusts the chemical environment to conditions which are just tolerable for growth at near-maximal rates. Any emerging genetic variant that can tolerate more challenging conditions will enjoy a selective advantage, causing the system to "call for nonpermissive medium" more frequently, and gradually displace its more poorly adapted progenitors. The underlying genetic alterations become rapidly fixed in the population.

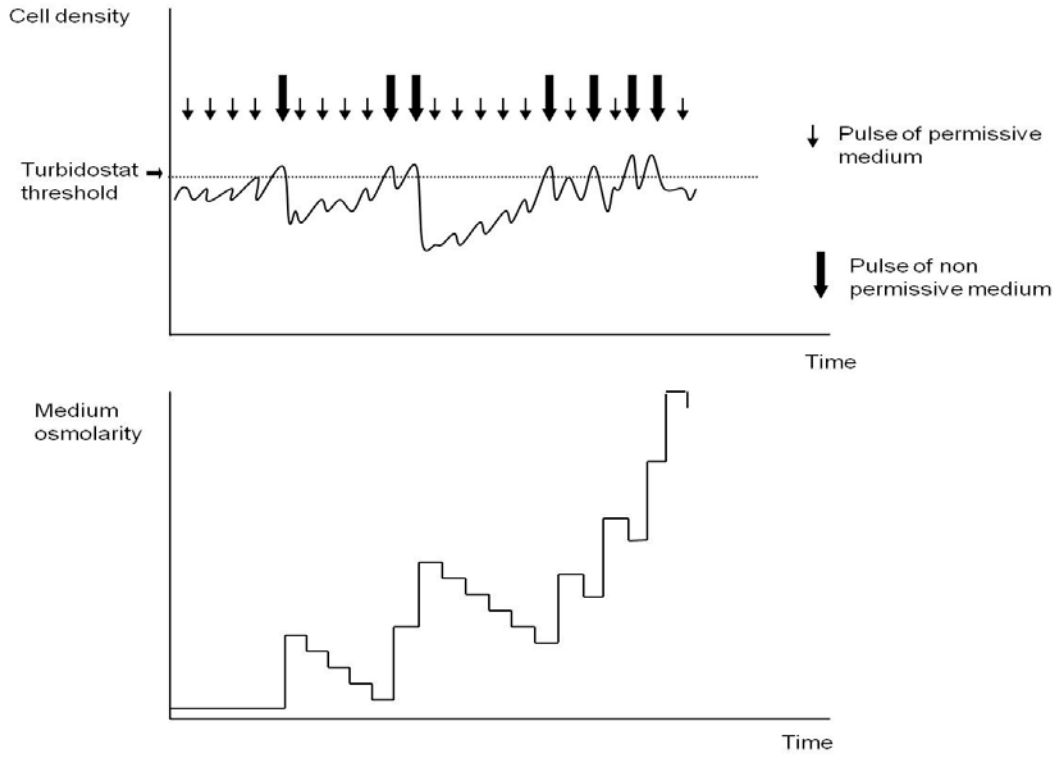

Figure 4: Schematic representation of continuous cultivation under a conditional pulse feed regime.

Populations of cells grow in an excess of nutrients at a rate close to their maximal growth rate and are diluted with a pulse of permissive medium of defined volume at regular, defined intervals. When the density of the culture passes the prefixed "turbidostat threshold", a pulse of nonpermissive medium of defined volume is applied instead.

Analysis of growth characteristics

From calibrated inflow rates and the opening times of the valves, which control the dilution of the culture with permissive and non permissive medium, the current salt concentration can be determined. The total number of pulses per time unit is determined by the preset rhythm (352 pulses/day).The number of pulses of

nonpermissive medium and the respective opening times of the valve commanding addition of nonpermissive medium are recorded on a personal computer running in the DOS mode with the help of a program ("snoopy", S. Brunke, unpublished) and from these data the supplied volumes of permissive and nonpermissive medium per day can be calculated.

Inoculation of the genetic machine

Initial cultures of MG1655 and ε2124 from previous work were used. These cultures were stored frozen in the presence of 10% (v/v) DMSO at -70°C. A one-ml aliquot was thawed at room temperature, inoculated in 30 ml MS-man/0.8 M NaCl medium in an Erlenmeyer flasks and incubated at 30ºC for 2 days. Cultures were inoculated via the machine's sampling port using a sterile 50 ml plastic syringe.

Preservation of bacterial strains

For preservation of bacterial strains, samples were withdrawn weekly from the machines. 100 µl DMSO were mixed with 900 µl of the bacterial culture, vortexed, and the samples were frozen at -70°C.

Control of the genetic identity of bacterial strains by PCR

The genetic identity of the evolving strains was checked every 200-250 generations by PCR amplification of a part of the *def/fmt* operon (Fig. 5). In ε2124 the operon is disrupted by insertion of a Chloramphenicol resistance cassette which renders the amplified DNA fragment larger (ca. 2000 bp) than in the wild type (MG1655) situation (ca. 1000 bp). For PCR, 1 ml of bacterial suspension grown to OD_{600}=0.8, was centrifuged at 8000 rpm for 5 min, and the pellet was resuspended in 0.5 ml MQ water. One µl of this suspension was used as a source of genomic DNA in the PCR reaction. PCR reaction was performed as follows:

25 µl assay mixture contained
12.5 µl Master mix (2x)
1 µl oligo 301 (10 µM)

1 µl oligo 308 (10 µM)

10.5 µl MQ water

1 µl of bacterial cell suspension

In the PCR reaction the following program was used:

Denaturation	95ºC 10 min	
Denaturation	95ºC 45 s	**30 x**
Primer annealing	58ºC 45 s	
Polymerization	72ºC 3 min	
Polymerization	72ºC 5 min	

Agarose gel electrophoresis

5 µl of PCR product were mixed with 1 µl of 6x concentrated loading dye and loaded onto a 1% agarose gel. Electrophoretic separation of DNA was carried out at 100 V for 30 min, then the gel was stained in 10 mg/ml Ethidium bromide for 15 min. The result documented in figure 6 shows an example of such a control experiment.

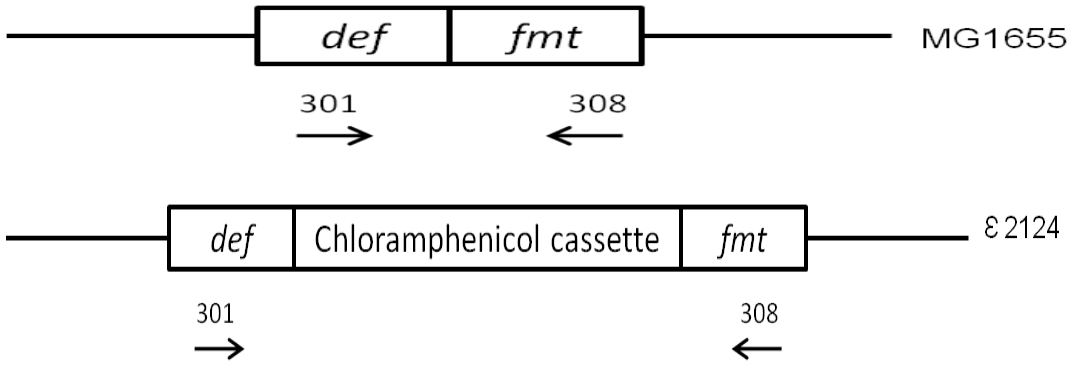

Figure 5: Schematic representation of the *def/fmt* region in *E. coli* MG1655 and the deletion of the *def/fmt* region in *E. coli* ε2124

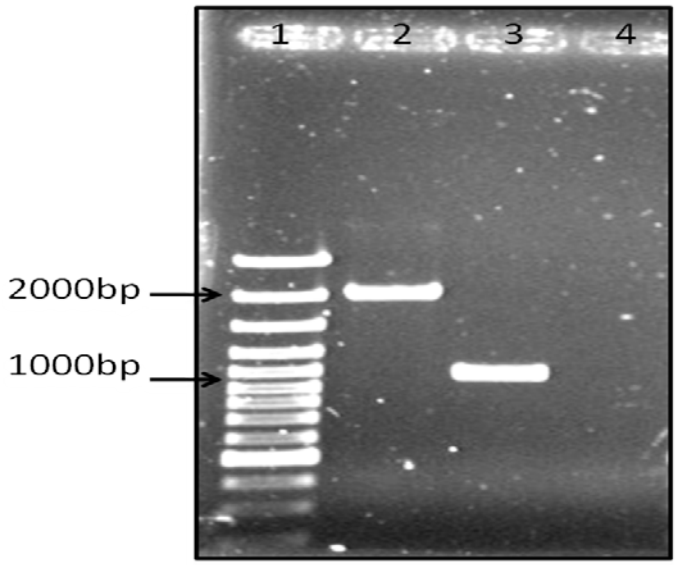

Figure 6: Control of the genetic identity of the bacterial strains by PCR

1, Gene Ruler 100 bp plus DNA-Ladder; 2, *E. coli* ε2124; 3, *E. coli* MG1655; 4, MQ water as a negative control.

2.2.2 Analysis of salt tolerance and sorbitol tolerance

To analyze the development of salt tolerance, growth of the evolving strains in media containing increasing concentrations of various salts (NaCl, KCl, KI and K_2SO_4) or sorbitol was measured.

30 ml MS-man medium with the indicated concentrations of salt were inoculated with 1 ml of a bacterial culture in a 100ml Erlenmeyer flask and incubated at 30ºC with shaking at 150 rpm for 24-48 h. Bacterial growth was determined by measuring the optical density at 600 nm (OD_{600nm}).

2.2.3 Determination of generation time

Generation times of evolving strains in the presence of various concentrations of salts (NaCl, KCl) and Sorbitol at different concentration were determined.

For this experiment, 30 ml MS-man medium with the indicated concentration of salt were inoculated in Erlenmeyer flasks with an appropriate volume of bacterial

culture to give an initial OD_{600nm} of 0.1 and incubated at 30 ºC with shaking at 150 rpm. Growth was determined by measuring OD_{600nm} at regular intervals.

2.2.4 Detection of intracellular trehalose by thin-layer chromatography

Cells were grown in MS-man medium with various concentrations of NaCl. 10 ml of bacterial culture were harvested when the cell density had reached OD_{600nm}=0.8 by centrifugation at 4000 rpm for 10 min, and the bacterial pellets were washed once in 1ml of MS-man medium containing the same salt concentration. The pellets were then resuspended with 8 µl of 15% TCA, incubated on ice for 10 min and centrifuged again at 8000 rpm for 10 min to remove insoluble material. Afterwards the extracts were transferred to a new Eppendorf tube.

TCA extracts (3 µl) were chromatographed on TLC silica gel 60 F254 thin-layer plates using 50% butanol/30% ethanol/20% water for 4 h, dried, sprayed with 80% methanol/20% sulfuric acid and the chromatograms were developed at 115ºC in an oven for 15 min.

2.2.5 Detection of intracellular amino acids by thin-layer chromatography

Cells were grown in MS-man medium with various concentrations of NaCl and bacterial extracts were prepared as described above (2.2.4). TCA extracts (3 µl) were chromatographed on TLC silica gel 60 F254 thin-layer plates using 50% chloroform/32% ethanol/10% acetic acid/8% water for 4 h, dried, sprayed with ninhydrin solution (Spray solution ready to use , Roth) and incubated at 115ºC in an oven for 15 min.

2.2.6 Quantification of intracellular amino acids by high-performance liquid chromatography (HPLC)

The detection and quantification of amino acids was performed by HPLC analysis on an Agilent 1200 SL system referring to an Agilent Application Note. To ensure specificity and sensitivity of the analysis, amino acids in the samples were first derivatized using a pre-column protocol [o-phthalaldehyde (OPA) for the primary and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids].

The derivatization was done using the following injection program for the automated liquid sampler:

1. Draw 2.5 µl from sodium borate vial
2. Draw 1 µl from sample vial
3. Mix in washport 5 times
4. Wait 12 s
5. Draw 0.5 µl from OPA vial
6. Mix in washport 10 times max speed
7. Draw 0.4 µl from FMOC vial
8. Mix in washport 10 times max speed
9. Draw 32 µl from Diluent injection vial
10. Take 20 µl and mix in washport 8 times
11. Inject
12. Wait 6 s
13. Valve bypass

After injection, the samples were separated on a ZORBAX Eclipse Plus C18 column at 40ºC using a binary mobile gradient of mobile phase A and mobile phase B.

The gradient program was as follows:

Time in min	% of mobile phase B
0	2
0.5	2
20	57
20.1	100
23.5	100
23.6	2
25	End
Flow (ml min^{-1})	0.64

The eluting amino acids were detected with a fluorescence detector (FLD).

Parameter

FLD: signal was monitored at a wavelength of 450 nm (excitation wavelength at 340 nm) after 18 min monitoring at 305 nm (excitation wavelength at 266 nm) for detection of secondary amino acids.

Quantification:

For quantitative analysis, a standard amino acid (AAS18) mix was used as a reference to identify and determine the concentrations of the amino acids in the cell extracts. The standard is composed of Alanine, Arginine, Aspartic acid, Cysteine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine and Valine.

2.2.7 Detection of total cellular proteome with the use of one-dimensional SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out after Laemmli (Laemmli, 1970).

Preparation of samples

Cells were grown in MS-man medium containing various concentrations of NaCl. 10 ml of culture were harvested at OD_{600nm}=0.4 by centrifugation at 5000 rpm for 10 min at 4ºC. Pellets were washed once with 1 ml of the same medium, resuspended in 200 µl of 2 x GSB, heated at 95ºC for 10 min and centrifuged again at 13000 rpm for 1 min to remove insoluble material. Supernatants were transferred to fresh tubes.

Gel electrophoresis

For gel electrophoresis a Protean II xi cell was used. Equal amounts of protein samples were loaded on 12.5 % polyacrylamide/0.1% SDS gels and proteins were separated under cooling at 75 V overnight.

Staining

Gels were stained with Coomassie-Brilliant blue G250 stain overnight, and then destained in destained solution for 4-6 h.

2.2.8 Western Blot

Preparation of protein samples

Cells were grown in MS-man medium supplemented with various concentrations of NaCl. 10 ml of each culture were harvested at OD_{600nm}=0.4 by centrifugation at 5000 rpm for 10 min at 4ºC. Pellets were washed once in 1 ml of the same medium, resuspended in 200 µl of 2 x GSB, heated at 95ºC for 10 min and centrifuged again at 13000 rpm for 1 min to remove any insoluble material. Supernatants were transferred to fresh tubes.

Separation and transfer of protein sample

Equal amounts of protein samples were loaded onto and separated on 12.5 % polyacrylamide/0.1% SDS gels. The transfer of proteins to nitrocellulose membrane was carried out with the "semi dry method" according to Kyhse-Andersen (1984).

2.2.9 Detection of outer membrane proteins by SDS-polyacrylamide gel electrophoresis

Preparation of the sample

The wild type MG1655 strain was grown in MS-man medium without salt and evolved strains with 0.8 M NaCl. Cultures were incubated with shaking at 150 rpm in 30ºC and harvested at OD_{600}=0.8.

Extraction of outer membrane proteins

Extraction of outer membrane proteins was carried out according to Lugtenberg et al. (1975), as follows:

1. 100 ml of growing cells were harvested by centrifugation at 5000 rpm, 4ºC for 10 min.

2. Pellets were washed with 40 ml of 0.9% NaCl and centrifuged in a Sorvall centrifuge using a GSA rotor at 5000 rpm, 4ºC for 10 min.

3. Pellets were resuspended in 40 ml of 50 mM Tris-HCl pH 8.5 containing 2 mM EDTA. After sonication (4 times 1 min under cooling), unbroken cells and large fragments were removed by centrifugation for 20 min at 5000 rpm in a Sorvall centrifuge using a GSA rotor

4. The supernatant was centrifuged for 90 min at 20000 rpm in a Sorvall centrifuge using a SS-34 rotor. The obtained pellet containing the envelopes was resuspended and washed once in 20 ml of 50 mM Tris-HCl pH 8.5 containing 2 mM EDTA and

centrifuged again for 90 min at 20000 rpm. The pellet was finally resuspended in 2 ml of 2 mM Tris-HCl, pH 7.8.

Gel electrophoresis

Equal amounts of protein sample were loaded onto and separated on 12.5 % polyacrylamide /0.1% SDS gels.

Staining

Gels were stained with the Page Silver™ Silver Staining Kit (# K0681) according to the manufacturer's instructions.

2.2.10 Analysis of the total cellular proteome by two-dimensional SDS gel electrophoresis (2-D PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a method commonly used in proteomics. The first dimension is isoelectric focusing (IEF), which is used to separate proteins according to their isoelectric point, while the second dimension is SDS-PAGE, which relies on the separation of proteins according to their molecular weight.

Preparation of protein samples for 2D-PAGE

Samples were prepared for 2D-PAGE according to Weichart et al. (2003) as follows:

The MG1655 wild type strain was grown in MS-man medium without salt and the $MG1655_{858}$ evolvant with 1.2 M NaCl. 10 ml of each sample were harvested during the logarithmic growth phase by centrifugation at 5000 rpm for 10 min at 4ºC. Pellets were washed once with 1 ml of the same medium, then twice with the wash buffer and centrifuged at 13000 rpm for 5 min in mini spin plus centrifuge. Pellets were then resuspended with 200 µl of SA buffer. The resuspended pellets were heated at 100 ºC for 2 min and subsequently kept at RT for 5 min. Then DNase I and RNase A

were added at concentrations of 0.064 mg ml^{-1} and 0.09 mg ml^{-1}, respectively, together with 58 mM $MgCl_2$. The mixture was vortexed vigorously for 1-2 min and nuclease digestion was allowed to proceed for 20 min.

Solubilization and denaturation of proteins

The pellet was resuspended in a lysis buffer to solubilize and denature proteins by intensive vortexing for 30 min at RT. After solubilization the sample was sonicated 4 times for 30 s and then centrifuged at 13000 rpm at RT for 5 min. The supernatant, which contains solubilized and denatured proteins, was collected and transferred into fresh 2 ml tubes.

Cleaning of proteins with the "2-D Clean-Up Kit"

Before applying to 2D-PAGE, proteins were precipitated and cleaned up with the use of a 2-D Clean-Up Kit (GE Healthcare) to reduce streaking on the gels and remove non protein contaminants. The protein sample was cleaned according to the 2-D Clean-Up Kit's manual (GE Healthcare).

Determination of protein concentrations

The protein concentration of the samples was determined with the use of RC/DC Protein Assay II (BioRad), according to the protocol provided by the company. The measurement of protein concentration with the RC/DC Protein Assay II is based on Lowry's method (Lowry et al., 1951).

Isoelectric focusing

200 µg of the purified proteins were separated during IEF using 18 cm Immobiline™ Dry Strips, pH 4-7 (GE Healthcare). Before IEF, the samples were incubated in 10 mM DTT solution at RT for 20 min to reduce the disulfide bonds of the proteins. Afterwards, the samples were incubated in 2% IPG buffer (pH 4-7) at RT for 20 min

to denaturate the proteins and minimize streaking which can appear on the gels. Then the volume of the sample was adjusted to 340 µl with rehydration buffer and the sample was applied on the dry strip. Isoelectric focusing was conducted according to the following program:

Step	Time	Volt
Rehydration	1h	0 V
Step1	5.5 h	30 V
Step2	8h	60 V
Step3	1h	200 V
Step4	1h	500 V
Step5	1h gradient	8000 V
Step6	4.5h	8000 V
Step7	5h	30 V

After IEF, the strips were either directly applied on the second-dimensional gel (SDS-PAGE) or stored frozen at -70 °C.

SDS-PAGE (Second dimension)

Equilibration of the strips

Before separation of the proteins according to their molecular weight, the IEF strips were first put in an equilibration tube and incubated in 15 ml of SDS equilibration buffer in the presence of 0.15 g DTT with gentle shaking for 20 min at RT for protein reduction. Then, the strips were transferred into another equilibration tube with 15 ml of SDS equilibration buffer and 0.375 g iodoacetamide and incubated with gentle shaking for 20 min at RT for protein alkylation.

SDS-PAGE

For gel electrophoresis a Protean II xi cell was used, and 12.5 % acrylamide/0.1% SDS gels (22cm x 20 cm) were prepared.

After equilibration, the strips were placed on the surface of the SDS polyacrylamide gel and covered with agarose sealing solution. Agarose sealing solution was used to protect the gels from drying during SDS-PAGE gel electrophoresis. Electrophoresis was conducted at 75 V for 19 h and 16 °C with 1 x SDS electrophoresis buffer.

Staining of the gels with SYPRO Ruby

SYPRO Ruby gel stain is a fluorescence dye used to detect proteins in an acrylamide gel. After SDS-PAGE gel electrophoresis, the 2D gels were fixed in a fixing solution (500 ml of fixing solution per gel) for 5 hour at RT. After fixing, the gels were washed twice in MQ-water (each washing for 10 min at RT) and then stained with SYPRO Ruby gel stain (500 ml per gel) overnight in the dark at RT. After staining, the gels were washed with washing solution (800 ml per gel) for 30 min, then with MQ-water two times for 5 min. The stained gels were then visualized using the FLA 2000 scanner (Fuji Photo Film) with 100 resolution, 16 bit degration and wavelength of 473 nm excitation and 510 nm emission.

3. Results

This thesis describes experiments to evolve halotolerant and halophilic derivatives of the mesophilic bacterium *Escherichia coli* under strictly controlled and highly selective laboratory conditions. Using the "Genemat" technology (Marlière and Mutzel, 2004), which allows for permanent proliferation of living organisms exclusively in suspension, two populations of *E. coli* were propagated under limiting osmolarities for approximately 8800 and 6700 generations, respectively. Populations of approx. 2.5 x 10^{10} cells were grown at 30 °C in minimal medium containing mannitol as a carbon source, and a growth rate of at least 0.25 was imposed by the dilution rate. A conditional pulse-feed regime was applied which automatically maintains the osmolarity in the growth medium at just tolerable levels. For this, the culture chamber was connected with two medium reservoirs, one containing "permissive" medium (compatible with growth of the cells), and the other containing "nonpermissive" medium with an osmolarity that is too high for growth. Pulses of permissive medium regularly dilute the culture, and the cell density is constantly monitored. Whenever cell density passes a pre-set threshold (ca. 10^9 cells/ml) the culture is diluted with a pulse of nonpermissive medium instead of permissive medium. Under this regime genetic variants, which tolerate higher osmolarity, are rapidly displacing their mesophilic progenitors. Pilot experiments have been reported in the Diploma theses of Linda Corvers (2007) and Markus von Scheibner (2006). The present work started with derivatives of *E. coli* MG1655 and ε2124 that had been selected during their work. The following chapters describe the evolutionary paths of both strains and their evolved traits.

3.1 MG1655

3.1.1 Evolutionary kinetics

Figure 7 shows the adaptive kinetics of strain MG1655 during the process of continuous selection for growth in medium with increasing NaCl concentration.

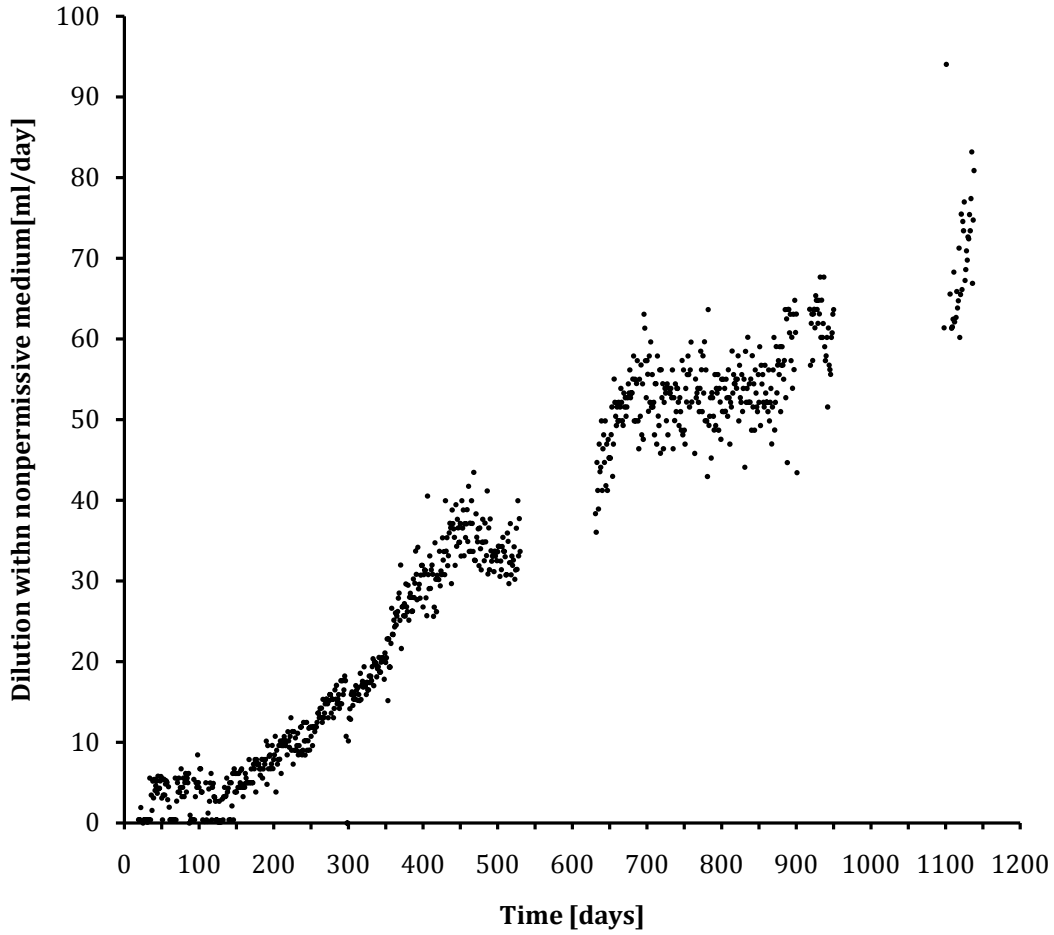

Figure 7: Evolutionary kinetics of MG1655 under the conditional pulse-feed regime for selection of halotolerance.

Cell cultures (26.5 ml, approximately $1x10^9$ cells/ml) were cultivated at 30 °C in minimal (MS-man) medium containing either 0.8 M NaCl (permissive medium) or 2 M NaCl (nonpermissive medium). Opening times for the magnetic valve commanding the inflow of nonpermissive medium were recorded and are summed up for 24 hours periods (dots). Data for the periods between days 530 and 631, and 950 and 1098, respectively, were lost due to failure of the recording personal computer.

An initial phase of steadily increasing halotolerance during the first 450 days of the experiment (approximately 2700 generations) was followed by a period of around

150 days where further adaptation appeared halted. Between 600 and 900 days of continuous cultivation the consumption of nonpermissive medium (2 M NaCl) showed a further 30% increase to remain again constant until around day 1100 when a new, sharp increase in the rate of dilution with nonpermissive medium was recorded which lasted until the end of the cultivation campaign reported here. From the ratio of the volumes of permissive and nonpermissive media consumed per day, the steady state concentration of NaCl in the culture after 1138 days of selection can be estimated to be 1.3 M. Including the initial experiment performed by L. Corvers (2007) MG1655 has been cultivated under challenging NaCl concentrations for approx. 8800 generations.

3.1.2 Evolution of halotolerance

Growth in NaCl

To determine the level of halotolerance of MG1655 derivatives selected during the course of the experiment, isolates from the genetic machine were tested for their ability to grow in batch cultures in media with various osmolarities. Figure 8 shows growth yields of successive evolvants after cultivation in batch culture in various NaCl concentrations along with the properties of wild type MG1655 populations. To get an idea of both growth rates and final yields as well as maximal halotolerance, the OD_{600nm} of the cultures was measured after incubation for 24 and 48 hours at 30 °C. Wild type MG1655 grew best in the absence of NaCl and up to 0.6 M of the salt, albeit with much reduced growth rates at the latter concentration; only occasionally significant growth at 0.8 M could be observed after 48 hours of incubation. This concentration thus delineates the limit of halotolerance of *E. coli* MG1655 (Fig. 8 A). As it could be expected from the adaptive kinetics (see above, Fig. 7), evolvants supported progressively higher salt concentrations. The last sample isolated during this work (day 1119, "$MG1655_{1119}$") grew to high yields at 1.4 M NaCl and showed significant growth up to 2.0 M (Fig. 8 D). Interestingly, growth at low salinities became poorer and poorer in populations after longer selection. Thus, the derivative isolated on day 858 grew only after prolonged incubation in medium without NaCl (Fig. 8C), isolates obtained at day 1119 did not show any growth even after incubation for 48 hours, and growth at moderate salt concentrations was severely impaired in isolates from later stages of the experiment (Fig. 8 D).

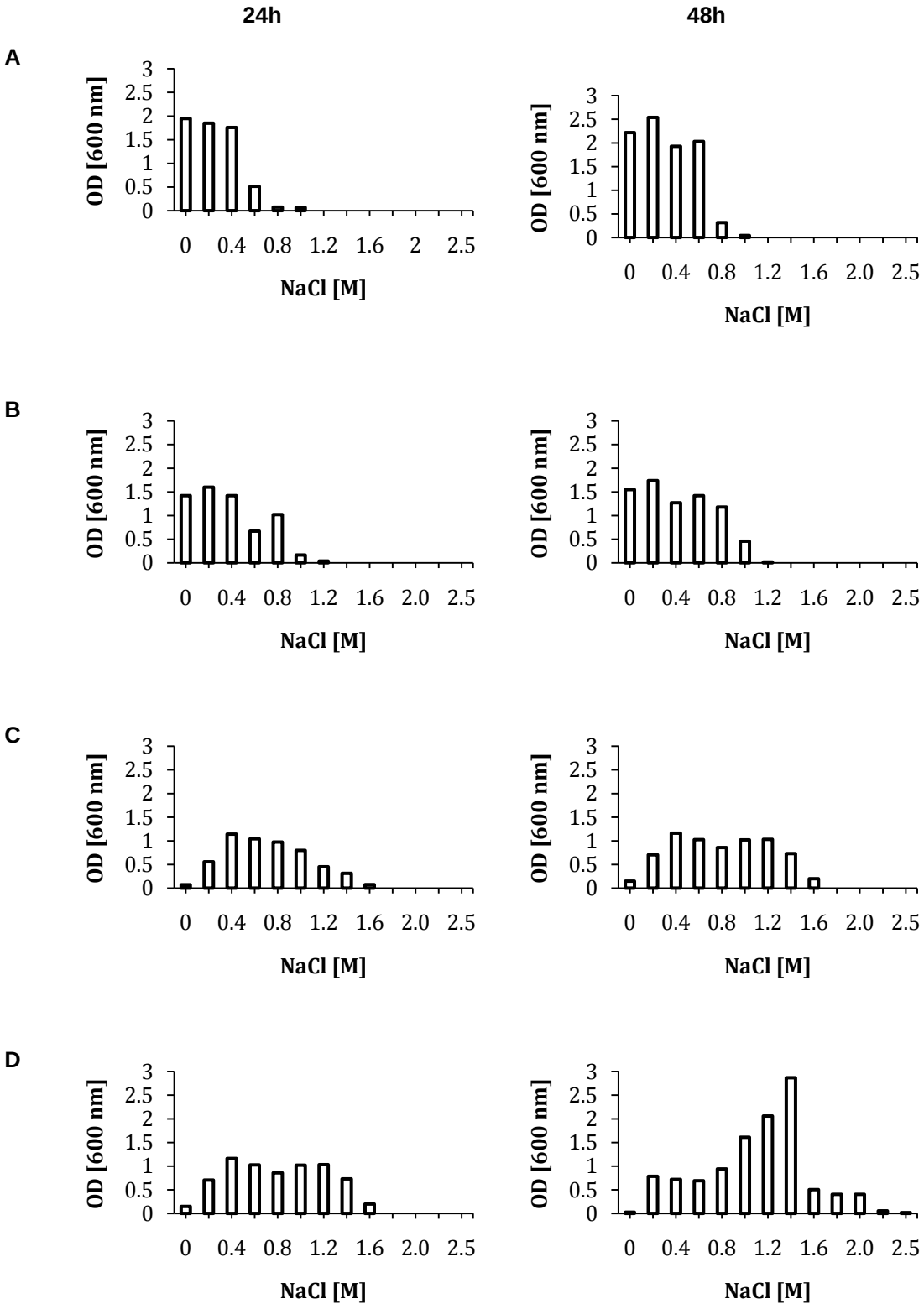

Figure 8: NaCl tolerance of *E. coli* MG1655 wild type cells and their evolved derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated NaCl concentrations from precultures grown at 0.0 M NaCl in case of wild type MG1655 and in 0.8 M NaCl for their evolved derivatives and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $MG1655_{262}$; C, $MG1655_{858}$; D, $MG1655_{1119}$.

Yet, it is still possible to re-adapt these derivatives to low salt concentrations by passaging them over decreasing salinities (data not shown). Taken together, these data suggest a shift in the physical niche of the bacteria; robust growth in media containing around 10% w/v salt is “paid for” by the failure to grow well in diluted media. At an intermediate salinity of 0.6 M (approx. 3.5% w/v) NaCl growth yields of wild type and evolved strains are comparable.

Tolerance towards cations, anions and osmolarity

In order to analyze whether the nature of the cations and anions as well as the total osmolarity of the medium influence growth of wild type and evolved MG1655 populations, NaCl was exchanged for KCl, K_2SO_4 and KI, whereas sorbitol was used as an uncharged, osmotically active extracellular solute.

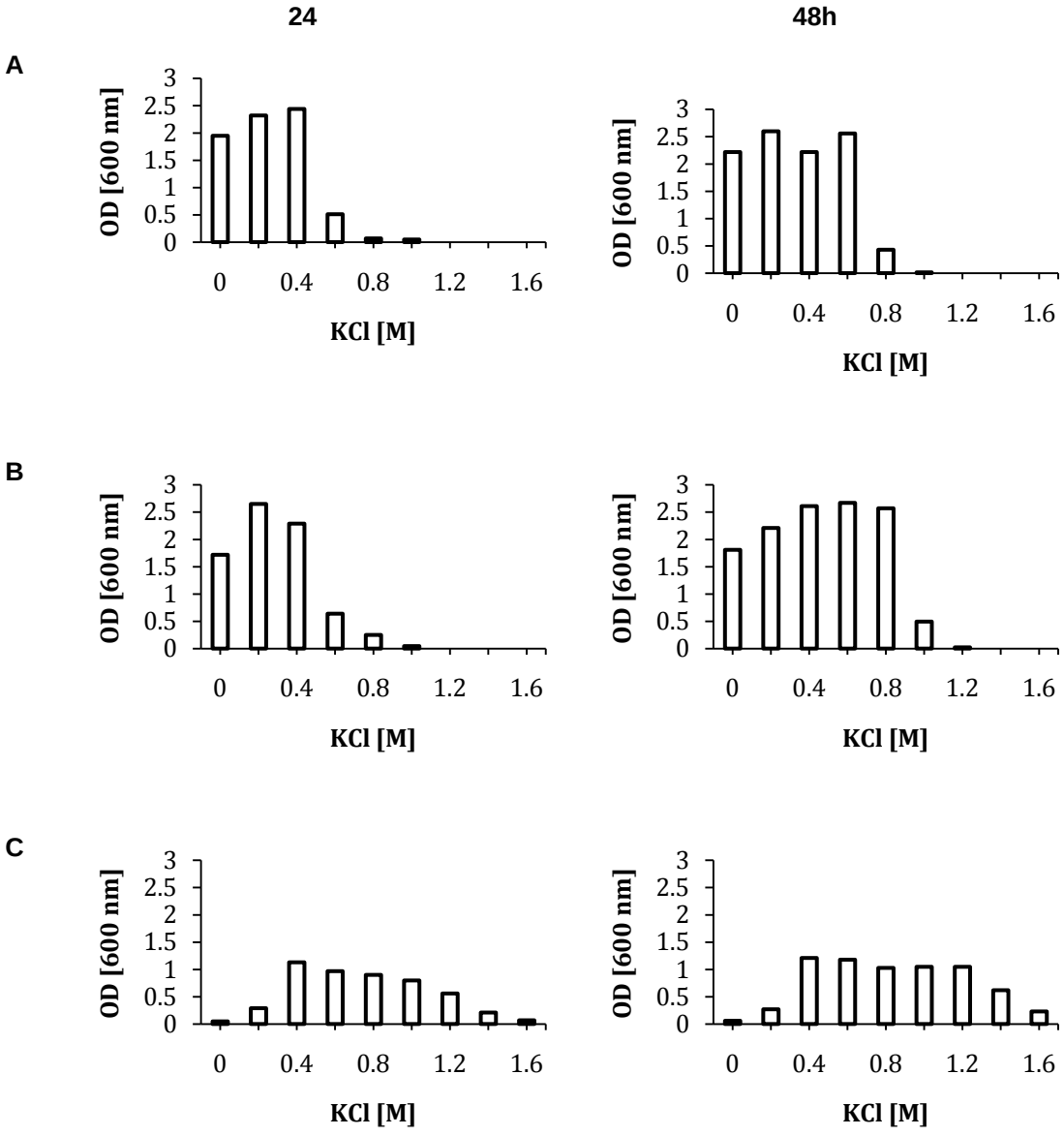

Figure 9: KCl tolerance of *E. coli* MG1655 wild type cells and their evolved derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated KCl concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $MG1655_{262}$; C, $MG1655_{858}$.

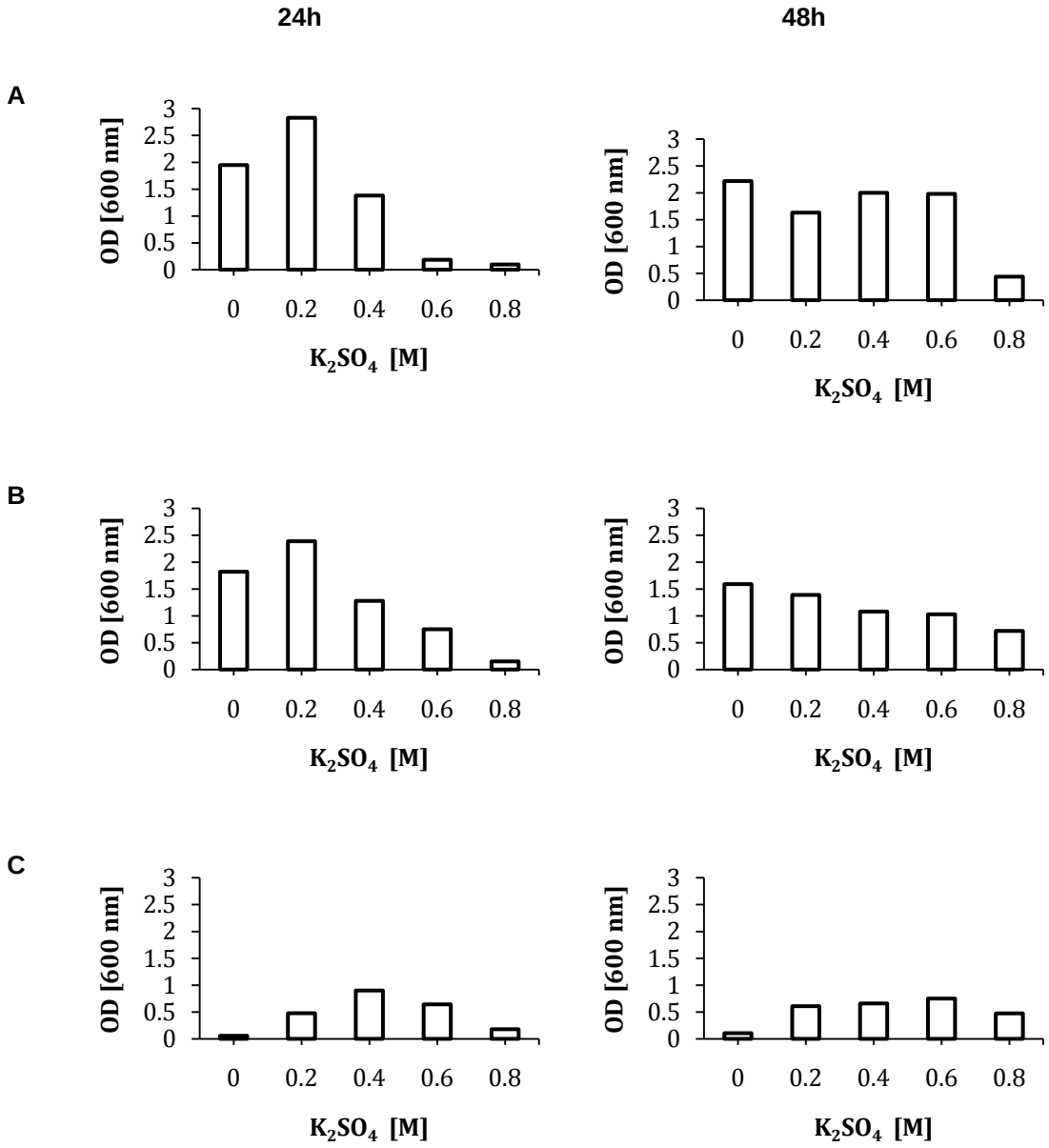

Figure 10: K_2SO_4 tolerance of *E. coli* MG1655 wild type cells and their evolved derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated K_2SO_4 concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). Concentrations of more than 0.8 M could not be tested, as the salt was insoluble in MS-man medium at these higher concentrations. The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $MG1655_{262}$; C, $MG1655_{858}$.

Growth in the presence of increasing concentrations of KCl (Fig. 9) and K_2SO_4 (Fig. 10) was similar to growth in the presence of increasing NaCl concentrations. It should be noticed that wild type MG1655 could still grow with good yield in presence of 0.6 M K_2SO_4 (i.e., with 1.2 M K^+ ions). Although the basic minimal medium contains 0.05 M K ions, this concentration was apparently not sufficient to support growth of

evolved derivatives ($MG1655_{858}$) in the absence of added salt. Similar growth in the presence of chloride or sulfate counter ions indicates that the nature of these anions is of minor importance for halotolerance of the strains.

This was different when iodide was used as the counter-ion. Iodide is toxic for bacterial cells and MG1655 cells can only grow up to 0.2 M KI, although slowly at this concentration (Fig. 11).

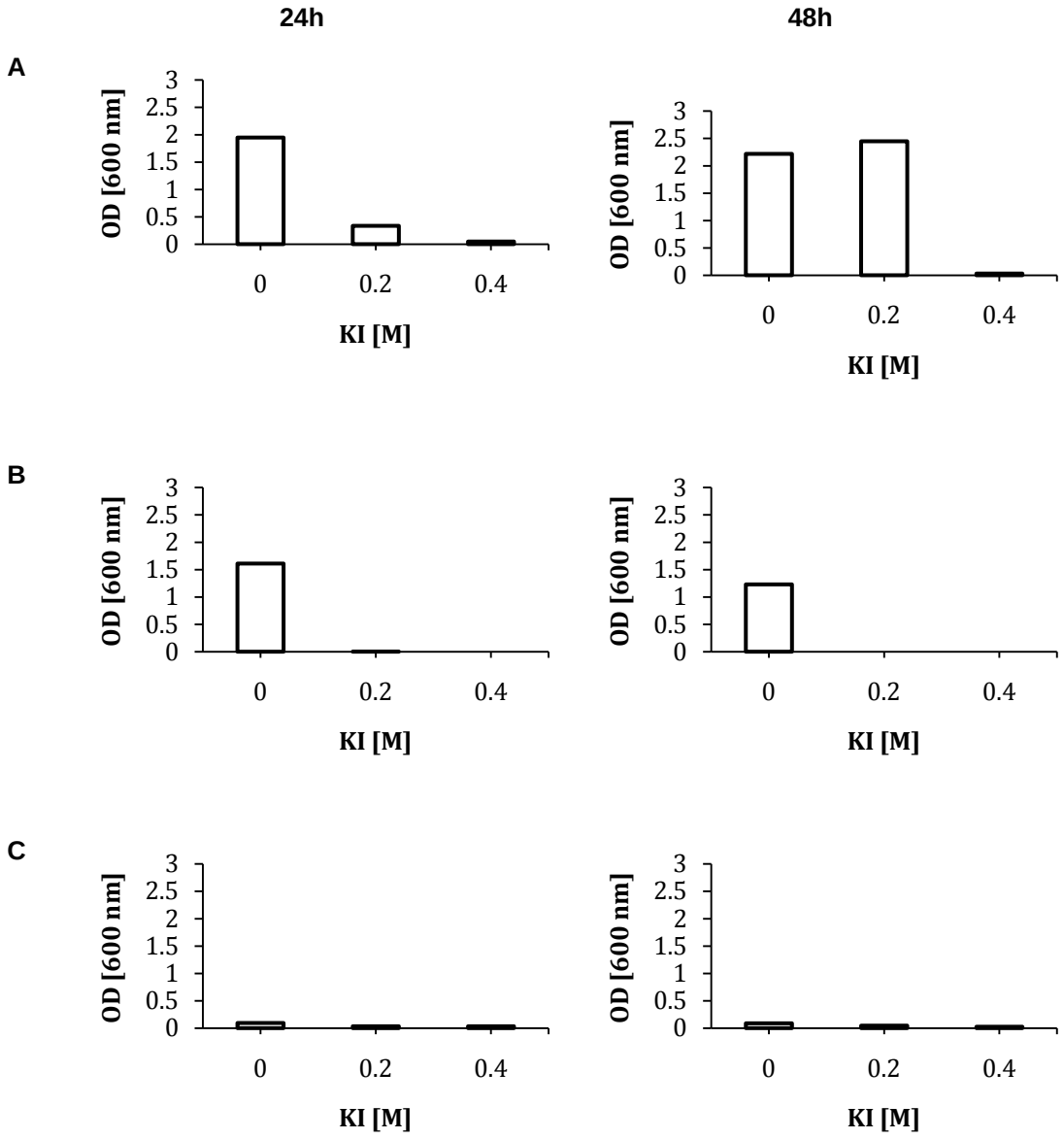

Figure 11: KI tolerance of *E. coli* MG1655 wild type cells and their evolved derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated KI concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $MG1655_{262}$; C, $MG1655_{858}$.

Neither of the tested evolved strains ($MG1655_{262}$ and $MG1655_{858}$), however, showed any growth even at the lowest added KI concentration. This indicates that evolved derivatives may depend on chronically elevated cytosolic K^+, as is found for halophilic organisms which accumulate molar intracellular amounts of K^+ for charge shielding of functional macromolecules (Lanyi, 1974). Evolved derivatives therefore could actively enrich K^+ from the extracellular milieu, which must be balanced by the uptake of anions. In the case of I^- the cell would at the same time accumulate the toxic iodide, which inhibits growth. Preliminary experiments with the evolved derivatives in which K^+-free media were used to measure osmotolerance indeed support the hypothesis that these strains require some extracellular K^+ for growth in the presence of other osmotically active salts (S. Vettermann & R. Mutzel, personal communication).

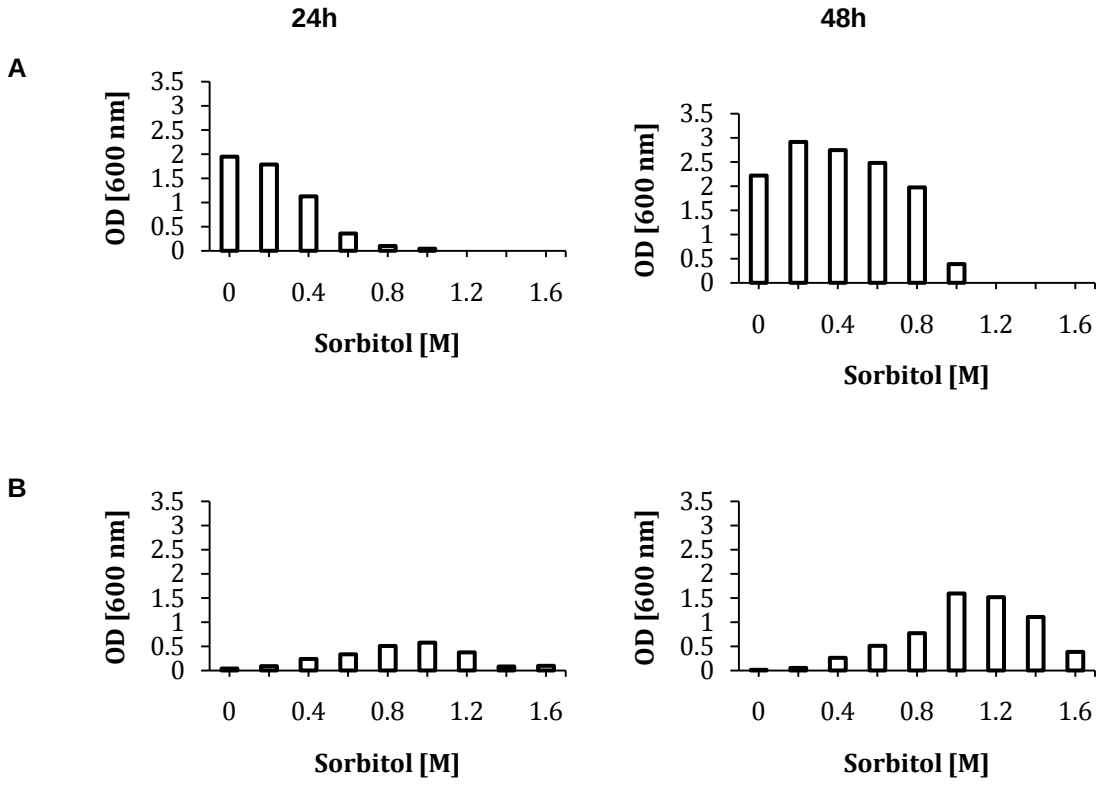

Figure 12: Sorbitol tolerance of *E. coli* MG1655 wild type cells and their evolved derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated sorbitol concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $MG1655_{858}$.

Figure 12 shows that evolved bacteria also display drastically increased tolerance against sorbitol, an uncharged, osmotically active carbohydrate. Wild type cells grew

best at low and intermediate concentrations of sorbitol, with some slow growth being observed up to 1 M. $MG1655_{858}$ cells, in contrast, required at least 0.4 M sorbitol to grow to low density, grew best at 1 M, and supported 1.6 M of the sugar.

In summary, with longer duration of the adaptation process, evolved cells showed increasing levels of tolerance against various osmotically active agents and became increasingly dependent on the presence of such agents in their extracellular milieu. The hypothesis can be put forward that these evolved cells need elevated levels of intracellular K^+ ions. Adopting a definition by Oren (2008) which states that halophilic organisms (i) grow best at concentrations of 0.85 M NaCl or higher, (ii) tolerate extracellular NaCl concentrations of at least 1.7 M and (iii) fail to grow in the absence of extracellular NaCl, these strains are (at least) close to becoming halophilic.

3.1.3 Determination of generation times

Generation times of wild type and evolved $MG1655_{858}$ in the presence of various concentrations of salts (NaCl, KCl) and sorbitol at different concentrations were determined. 30 ml MS-man medium with the indicated concentration of salt were inoculated in Erlenmeyer flasks with an appropriate volume of bacterial culture to give an initial OD_{600nm} of 0.1 and incubated at 30 ºC with vigorous agitation. Growth was determined by measuring the OD_{600nm} at regular intervals and generation times were determined (Tables 1, 2).

Table 1: Generation times of MG1655

Molarity	NaCl	KCl	Sorbitol
0.0 M	3.1 h	3.0 h	3.0 h
0.2 M	2.4 h	3.0 h	3.0 h
0.4 M	3.0 h	3.2 h	3.0 h
0.6 M	4.5 h	5.5 h	5.5 h

MG1655 cells grew in the minimal medium at 30°C with generation times of about 3 h in NaCl, KCl and sorbitol concentrations of up to 0.4 M. The fastest growth of the wild type was observed at 0.2 M NaCl. At higher osmolarities growth rates decreased to 0.22 (for 0.6 M NaCl) and 0.18 (for 0.6 M KCl and sorbitol, respectively). Growth rates at higher osmolarities could not be measured since the strain barely grows at concentrations above 0.6 M.

Table 2: Generation times of $MG1655_{858}$

Molarity	NaCl	KCl	Sorbitol
0.2 M	2.4 h	2.1 h	3.1 h
0.4 M	2.2 h	2.2 h	2.7 h
0.6 M	2.6 h	2.5 h	3.0 h
0.8 M	4.0 h	2.7 h	3.2 h
1.0 M	4.4 h	4.0 h	4.0 h
1.2 M	5.0 h	5.2 h	5.5 h

Growth rates of $MG1655_{858}$ populations were higher than 0.22 at concentrations up to 1.0 M NaCl and only decreased below 0.2 at 1.2 M NaCl. These results show that the evolved cells grow robustly even at elevated osmolarities.

3.1.4 Mechanisms of halotolerance in wild type and evolved MG1655 cells

During acute osmotic stress *E. coli* cells accumulate K^+ ions that trigger the accumulation of glutamate. This is transient as K^+ interferes with protein function and DNA-protein interactions (Kempf and Bremer, 1998). K^+ ions are therefore transported out of the cell and a second answer ensues, which consists either in the accumulation of extracellular osmoprotectants such as proline or glycine-betaine (if they are available) or in the induction of the genes for synthesis of the intracellular osmoprotectant trehalose (Kempf and Bremer, 1998). *E. coli* cells under chronic osmostress in minimal media accumulate intracellular trehalose.

To analyze intracellular trehalose accumulation in wild type and evolving cells cleared tricholoracetic acid extracts were analyzed by thin-layer chromatography.

In wild type MG1655 cells no intracellular trehalose could be detected when the population was grown without external salt and low amounts were found at 0.2 M extracellular NaCl. However, trehalose accumulated to high concentrations during growth at 0.4, 0.6 and 0.8 M NaCl (Fig. 13 A).

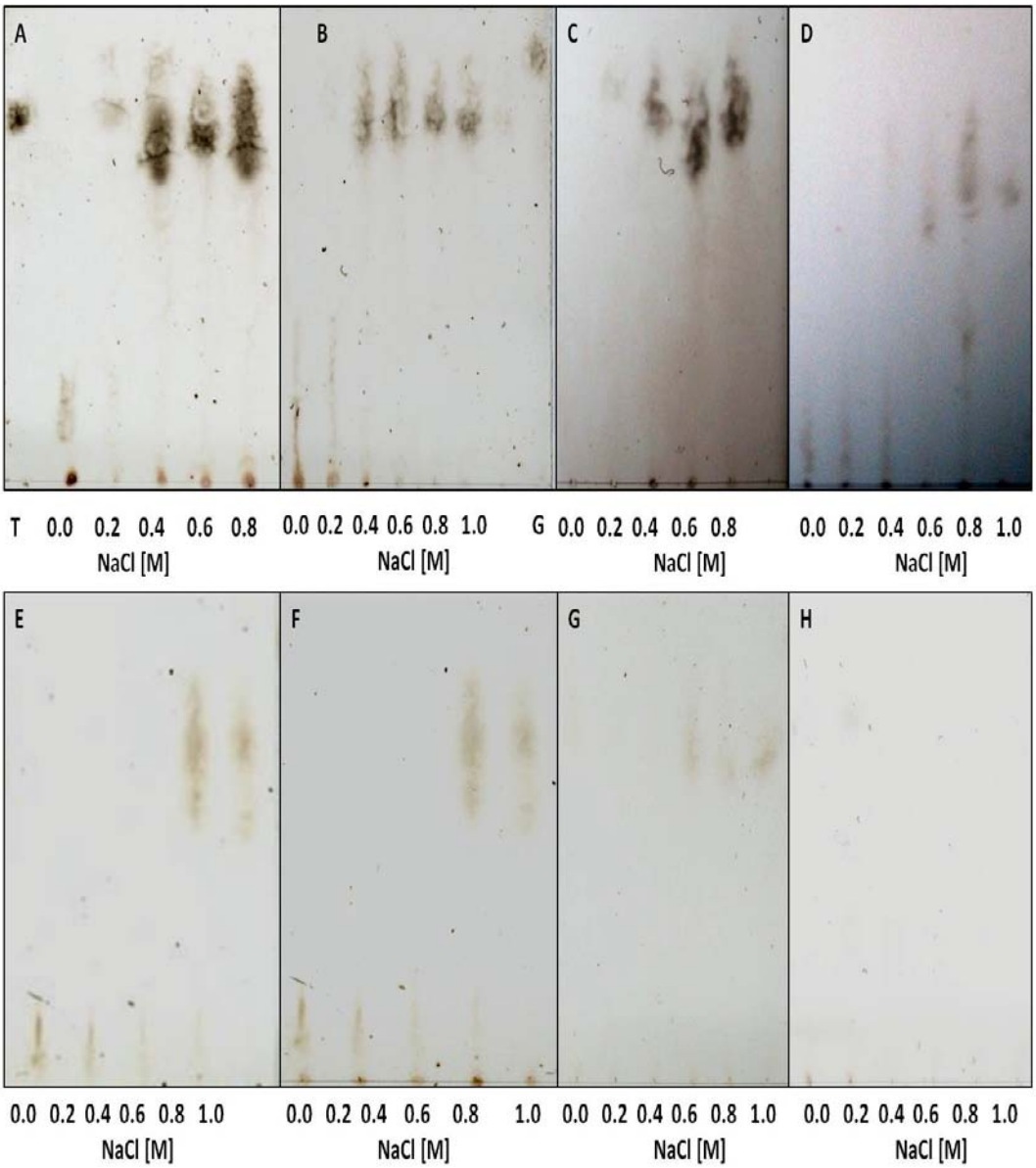

Figure 13: Intracellular trehalose accumulation of *E. coli* MG1655 wild type cells and their evolved derivatives.

TCA extracts (3µl) were chromatographed on TLC silica gels after growth of the cells in various NaCl concentrations. T: 10 mM trehalose; G, 10 mM glucose; A, MG1655; B, $MG1655_{185}$; C, $MG1655_{220}$; D, $MG1655_{262}$; E, $MG1655_{304}$; F, $MG1655_{375}$; G, $MG1655_{465}$; H, $MG1655_{481}$.

In early isolates from the evolution experiment ($MG1655_{185}$ and $MG1655_{220}$) the situation was similar (Fig. 13 B, C). In isolates from day 262 and thereafter, however, trehalose could only be detected after growth in 0.4 M NaCl ($MG1655_{262}$, Fig. 13 D) or 0.6 M NaCl and higher ($MG1655_{304}$, Fig. 13 E; $MG1655_{375}$, Fig. 13 F; $MG1655_{465}$, Fig. 13 G). Even more important, as compared to control chromatograms with wild type extracts that were routinely run in parallel (not shown), the signals became

gradually weaker. In extracts from isolates taken at 481 days (Fig. 13 H) and thereafter, trehalose was undetectable by the method applied.

Figure 14 shows the final situation in MG1655$_{858}$ as compared to the wild type: over the entire range of salt concentrations in which the evolvant is able to grow (0.2 - 1.6 M NaCl) no accumulation of intracellular trehalose was observed (Fig. 14B).

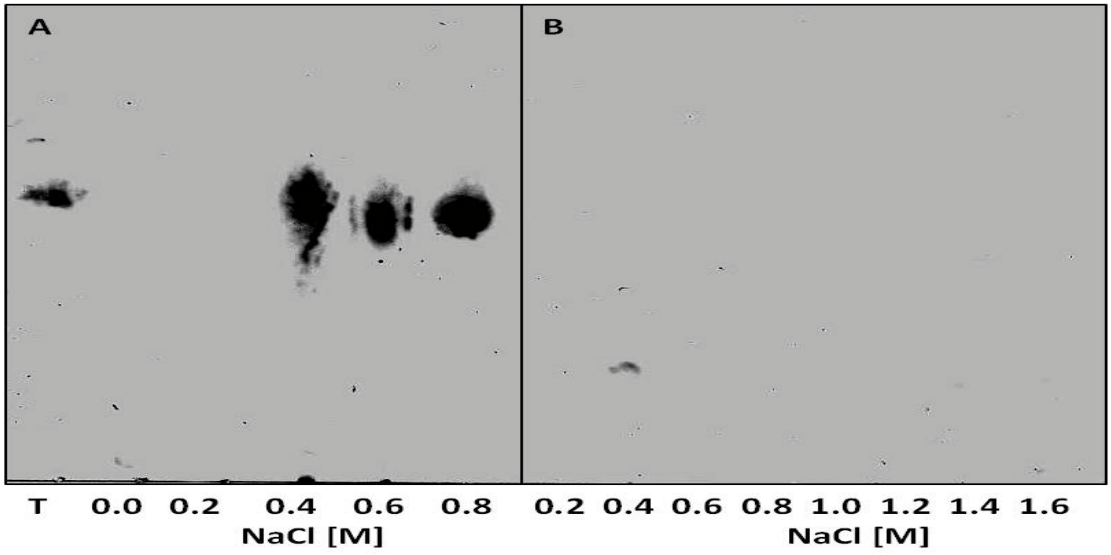

Figure 14: Intracellular trehalose accumulation in *E. coli* MG1655 wild type cells and MG1655$_{858}$ after growth in various salt concentrations.

TCA extracts (3µl) were chromatographed on TLC silica gels after growth of the cells in various NaCl concentrations. T: 10 mM trehalose; A, MG1655; B, MG1655$_{858}$

Thus, in the early stages of the evolution experiment, the population relied on the well-characterized intracellular accumulation of the disaccharide trehalose as an osmoprotectant (Dinnbier et al., 1988; Rod, 1988). At later stages, lower and lower amounts of trehalose accumulated in the cells even at limiting extracellular osmolarities, and eventually, synthesis of trehalose became undetectable under all tested salt concentrations.

This result was unexpected and therefore a question arose: how do the evolving cells protect themselves from the loss of intracellular water during growth at high external osmolarities?

3.1.5 A switch in the synthesis of osmoprotectants during evolution of salt tolerance

It can be hypothesized that the role of trehalose as an osmoprotectant was taken over by some other intracellular compatible solute. The nature of this hypothetical osmoprotectant was completely obscure. Possible candidates include polyalcohols such as glycerol or more complex sugars, or amino acids and their derivatives (Roberts, 2005). Since thin layer chromatographs did not reveal the accumulation of intracellular saccharides that would be detected by the applied method, it was hypothesized that during the adaptation process usage of an amino acid had been selected to replace trehalose. In a first step a thin-layer chromatographic detection system was established and validated using authentic amino acids as standards and the ninhydrin method for detection (Friedman, 2004).

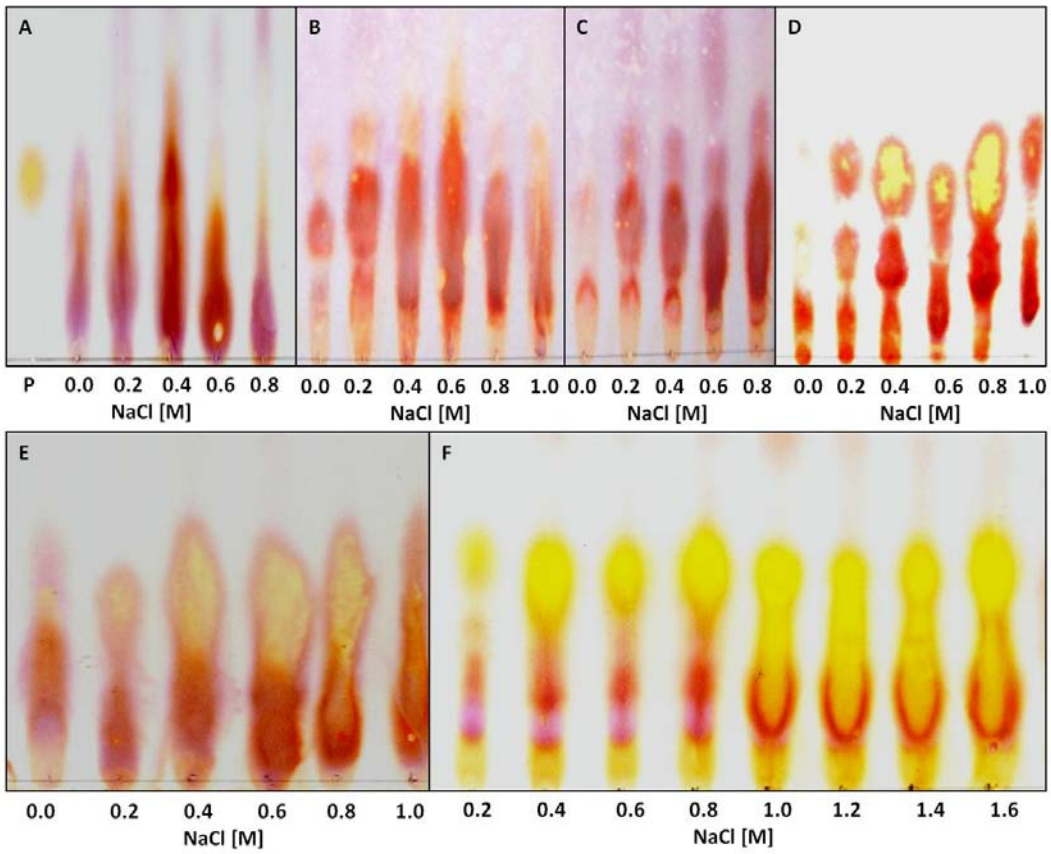

Figure 15: Intracellular proline accumulation of *E. coli* MG1655 wild type cells and their evolved derivatives.

TCA extracts (3 µl) were chromatographed on TLC silica gels after growth of the cells in various NaCl concentrations. P: 10 mM proline; A, MG1655; B, $MG1655_{185}$; C, $MG1655_{220}$; D, $MG1655_{262}$; E, $MG1655_{465}$; F, $MG1655_{858}$.

As shown in Figure 15 ninhydrin-stained solutes accumulated to higher levels in evolvants from later stages in the experiment, most prominently a brightly yellow stained product that comigrated with authentic proline (see Fig. 15 A, lane "P"). In addition, a second amino acid migrating at lower R_f values was increasingly accumulated. This spot comigrated with glutamate (not shown). When extracts from cells producing these two compounds were diluted and mixed with authentic glutamate and proline, no separation of the authentic amino acids and the products in the cell extracts could be observed, suggesting that these two compounds represent indeed proline and glutamate (data not shown).

Figure 15 shows that the signals of proline and glutamate became stronger in later evolvants, the kinetics of this change being roughly anti-parallel to the gradual disappearance of trehalose in the extracts (compare Fig. 13 above) with a remarkable increase in the evolvant isolated on day 262 as compared to $MG1655_{220}$ (Fig. 15 C, D). This is in agreement with the hypothesis that utilization of trehalose as an osmoprotectant was replaced by utilization of free intracellular amino acids. It should be noted that accumulation of these amino acids was regulated by the osmolarity of the medium, as can be judged from stronger staining in extracts prepared form cells grown at higher NaCl concentrations.

3.1.6 Quantification of intracellular amino acids by high-performance liquid chromatography (HPLC)

To quantitatively measure the intracellular accumulation of free amino acids in more detail, cell extracts from wild type MG1655 and evolved ($MG1655_{858}$) cells grown in the presence of various concentrations of NaCl were analyzed by HPLC.

Wild type MG1655

Figure 16 shows that in extracts from wild type cells most of the canonical amino acids could be found in low millimolar concentrations, with glutamate levels being up-regulated during growth in higher NaCl concentrations, reaching 15 mM in extracts from cells grown at 0.6 M NaCl. Arginine was the next abundant amino acid, but its levels did not show pronounced dependence from the osmolarity of the medium. Low concentrations of proline (0.2 mM) were detected under all osmotic conditions.

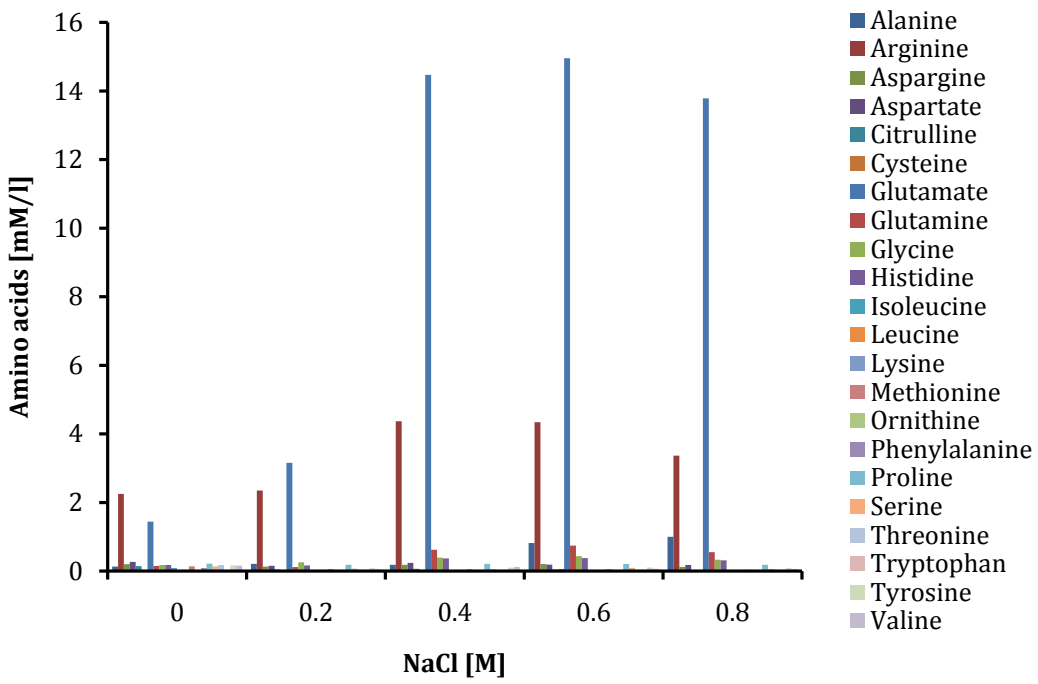

Figure 16: Quantification of intracellular amino acids of wild type MG1655 strain by HPLC.

Detection and quantification of amino acids was performed by HPLC analysis on an Agilent 1200 SL. Amino acids in the samples were first derivatized using a pre-column protocol [o-phthalaldehyde for the primary and 9-fluorenylmethyl chloroformate for secondary amino acids]. After injection, the samples were separated on a ZORBAX Eclipse Plus C18 column at 40 ºC using a binary gradient mobile phase. The eluting amino acids were detected with a fluorescence detector (FLD) and quantified with the use of a standard amino acid (AAS18).

MG1655$_{858}$

In extracts from MG1655$_{858}$ cells grown in the presence of various NaCl concentrations most of the canonical amino acids could be found in low millimolar concentrations. Arginine, glutamine and histidine increased somewhat during growth at higher salinity (Fig. 17 A).

A dramatic increase in the intracellular concentrations of glutamate and proline was measured in cells grown at high osmolarities (Fig. 17 B) with glutamate accumulating to approximately 20 mM and proline to more than 190 mM in cells grown in 1.2 M NaCl. As could be predicted from the more qualitative detection of these amino acids by the TLC technique, their synthesis was regulated by the medium osmolarity.

A rough calculation of the intracellular concentration of proline (assuming that an *E. coli* cell is a cylinder of 1 µm diameter and 2 µm lengths) yields values of 600 mM during growth at high NaCl concentrations.

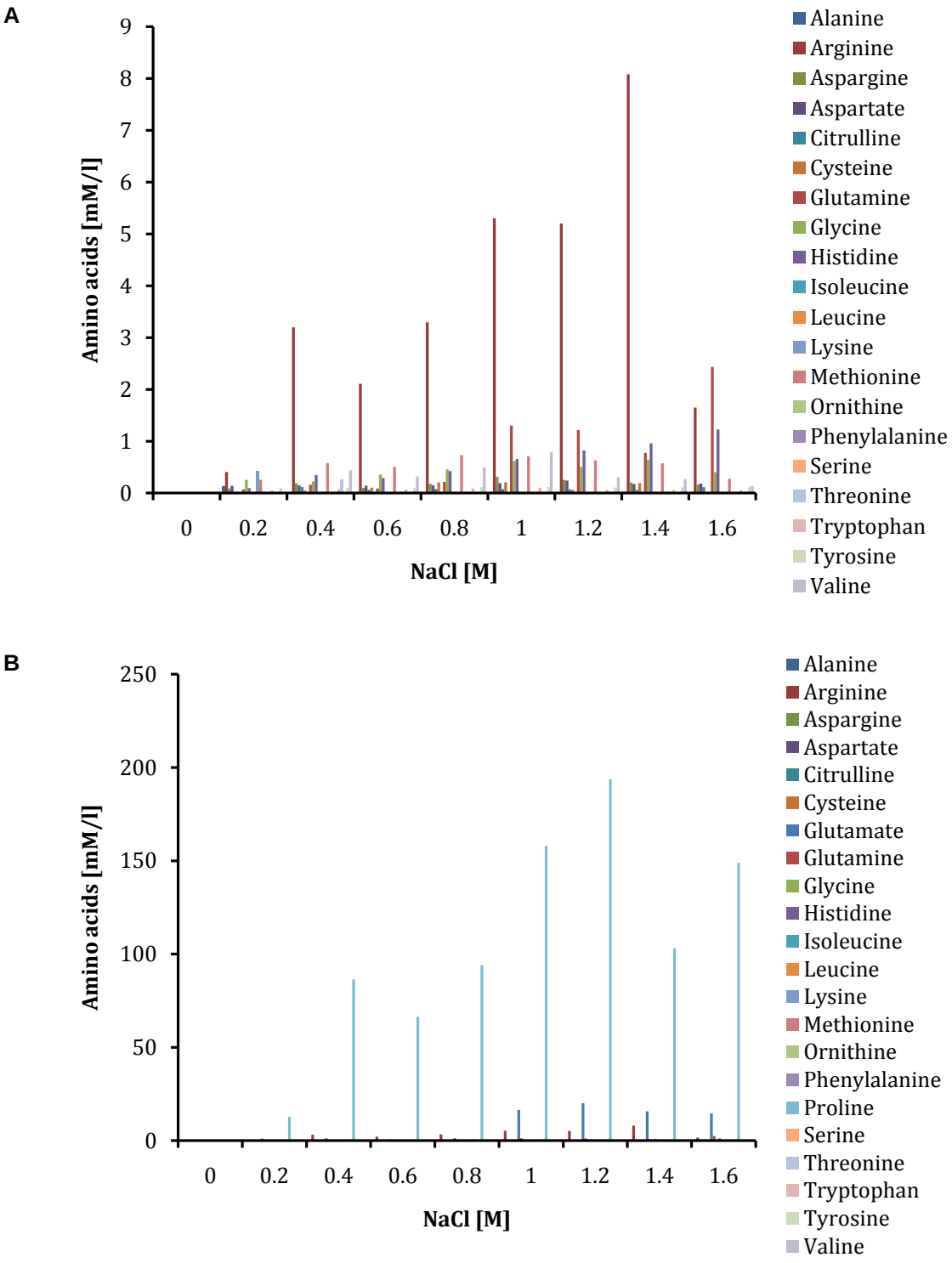

Figure 17: Quantification of intracellular amino acids in MG1655$_{858}$ by HPLC.

Detection and quantification of amino acids was performed by HPLC analysis on an Agilent 1200 SL. Amino acids in the samples were first derivatized using a pre-column protocol [o-phthalaldehyde for the primary and 9-fluorenylmethyl chloroformate for secondary amino acids]. After injection, the samples were separated on a ZORBAX Eclipse Plus C18 column at 40 ºC using a binary gradient mobile phase. The eluting amino acids were detected with a fluorescence detector (FLD) and quantified with the use of a standard amino acid (AAS18). A, intracellular amino acids except for glutamic acid and proline; B, all intracellular amino acids scaled to show the quantities of proline and glutamic acid.

In summary, under osmotically challenging conditions evolving populations of MG1655 derivatives switched their haloprotective strategy from the synthesis and intracellular accumulation of the disaccharide trehalose to the accumulation of the amino acids glutamate and proline. It should be stressed that (i) synthesis of trehalose ceased gradually in favor of increasing enrichment of proline and glutamate and (ii) accumulation of the amino acids is dependent on the osmolarity of the medium.

3.1.7 Stress response in evolving populations

The general response of *E. coli* cells to a wide variety of stress conditions is transcriptional and translational up-regulation as well as stabilization of the "stress sigma factor" RpoS (σ^S) which regulates about 10% of the total *E. coli* transcriptome (Hengge, 2009; Weber et al., 2005). This is also the case during osmotic stress where genes encoding proteins of the trehalose biosynthesis pathway are under control of RpoS (Hengge et al., 1991).

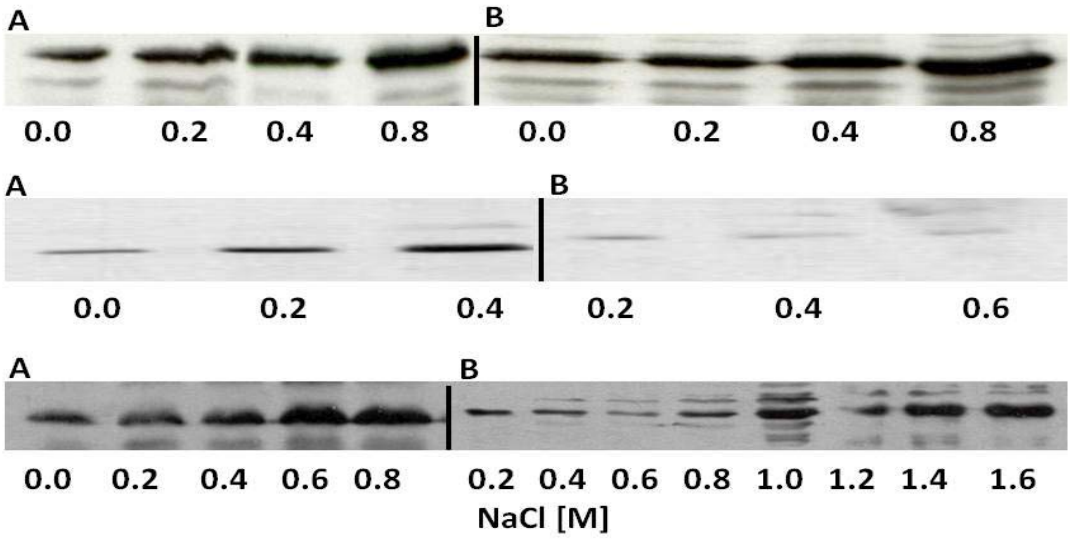

Figure 18: Expression of RpoS in *E. coli* MG1655 wild type cells and their evolved derivatives.

Extracts were prepared from cells growing at the indicated NaCl concentrations when the cell density had reached an OD_{600nm} of 0.4. Equal amounts of proteins were separated on 12.5% polyacrylamide/0.1% SDS gels and transferred to nitrocellulose membranes which were subsequently probed with 1:5000 diluted anti-RpoS antibodies, developed with ECL reagent, and the chemiluminescence was documented by exposure of the blots on Kodak BioMax light autoradiography films. Only parts of the blot (molecular mass of 38 kDa) are shown. Panel A, MG1655; Panel B in the upper part, $MG1655_{220}$; Panel B in the middle part, $MG1655_{647}$; Panel B in the lower part, $MG1655_{858}$.

On Western Blots from logarithmically growing MG1655 cells (OD_{600nm} = 0.4) probed with antibodies against RpoS, expression of the sigma factor increased steadily with growth in the presence of increasing NaCl concentrations (panel A in Fig. 18) to maximal levels at the highest tolerable salt concentration. This situation was similar in $MG1655_{220}$. In the derivative isolated after 647 days of selection, RpoS expression was lower at all tested osmolarities than in wild type cells tested in parallel (Fig. 18 middle part). This trend was even more pronounced in $MG1655_{858}$, where a significant increase in the level of RpoS could only be measured at NaCl concentrations of 1 M and higher (Fig. 18 lower part). Interestingly, expression of the sigma factor even appeared to decrease somewhat in cultures growing at 0.4 M and 0.6 M NaCl as compared to samples grown at 0.2 M NaCl (expression in the absence of salt could not be measured since this strain failed to grow at 0.0 M NaCl).

As already suggested by the results of the analysis of growth yields in the presence of various NaCl concentrations, this shift in the expression pattern of the stress sigma factor RpoS indicates that a shift in the "ecological niche" of the evolvants has taken place during the adaptation process: first, osmolarities which induce the stress response in wild type cells are apparently no longer perceived as stressful in the evolving derivatives, second, the tendency of higher expression of RpoS in media with low osmolarity could mean "stress" for these strains. Consequently, signal transduction reactions that sense stressful conditions and regulate the expression of RpoS must have changed during evolution to high salt tolerance.

3.1.8 Detection of outer membrane proteins by SDS-polyacrylamide gel electrophoresis

Proteins which face the extracellular space (outer membrane proteins, periplasmic proteins and the periplasmic domains of inner membrane proteins) have to function under the osmotic conditions of the extracellular milieu. To get an idea of possible changes in the nature and structure of such proteins in evolving MG1655 cells, outer membrane proteins were isolated according to the procedure developed by Lugtenberg and co-workers (1975) and analyzed by SDS polyacrylamide gel electrophoresis.

Figure 19 shows that even by this low-resolution approach dramatic change in the expression pattern of outer membrane proteins could be revealed. Thus, as

compared with MG1655 wild type cells grown at low osmolarity, several high molecular mass proteins as well as a number of proteins in the molecular mass range of approx. 18 to 25 kDa are less abundant in the outer membrane of $MG1655_{382}$ cells grown at high osmolarity (arrows in Fig. 19). The identity of these proteins is so far unclear.

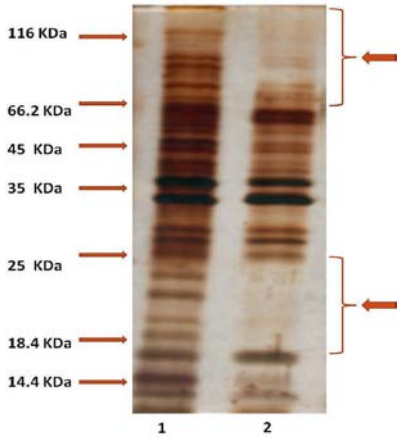

Figure 19: Separation of outer membrane proteins of *E. coli* MG1655 wild type cells and $MG1655_{382}$.

Outer membrane proteins were extracted from MG1655 cells grown in 0.0 M NaCl lane (1) and from $MG1655_{382}$ cells grown in 0.8 M NaCl lane (2) when the cell density had reached an OD_{600nm} of 0.8. Equal amounts of proteins were separated on 12.5% polyacrylamide/0.1% SDS gel and stained with the Page Silver™ Silver Staining Kit. The position and sizes of marker proteins are indicated to the left.

3.1.9 Analysis of the total cellular proteome by two-dimensional SDS gel electrophoresis (2-D PAGE)

Since SDS polyacrylamide gel electrophoresis of outer membrane proteins (Fig. 19) and of total cells extracts (not shown) had indicated drastic changes in the cellular proteome, an effort was made to analyze the proteome of wild type and evolved populations by two-dimensional polyacrylamide gel electrophoresis. To give a hint on both regulatory and molecular adaptations (i.e., to reveal the maximal change in the proteome), extracts from wild type cells grown in the absence of NaCl and extracts from $MG1655_{858}$ cells grown in the presence of 1.2 M NaCl were compared.

Cellular proteins were separated by isoelectric focusing (pH 4-7) in the first dimension, then on 12.5% polyacrylamide gels in the presence of 0.1% SDS according to their apparent molecular masses (Fig. 20).

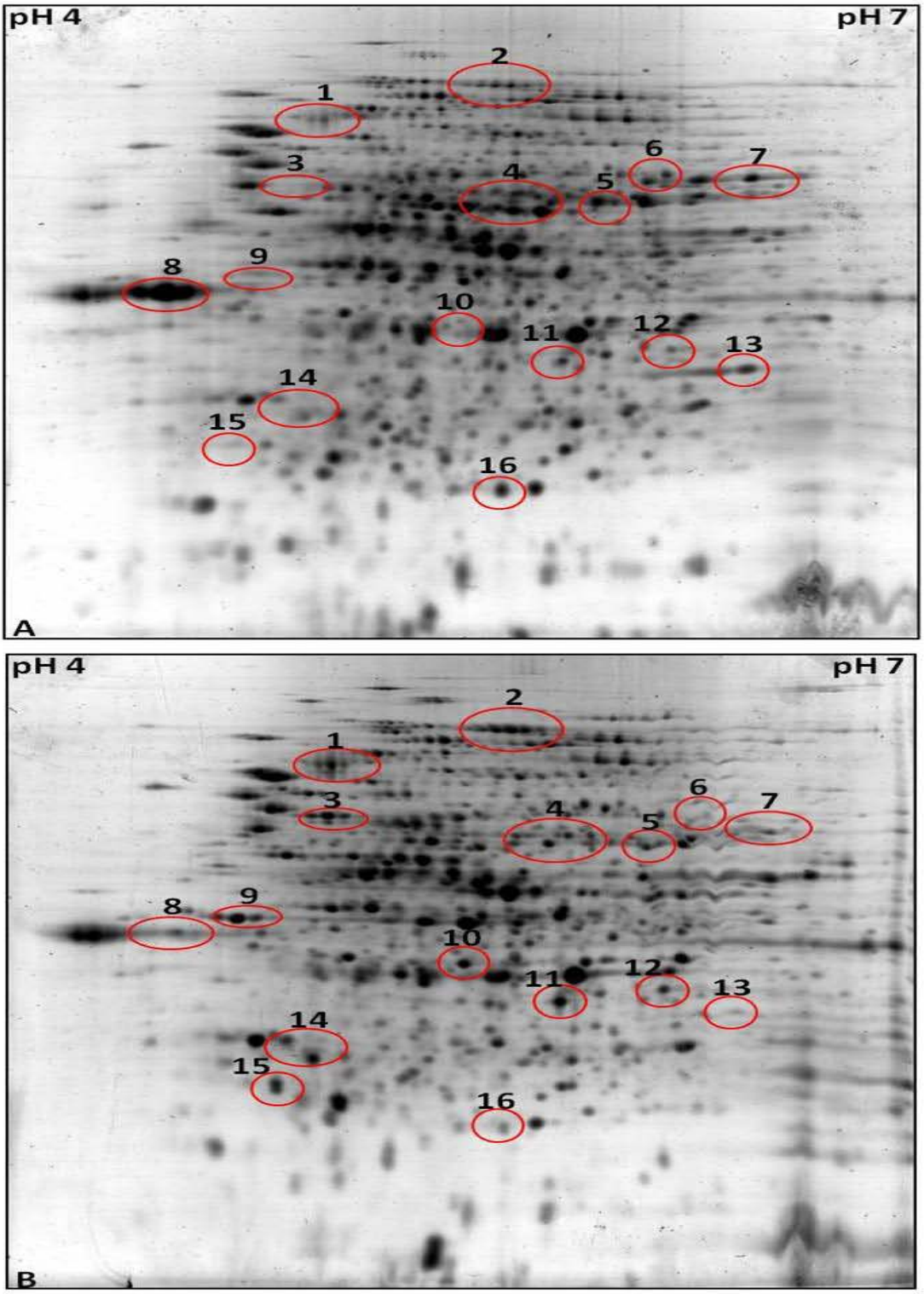

Figure 20: Total cellular proteomes of wild type MG1655 and evolved MG1655 cells.

Cellular proteins were extracted from MG1655 cells grown in 0.0 M NaCl (A) and from $MG1655_{858}$ cells grown in 1.2 M NaCl (B) when the cell density had reached an OD_{600nm} of 0.8. Before applying to 2D-PAGE, proteins were precipitated and cleaned up with the use of the 2-D Clean-Up Kit. 200 µg of the purified proteins were separated by isoelectric focusing pH 4-7 (18 cm dry strips) in the first dimension, then on 12.5% polyacrylamide gels in the presence of 0.1% SDS according to their apparent molecular masses. Second dimensional gels were stained with SYPRO Ruby stain.

Comparison of the two-dimensional gels reveals a number of major changes in the total proteome. For example, the spots marked 1, 2, 3, 9, 10, 11, 12, 14, and 15 were stained much more intensely in gels from the proteome of evolved cells, while the spots marked 4, 5, 6, 7, 8, 13, and 16 stained much weaker in these extracts (Fig. 20). Spots 9 and 10 are barely visible in gels from wild type cells. The nature of these proteins is unknown at present. Their identity should be analyzed by mass-spectrometric analysis in further studies which should also aim at investigating their expression in MG1655 and its evolved derivatives under various medium osmolarities.

3.2. ε2124

Strain ε2124 is a derivative of *E. coli* MG1655 which carries deletions of the genes *fmt* and *def* (*fmt* and *def* encode Met-tRNAi transformylase and polypeptide deformylase, respectively) and, in addition, a mutation in the DNA repair gene, *mutD*, which leads to a mutator phenotype. Deletion of the *fmt/def* genes causes a severe growth defect, increasing the generation time of the mutant by a factor of 5-10 (Mazel et al., 1994). In previous work (Marlière et al., 2005) this growth defect was overcome by continuous cultivation of the mutant in the GM device under a turbidostat regime. Unlike all other known eubacteria the resulting evolvant initiates protein synthesis with unformylated methionine. It might prove useful for biotechnological applications, e.g. the production of recombinant proteins. To expand the spectrum of possible applications for this strain, it was selected for higher salt tolerance in a parallel experiment to the work described for the prototypic MG1655 in chapter 3.1. Evolution of robust growth under the turbidostat regime had been demonstrated to be significantly faster in the *mutD* version of Δ*def/fmt* strains (Marlière et al., 2005). It was thus interesting to analyze whether this would also be the case for evolution of halotolerance and to see whether higher genetic variability is a general advantage for adaptation to stressful conditions.

3.2.1. Evolutionary kinetics

Figure 21 shows the adaptive kinetics of strain ε2124 during the process of continuous selection for growth in medium with increasing NaCl concentration over 962 days (ca. 6700 generations). Whereas adaptation proceeded rather smoothly with MG1655 cells (see chapter 3.1), the continuous culture of ε2124 showed massive and sustained oscillations in the consumption of nonpermissive medium during most of the adaptation campaign and did not reach the high steady state concentration of 1.3 M in the MG1655 derivative but led only to tolerance of around 1 M NaCl during growth with the imposed generation time of 4 h. Although both experiments were initially conducted using identical genetic machines in the same environment kept at 28 - 30°C, differences in the functioning of the setup could play a role in the different behavior of the two cultures.

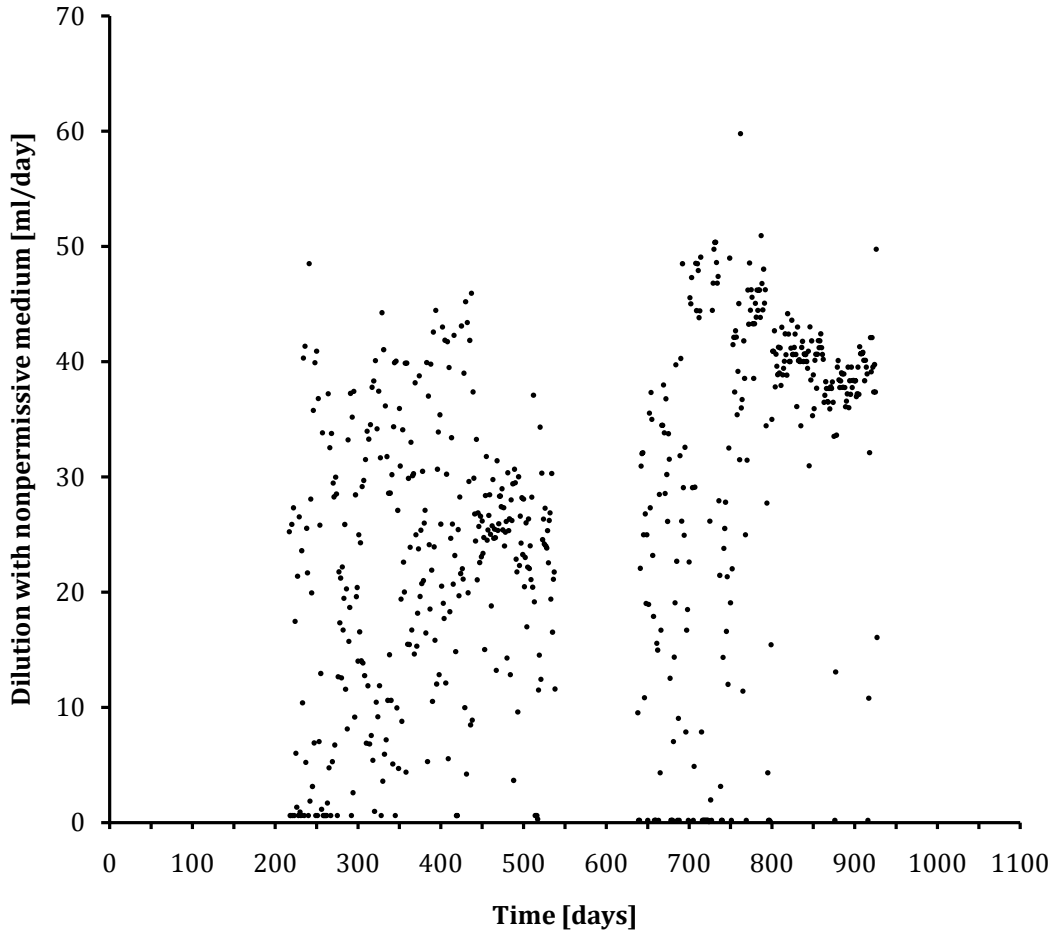

Figure 21: Evolutionary kinetics of ε2124 under the conditional pulse-feed regime for selection of halotolerance.

Cell cultures (26.5 ml, approximately $1x10^9$ cells/ml) were cultivated at 30 °C in minimal (MS-man) medium containing either 0.8 M NaCl (permissive medium) or 2 M NaCl (nonpermissive medium). Opening times for the magnetic valve commanding the inflow of nonpermissive medium were recorded and are summed up for 24 hours periods (dots). Data for the periods between days 1 and 217, and 538 and 638, respectively, were lost due to failure of the recording personal computer.

Therefore, at day 217 the experiment was transferred to another Genemat device which was equipped with a thermostat that keeps the culture temperature constant at 30°C. Although dilution rates with nonpermissive medium increased under these conditions, adaptation to higher salt concentrations still appears slower than in case of MG1655, and the oscillations in the consumption of nonpermissive medium persisted or even became more pronounced (days 250 to 500) with a slight tendency to smoothen around day 450. By day 589 the experiment was transferred back to the initial setup. Oscillations in the dilution rate with nonpermissive medium persisted for another period of around 275 days, and then dampened during the final phase of the

present experimental campaign. Thus, these oscillations appear not to be caused by the experimental setup, but rather by some biological trait of the culture, most probably its mutator phenotype.

In similar experiments, as shown above for MG1655 the properties of the evolving populations were investigated and compared with those of the prototypic MG1655 strain. The results of this analysis are presented below.

3.2.2 Evolution of halotolerance

Growth in NaCl

To determine the level of halotolerance of ε2124 derivatives selected during the course of the experiment, isolates from the genetic machine were tested for their ability to grow in batch cultures in media with various osmolarities. Figure 22 shows growth yields of successive evolvants after cultivation in batch culture in various NaCl concentrations along with the properties of wild type MG1655 populations. ε2124 populations isolated after 273 days of selection displayed both better growth at higher NaCl concentrations and reduced yields in the absence of extracellular salt (Fig. 22 B). This trend continued in samples from later stages of the experiment (Fig. 22 C), and cells isolated on day 962 failed to grow in the absence of NaCl, grew to highest yields at concentrations of around 1 M and showed some growth even at concentrations higher than 1.4 M (Fig. 22 D).

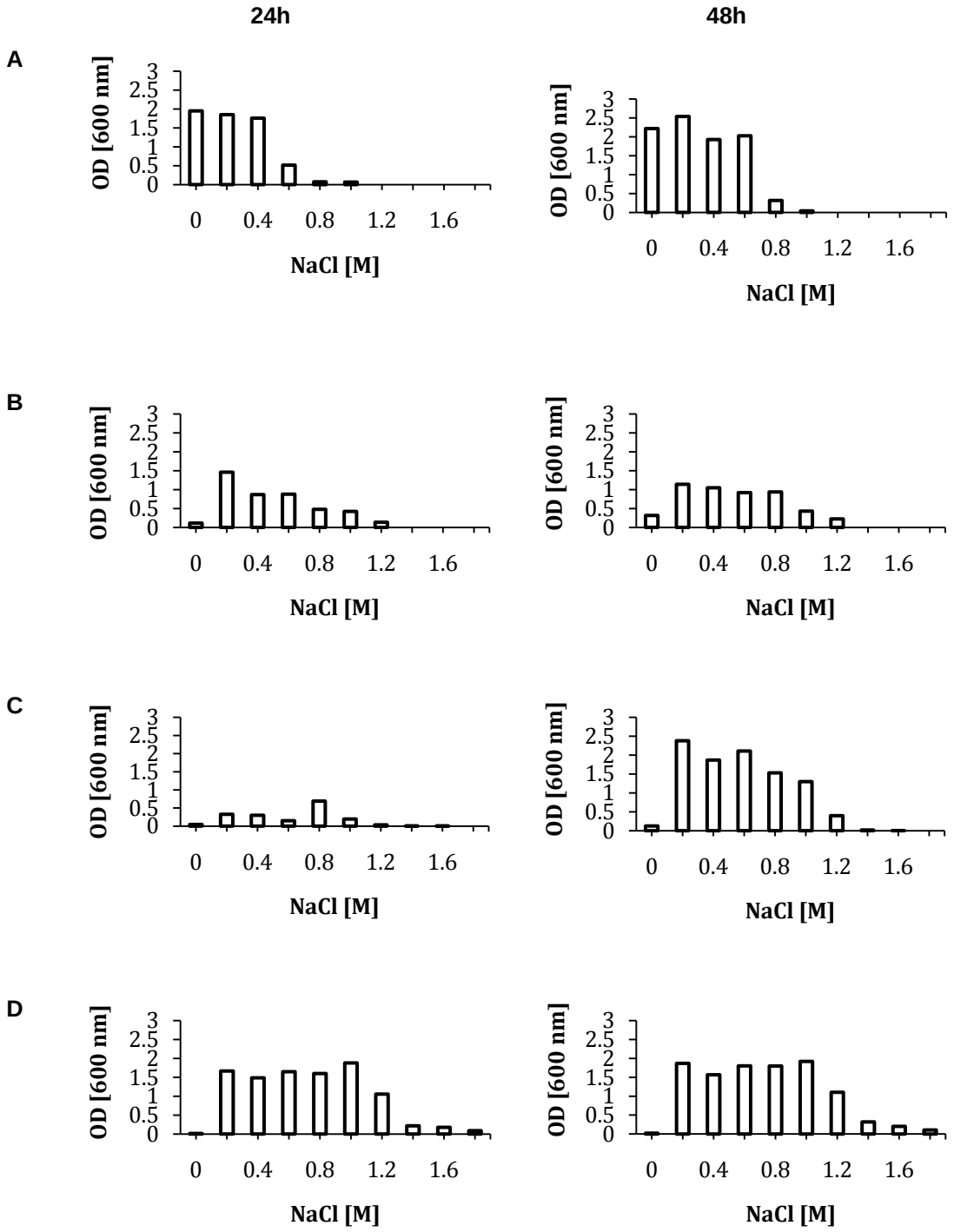

Figure 22: NaCl tolerance of *E. coli* MG1655 wild type cells and evolved ε2124 derivatives.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated NaCl concentrations from precultures grown at 0.0 M NaCl in case of wild type MG1655 and in 0.8 M NaCl in evolved ε2124 derivatives and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $\varepsilon2124_{273}$; C, $\varepsilon2124_{601}$; D, $\varepsilon2124_{962}$.

Tolerance towards cations and anions

In order to analyze whether the nature of the cations and anions influences growth of the evolved ε2124 populations, NaCl was exchanged for KCl, K_2SO_4 and KI. Results of these experiments are summarized in figures 23 to 25 and proved to be very similar to those obtained with MG1655 evolvants. ε2124 derivatives isolated after 273 days of selection tolerated higher concentrations of both KCl and K_2SO_4 and were also sensitive against the lowest concentration of KI tested, suggesting that ε2124 may also chronically enrich intracellular K^+ ions.

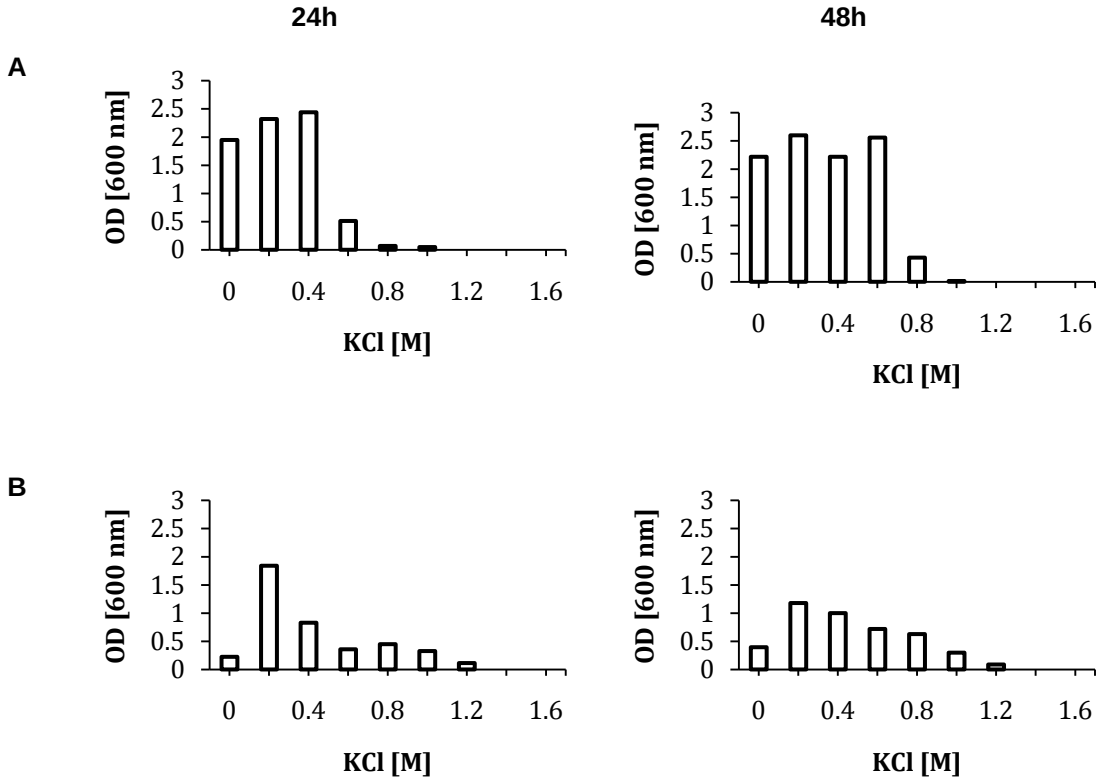

Figure 23: KCl tolerance of *E. coli* MG1655 wild type cells and an evolved ε2124 derivative.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated KCl concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $\varepsilon 2124_{273}$.

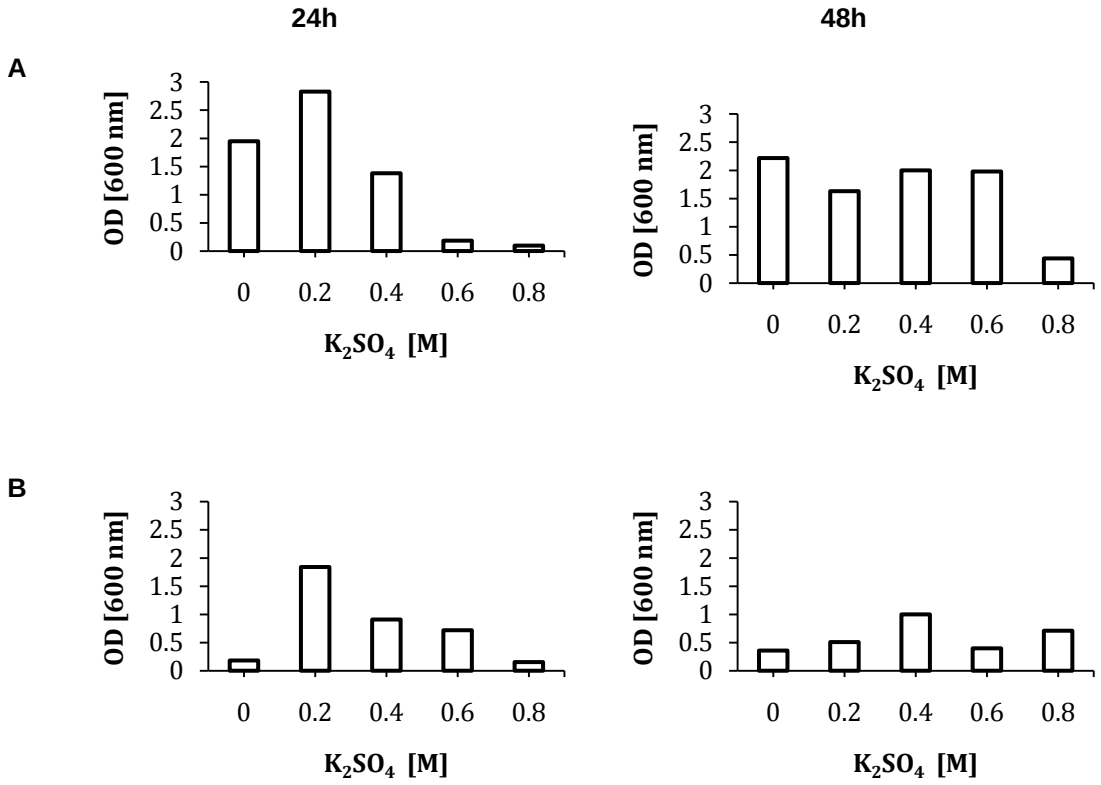

Figure 24: K_2SO_4 tolerance of *E. coli* MG1655 wild type cells and an evolved ε2124 derivative.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated K_2SO_4 concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). Concentrations of more than 0.8 M could not be tested, as the salt was insoluble in MS-man medium at these higher concentrations. The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $\varepsilon 2124_{273}$.

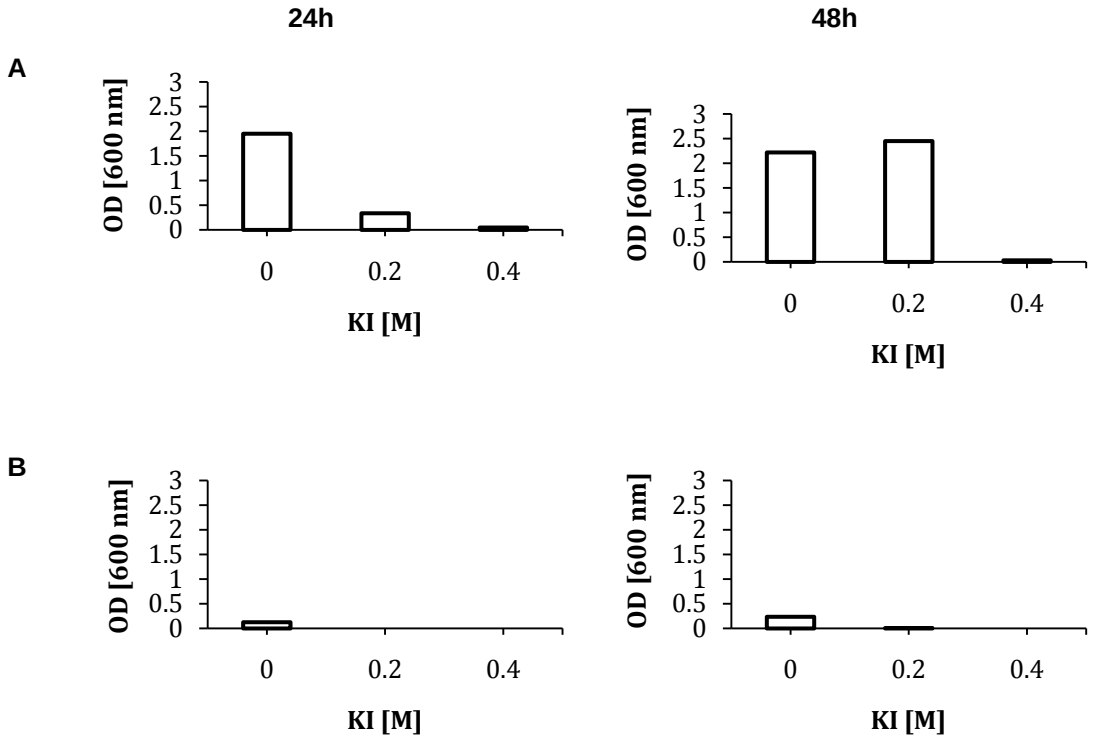

Figure 25: KI tolerance of *E. coli* MG1655 wild type cells and an evolved ε2124 derivative.

Batch cultures were inoculated to initial OD_{600nm} of 0.05 in 30 ml minimal (MS-man) medium containing the indicated KI concentrations and incubated with vigorous agitation at 30 °C. OD_{600nm} of the cultures was measured after 24 (left panel) and 48 h (right panel). The basal MS-man medium contained 50 mM of K^+ added as K_2HPO_4. A, wild type MG1655; B, $\varepsilon 2124_{273}$.

3.2.3 Mechanism of evolved halotolerance in ε2124 evolvants

To investigate the evolution of halotolerance mechanisms in ε2124 derivatives, the intracellular accumulation of trehalose and amino acids during growth at various NaCl concentrations was measured. Trehalose was detected by thin-layer chromatography; intracellular amino acids were analyzed both by thin-layer chromatography and by HPLC analysis. Results for MG1655 wild type cells are shown in chapters 3.1.4 - 3.1.6.

In extracts from ε2124 cells isolated at progressive stages during the adaptation process and grown in the presence of various extracellular salt concentrations, osmotically regulated trehalose accumulation could be detected throughout the course of the experiment. Although a tendency for reduced trehalose production in later isolates was apparent, trehalose was still detectable in cells of $\varepsilon 2124_{847}$, and was regulated by the osmolarity of the medium (Fig. 26 D).

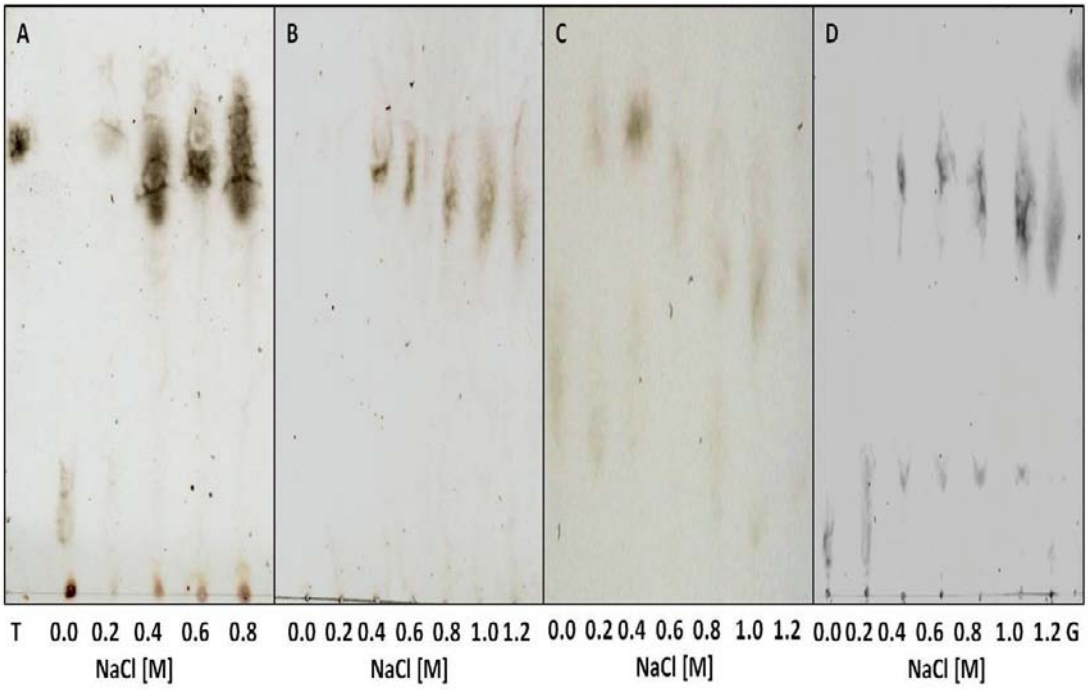

Figure 26: Development of intracellular trehalose accumulation of *E. coli* MG1655 wild type cells and evolved ε2124 derivatives.

TCA extracts (3 µl) were chromatographed on TLC silica gels after growth of the cells in various NaCl concentrations. T: 10 mM trehalose; G, 10 mM glucose; A, MG1655; B, $\varepsilon2124_{524}$; C, $\varepsilon2124_{670}$; D, $\varepsilon2124_{847}$.

3.2.4 Accumulation of intracellular amino acids during growth in high salt media

Since trehalose appeared to accumulate to lower levels in further evolved ε2124 cells during growth at high extracellular NaCl concentrations, intracellular free amino acids were measured by thin-layer chromatography as shown above (3.1.5) for evolved MG1655 derivatives. As for this line of descendants, ε2124 evolvants also progressively switched to massive synthesis of proline under chronic salt stress (Fig. 27).

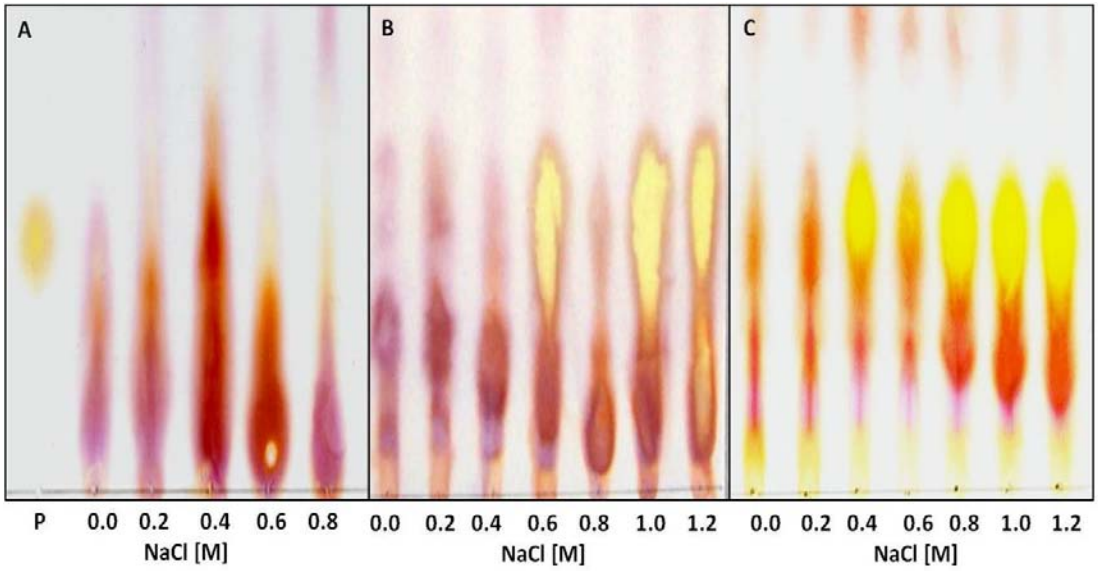

Figure 27: Intracellular proline accumulation in *E. coli* MG1655 wild type cells and evolved ε2124 derivatives.

TCA extracts (3 µl) were chromatographed on TLC silica gels after growth of the cells in various NaCl concentrations. P: 10 mM proline; A, MG1655; B, $\varepsilon2124_{670}$; C, $\varepsilon2124_{847}$.

Figure 27 shows the result of this analysis. Proline accumulated to high levels upon growth of the bacteria at limiting salt concentrations (> 1 M). Its synthesis was regulated by the NaCl concentration of the growth medium, with a significant increase at 0.4 M NaCl. However, intracellular glutamate concentrations remained rather low up to the highest salt concentration where about 22 mM of the amino acid could be measured.

3.2.5 Quantification of intracellular amino acids by high-performance liquid chromatography (HPLC)

$\varepsilon2124_{847}$

In extracts from $\varepsilon2124_{847}$ cells grown in the presence of various NaCl concentrations (Fig. 28 A) most of the canonical amino acids could be found in low millimolar concentrations (arginine 8 mM, glutamine 4 mM, valine 2.5 mM and threonine 1mM).

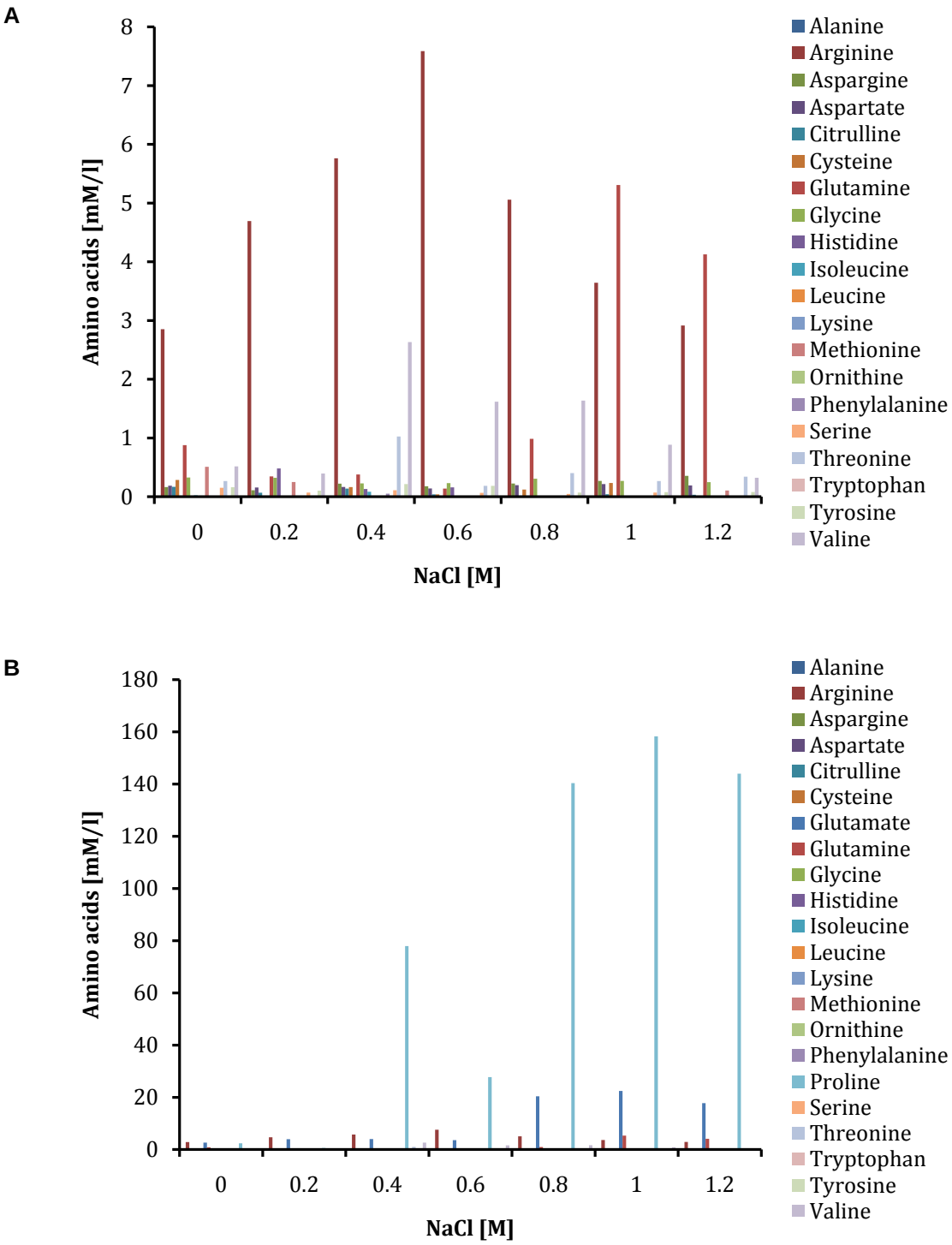

Figure 28: Quantification of intracellular amino acids in ε2124$_{847}$ by HPLC.

Detection and quantification of amino acids was performed by HPLC analysis on an Agilent 1200 SL. Amino acids in the samples were first derivatized using a pre-column protocol [o-phthalaldehyde for the primary and 9-fluorenylmethyl chloroformate for secondary amino acids]. After injection, the samples were separated on a ZORBAX Eclipse Plus C18 column at 40ºC using a binary gradient mobile phase. The eluting amino acids were detected with a fluorescence detector (FLD) and quantified with the use of a standard amino acid (AAS18). A, intracellular amino acids except for glutamic acid and proline; B, all intracellular amino acids scaled to show the quantities of proline and glutamic acid.

A dramatic increase also in the intracellular concentrations of glutamate and proline was measured in cells grown at high osmolarities (Fig. 28 B) with glutamate accumulating to approximately 22 mM and proline to around 160 mM in cells grown in 1 M NaCl. As could be predicted from the more qualitative detection of these amino acids by the TLC technique, their synthesis was regulated by the medium osmolarity.

3.2.6 Stress response in evolving ε2124 populations

In similar experiments as described for MG1655 and its evolved derivatives (see 3.1.7) the stress response of ε2124 derivatives was compared with that of wild type MG1655 cells by immunodetection of the RpoS stress sigma factor during growth in various NaCl concentrations.

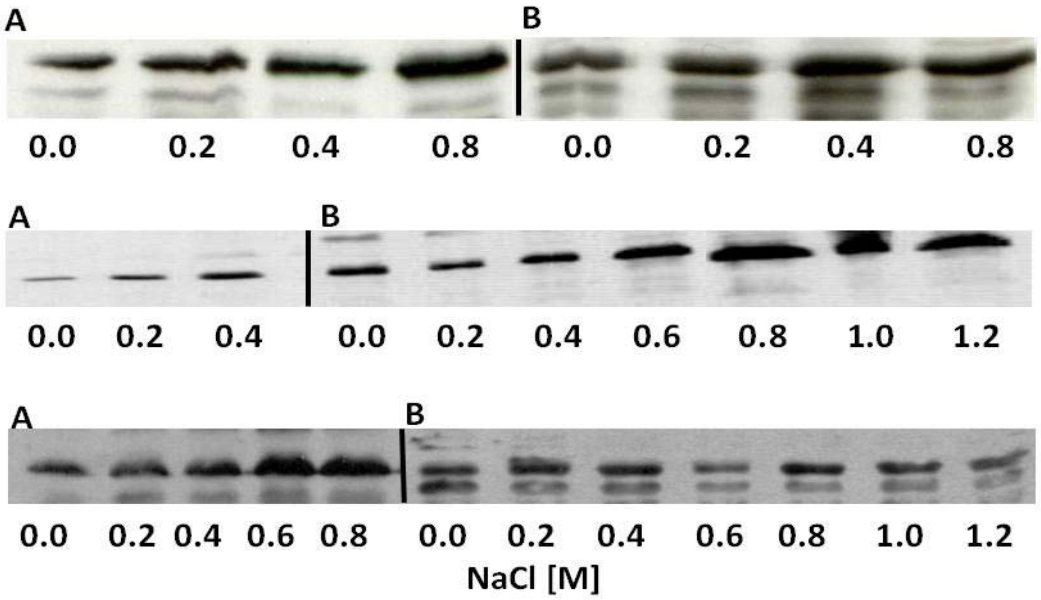

Figure 29: Expression of RpoS in *E. coli* MG1655 wild type cells and evolved ε2124 derivatives.

Extracts were prepared from cells growing at the indicated NaCl concentrations when the cell density had reached an OD_{600nm} of 0.4. Equal amounts of proteins were separated on 12.5%polyacrylamide/0.1% SDS gels and transferred to nitrocellulose membranes which were subsequently probed with 1:5000 diluted anti-RpoS antibodies, developed with ECL reagent, and the chemiluminescence was documented by exposure of the blots on Kodak BioMax light autoradiography films. Only parts of the blot (molecular mass of 38 kDa) are shown. Panel A, MG1655; Panel B in the upper part, ε2124 $_{231}$; Panel B in the middle part, ε2124 $_{656}$; Panel B in the lower part, ε2124 $_{847}$.

Figure 29 shows that ε2124$_{231}$ displayed a similar pattern of RpoS expression as wild type cells with an easily detectable increase in the amount of RpoS at 0.4 M NaCl. ε2124$_{656}$ cells showed increased RpoS expression at 0.6 M NaCl and higher salt concentrations. The evolvant isolated after 847 days of selection showed an almost constant level of the protein which appeared generally lower than in wild type cells.

3.2.7 Changes in the outer membrane and total proteome of ε2124 derivatives

Proteins of the outer membrane of ε2124$_{393}$ and total cellular proteins of ε2124$_{847}$ were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. On silver-stained gels containing outer membrane proteins (Fig. 30) a number of differences can be seen when the pattern of ε2124$_{393}$ is compared to that of wild type cells. Thus, several proteins both in the high and low molecular mass range are expressed at much lower levels or are even absent.

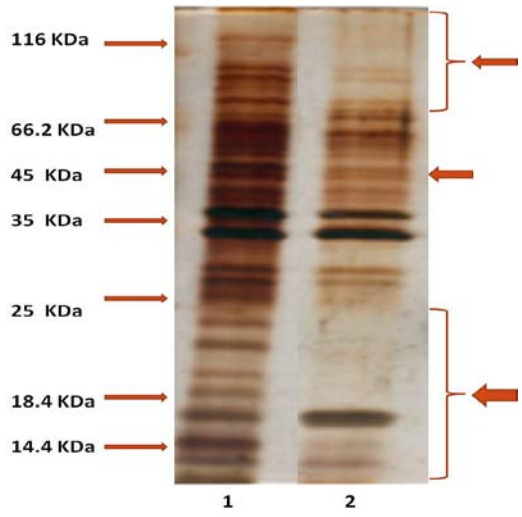

Figure 30: Separation of outer membrane proteins of *E. coli* MG1655 wild type cells and evolved ε2124 derivative.

Outer membrane proteins were extracted from MG1655 cells grown in 0.0 M NaCl lane (1) and from ε2124$_{393}$ cells grown in 0.8 M NaCl lane (2) when the cell density had reached an OD$_{600}$nm of 0.8. Equal amounts of proteins were separated on 12.5% polyacrylamide/0.1% SDS gel and stained with the Page Silver™ Silver Staining Kit. The position and sizes of marker proteins are indicated to the left.

Differences between wild type and evolved cells are also apparent in the total proteome (arrows in Fig. 31). As in the case of differences observed for MG1655 evolvants, the nature of these proteins is presently unknown. Further studies should aim at identifying these proteins by two-dimensional gel electrophoresis followed by mass-spectrometric analyses.

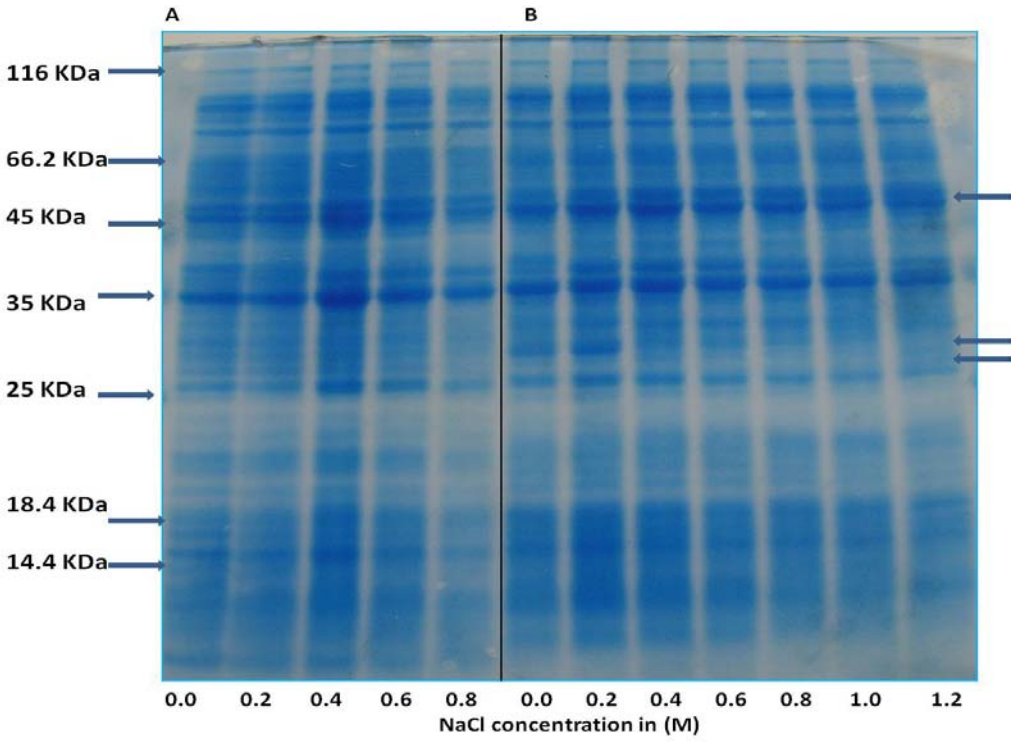

Figure 31: Separation of total cellular proteome of *E. coli* MG1655 wild type cells and the ε2124$_{847}$ derivative.

Total cellular proteins were extracted from cells growing at the indicated NaCl concentrations when the cell density had reached an OD_{600nm} of 0.4. Equal amounts of proteins were separated on 12.5% polyacrylamide/0.1% SDS gels and stained with Coomassie-Brilliant blue G250. A, MG1655; B, ε2124$_{847}$. The position and sizes of marker proteins are indicated to the left.

4. Discussion

4.1 Technology for experimental evolution

The technology of continuous culture developed more than sixty years ago independently by Monod (Monod, 1950) and Novick and Szilard (Novick and Szilard, 1950) is an excellent tool for experimental evolution of huge populations of cells under strictly controlled, selective laboratory conditions. In such culture devices a suspension of organisms is continuously renewed by a constant or conditional flow of fresh growth medium, and the cells have to compensate the concomitant loss of individuals by growing with a rate that is at least equal to the dilution rate. Theory predicts that under these conditions better growing variants will be selected which rapidly displace their less-adapted progenitors (Kubitschek, 1970). However, this technology was soon abandoned as a tool for experimental evolution because it also rapidly selects adhesive, biofilm-forming variants which colonize inner surfaces of the culture apparatus and are not constrained to compete with their pelagic counterparts (Chao and Ramsdell, 1985). The most extensive experiments on the evolution of bacterial populations over 50000 generations during a period of more than 20 years therefore relied on serial subculturing of cells (Barrick et al., 2009; Lenski, 2011), a tedious, contamination-prone technology which suffers several major disadvantages such as discontinuous growth, frequent loss of advantageous mutations due to regular forcing of the population through narrow bottlenecks of small numbers of individuals, and the impossibility to apply constant, harsh selection pressure.

In this work, two lines of halotolerant descendants of the prototypic *E. coli* strain MG1655 were created by continuous cultivation of huge bacterial populations under challenging medium osmolarities. Selection for constantly increasing halotolerance was achieved by using the Genemat technology developed about a decade ago by Marlière and Mutzel (Marlière and Mutzel, 2004). This technology overcomes the main obstacle of permanent proliferation of suspended living cells in continuous culture devices, rapid selection of adhesive, adaptively static variants, by alternatively cultivating the population in twin culture vessels which are periodically sterilized by a lethal chemical agent.

A conditional pulse feed regime was applied which automatically maintains the osmolarity of the growth medium at a level that is just tolerable for the best-adapted variants. Here, the culture is periodically diluted with a growth medium containing a salt concentration that is compatible with growth of the cells ("permissive medium"), and the population density is constantly monitored. When cell density passes a pre-set threshold, the culture is diluted with a pulse of "nonpermissive medium" (of an osmolarity that is too high for growth) instead. Genetic variants tolerating higher osmolarity enjoy a selective advantage under these conditions and will rapidly displace their less salt-tolerant progenitors, to be later replaced by their own, more salt-tolerant progeny.

The Genemat technology and the conditional pulse feed regime have recently proven their power with the quasi-total replacement of the canonical DNA base thymine in the genome of *E. coli* for the non-natural analogue, 5-chlorouracil, by Marlière and his co-workers (Marlière et al., 2011). It can be foreseen that this technology will become a standard tool in synthetic biology, enabling to deeply modify the chemical and metabolic constitution of microbial cells (Acevedo-Rocha and Budisa, 2011) and expand the range of physical environments in which experimentally evolved cells can thrive, as demonstrated in the present work.

4.2 Experimental evolution of halotolerance and evolved mechanisms of halotolerance

Both developed lineages greatly enhanced tolerance against the monovalent cations, Na^+ and K^+, as well as against the uncharged sugar, sorbitol. Cl^- and SO_4^{2-} could act as counter-ions whereas I^- was toxic at lower concentrations than for wild type cells. Perhaps the most spectacular result of this experiment is a switch in the intracellular osmoprotective strategy of evolved cells from the accumulation of the disaccharide trehalose to accumulation of several hundred mM of the amino acid proline, which is a well-known osmoprotectant in other organisms such as *Bacillus* species (Whatmore et al., 1990) but has so far not been reported to be part of the osmoprotective system of *E. coli*.

In wild type *E. coli* strains the response to osmotic stress consists in the rapid uptake of K^+ ions from the extracellular milieu, which triggers an increase in the synthesis of glutamate. Next, osmoprotectants such as glycine-betaine or proline can be taken up

from the medium, if they are present, or the induction of the *otsBA* trehalose synthetic operon is induced by the stress sigma factor RpoS. The precursors glucose-6-phosphate and UDP-glucose are condensed to trehalose-6-phosphate by trehalose-6-phosphate synthase (the product of *otsA*), which is then dephosphorylated by trehalose-6-phosphate phosphatase (the *otsB* gene product), and K^+ ions are subsequently exported from the cell because they interfere with protein function. Addition of extracellular osmoprotectants relieves the stress situation and, consequently, lowers intracellular trehalose concentrations (Dinnbier et al., 1988). Upon osmotic downshift, trehalose can be metabolized by the cell after export and re-import as trehalose-6-phosphate via the *treB* gene product and degraded to glucose and glucose-6-phosphate by the product of *treC*. Cell growth, which is halted during the initial phase of adaptation, can resume (Kempf and Bremer, 1998).

During our evolution experiment cells experience chronic and constantly growth-limiting osmotic stress. There are no osmoprotectants in the mineral medium supplied and the cells have to rely on the production of intracellular trehalose. This was indeed found in isolates analyzed during the early stages of adaptation, which showed the osmotically regulated synthesis of trehalose along with increased levels of RpoS. Both populations then switched to proline synthesis. In the MG1655 experiment this was accompanied by a distinct phase where the consumption of nonpermissive medium per day did not increase during 150 days between days 450 and 600 (compare Fig. 7); for ε2124 this cannot be assessed as this culture showed massive oscillations in the consumption of nonpermissive medium. In a roughly anti-parallel manner the synthesis of intracellular trehalose in response to high salt concentrations decreased, to be finally undetectable in MG1655 cells, whereas ε2124 evolvants retained synthesis of the sugar, although at much lower levels than wild type cells.

The fact that $MG1655_{858}$ evolvants did not show any synthesis of trehalose even at the highest tested salinities and when RpoS was clearly up-regulated (Fig. 13 and 18), indicates that the enzymatic machinery for trehalose synthesis is either completely uncoupled from the regulatory answer to osmostress (and no longer under control of RpoS), or that its enzymatic function is defective in these cells due to one or several mutations in the corresponding genes. This question could be answered by PCR amplification and sequence determination of these genes on the one hand and, on the other hand by exposure of the evolved cells to cold shock, a

condition that has been reported to trigger a similar increase in the intracellular trehalose concentration as osmotic shock (Kandror et al., 2002). The gradual decrease in trehalose accumulation probably reflects a gradual increase in proline synthesis, such that the cells experience lower stress levels (Dinnbier et al., 1988). An eventual "knock out" of trehalose synthesis might then be tolerated by the cells or even be beneficial since the trehalose system is no longer required. The situation might be similar in ε2124 evolvants, where low levels of osmotically regulated trehalose accumulation could be measured even in later stages, together with a moderate up-regulation of RpoS. Here, however, both trehalose and proline may still contribute to osmoprotection.

The intracellular proline concentration in *E. coli* is regulated by feedback inhibition of the gene product of *pro*B, the γ-glutamylkinase (Baich, 1969; Smith et al., 1984). A number of mutations in the catalytic domain are known to relieve this feedback inhibition, causing intracellular accumulation of the amino acid (Perez-Arellano et al., 2005). In addition, the C terminus of γ-glutamylkinase has been identified as a regulatory domain, and truncation of this C terminal domain also leads to relief of feedback inhibition by proline (Perez-Arellano et al., 2005). Such mutants constitutively over-produce proline, and its synthesis is apparently not regulated by the medium osmolarity.

First results from an ongoing project on the identification of mutations in the proline synthesis pathway show that the *proB* genes from both $MG1655_{1119}$ and $\varepsilon2124_{962}$ evolvants harbour two point mutations each in the part coding for the catalytic domain of the enzyme, and that in the ε2124 evolvant an additional frame shift mutation introduces a premature stop codon, so that the resulting gene encodes a C terminally truncated variant of the enzyme (F. Scholz, personal communication). In both strains therefore, proline synthesis should be unregulated by the concentration of proline. It will be interesting to determine, by analyzing the "fossil record" of weekly samples secured at -70 °C, at which point during the adaptation process these mutations were fixed in the populations.

4.3 Osmoregulation of proline synthesis

Synthesis of proline is regulated by the medium osmolarity in both evolved strains, as can be judged both from TLC and HPLC analyses. Yet, synthesis of proline does not appear to be regulated via the canonical RpoS-dependent stress response since

huge amounts of the amino acid can be found at salinities where no enhanced expression of RpoS is observed. How is proline synthesis regulated then?

A hint to answer this question may come from the observation that both evolved strains are sensitive to KI concentrations which the wild type strain tolerates. Starting from the hypothesis that evolved MG1655 and ε2124 cells may chronically enrich intracellular K^+ (with I^- being co-imported as a counter-ion), Susanne Vettermann in this laboratory recently tested the osmotolerance of the $MG1655_{1119}$ derivative in K^+-free medium. Her results show that under these conditions the strain fails to grow at 1 M NaCl or higher while it can grow up to 2 M NaCl in the presence of 50 mM K^+. Tolerance against challenging sorbitol concentrations is similarly impaired in the absence of K^+ (S. Vettermann, personal communication).

In wild type *E. coli* cells, a rapid, transient uptake of K^+ triggering the synthesis of glutamate is part of the acute response to increased osmolarity. Since the cation interferes with protein function, the cell has to remove it from the cytosol after induction of trehalose synthesis in order to resume growth. Glutamate is the direct precursor for proline, and deregulation of the *proB* gene product in the evolved derivatives (see above) would direct glutamate into this pathway. Chronically high intracellular K^+ would trigger constitutively high rates of glutamate synthesis, and the actual rate of synthesis of glutamate could directly correlate with the final cellular concentration of proline. Whether the intracellular K^+ concentration in the evolved cells directly correlates with the medium osmolarity and whether the level of intracellular K^+ regulates the rate of glutamate synthesis is a matter of speculation at this stage. To address this, intracellular K^+ concentrations in cells growing under various osmolarities should be measured in follow-up work. If the intracellular K^+ concentration indeed reflected the medium osmolarity, adaptation to various osmolarities in the evolved cells could bypass the canonical, RpoS-dependent, stress pathway. Chronically elevated K^+ concentrations, however, are incompatible with protein function in wild type cells. It is therefore to be expected that the adaptation process led to mutations in the genes for proteins that do not function in wild type cells in the presence of elevated intracellular K^+. This process would probably be slow because natural selection would have to "test" various mutants for their ability to function in high K^+, and this "search for better adapted variants" could explain the phase of stalled increase in the consumption of nonpermissive medium observed between days 450 and 600 in the MG1655 experiment. It might be mentioned here that there was a second phase where the consumption of nonpermissive medium did

only slowly increase (days 700 - 900) which was followed by a steep increase in the last period of cultivation conducted during this work. This might be another instance of such a search for rare mutations or combinations of mutations.

4.4 Impaired growth of evolved cells in the absence of salt

According to a definition proposed by Oren (2008) halophilic organisms grow best at NaCl concentrations over 0.85 M, grow up to 1.7 M, and fail to grow in the absence of external salt. In this respect, at least $MG1655_{1119}$ would be close to halophily. Failure to grow when diluted from 0.8 M NaCl into salt-free minimal medium was first observed in samples from the MG1655 and ε2124 experiments withdrawn at days 858 and 601, respectively. Still, even after long durations of continuous selection both strains can be re-adapted to grow in the absence of extracellular NaCl (notice that the medium contains 50 mM K-phosphate), either by passaging the population over media of decreasing salinities (where they grow within 24 hours after each passage to lower salinity) or upon direct inoculation from 0.8 M NaCl to 0 M NaCl, where they grow after lag periods of three to five days. This long lag phase could be due to very slow adaptation of all cells in the population to the low salt environment. It is also possible that only a tiny fraction of the cells can survive the osmotic downshift, leaving a small population of viable cells which grows with a fair rate but has to undergo multiple generations before growth becomes evident for the observer. We attempted to discriminate between these two possibilities by quantifying cell survival rates after dilution on agar plates (both LB and MS man agar), yet this approach had to be abandoned because evolved cells grow with very low yields on solid media even if they are plated at the salinity of the liquid pre-culture. This failure to grow on solid media may be explained by the fact that the cells were cultivated in the genetic machine for thousands of generations in an environment that effectively selects against adhesion to surfaces.

4.5 Proteome evolution

As long as the intracellular milieu can be osmotically balanced by massive synthesis of compatible solutes, proteins that are in contact with the extracellular space (outer membrane proteins, periplasmic proteins, and the outer face of inner membrane

proteins) should be subjected to selection for proper function in high salinities. Only when intracellular osmoprotectant production proves to be insufficient to compensate the loss of water under increasing extracellular salt concentrations, adaptations of the structure of intracellular proteins such as those found in the proteins of halophilic organisms (Ventosa et al., 1998) should occur. Adaptations such as the construction of "charge shields" - the enrichment of the outer surfaces of proteins in glutamic acid and aspartic acid (Zaccai and Eisenberg, 1990) - will require multiple mutations in the genes for those proteins and are expected to accumulate over many generations. For example, the differences in the amino acid composition of total cellular proteins would amount to at least 5% of the genome, as suggested by a comparison of the amino acid compositions of the halophile *Haloferax mediterranei* and the mesophilic *E. coli* (Ghanbir et al., 1995). Initial proteomic analysis of outer membrane proteins and total cell extracts of the evolvants isolated during our work suggest that the cellular proteome is indeed changing. So far, these changes appear to affect the *expression* of individual proteins rather than their *structures* since at least in the case of MG1655 evolvants shifts in the isoelectric points of individual proteins are not observed. A shift in the pI of a protein to lower values would indicate an enrichment of negatively charged amino acids.

4.6 Influence of genetic variability on halophilic adaptation

ε2124 carries mutations in both the *def/fmt* operon and the *mutD* gene. The original Δ*def/fmt* mutation led to a drastic increase in the generation time, and subsequent cultivation under a turbidostat regime rapidly restored near wild type growth rates (Marlière et al., 2005; see below, Fig. 32). Resurgence from the "crippling" effect of the *def/fmt* deletion was much faster in the *mutD* derivative than in the non-mutator parental strain (Fig. 32).

The haloadaptation experiment with ε2124 was initiated hypothesizing that higher mutation rates would speed up evolution similarly. However, whereas adaptation to higher osmolarities proceeded smoothly with MG1655 cells as revealed by regular increases in the consumption of nonpermissive medium, ε2124 cultures displayed massive and sustained oscillations in the consumption of nonpermissive medium during most of the experiment, and halotolerance did not reach the same level as in MG1655.

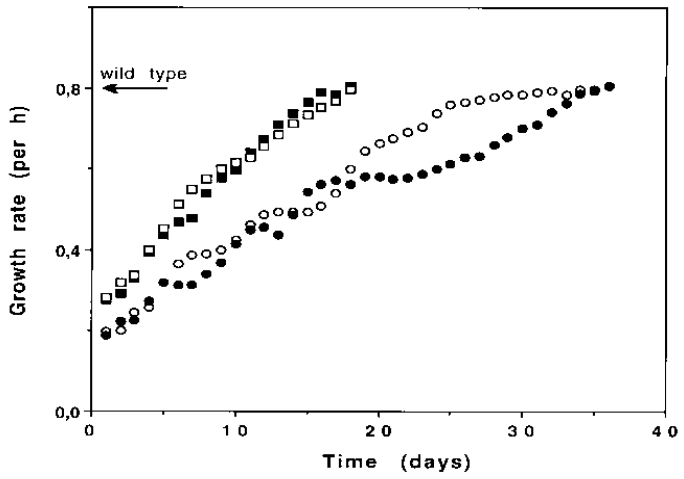

Figure 32: Resurgent evolution of *E. coli* Δ*def/fmt* strains in the "Genemat" (courtesy of R. Mutzel).
Two independent turbidostat runs (open and closed symbols) each are shown for input strains with wild type mutation rates (circles) and *mutD* derivatives (squares). The initial growth rate of the parental (*def*$^+$/*fmt*$^+$) strain is approximately 0.85 under these conditions and increases to 1.05 after 200 generations of growth in the turbidostat.

This appears not to be due to differences in the physical or chemical environment of the population since oscillations originally occurred in one of two identical setups run in the same laboratory, did not stop when the experiment was transferred to another setup, continued when it was re-transferred to the original machine and only started to dampen out during the last phase of the selection campaign. Therefore, these oscillations appear to reflect some biological property of the evolving population, most probably the absence of a functional *mutD* gene. The experiment is presently being continued, and oscillations in the consumption of nonpermissive medium are no longer observed, as could be predicted from the behaviour of the culture during the last 100 days of cultivation reported here (see Fig. 21). Nevertheless the evolving ε2124 population has not lost its mutator phenotype as recently shown by determination of the frequency of generation of Streptomycin-resistant mutants which is around 1000-fold higher than in MG1655 cells (L. Löwenau, personal communication).

Interestingly, a very similar "inhibitory" effect of a mutator allele was observed during adaptation to incorporation of 5-chlorouracil into DNA (P. Marlière, personal communication via R. Mutzel).

In the case of deletion of the *def/fmt* operon a very limited number of crucial adaptive mutations appear to be sufficient for restoring near-wild type growth rates, since the

genomes of evolved derivatives from four independent turbidostat experiments show only a small number of common mutations hidden in a "noisy background" of several hundred mutations in each of the Δ*def/fmt/mutD* evolvants (D. Mazel, personal communication via R. Mutzel). Both for adaptation to 5-chlorouracil usage and for haloadaptation a large number of mutations which individually have no big effect on the overall fitness of the evolving strain, but may be together necessary for better growth, (in fact, resurgence of the *def/fmt* mutation required only approx. 300 generations in case of MG1655 Δ*def/fmt* cells, while adaptation to high osmolarity is under way since thousands of generations; and adaptation to 5-chlorouracil usage has been shown to be accompanied by large numbers of point mutations and chromosomal rearrangements (Marlière et al., 2011). It is possible that *mutD* cells harbouring different adaptive mutations of small selective value coexist in the culture subjected to osmostress. During re-adaptation of Δ*def/fmt* variants to robust growth, on the other hand, individual adaptive mutations appear to confer large fitness gains to cells acquiring them, which should lead to very rapid displacement of progenitor populations which do not enjoy these gains in fitness.

4.7 Outlook

The focus of the work presented here was on the "constructive" aspects of experimental evolution of halophily, and it is planned to continue the continuous cultures under selection until no further adaptation can be observed. In-depth analysis of molecular, biochemical and physiological properties of the evolved bacteria obtained until now is far from being complete. A number of follow-up analyses may be proposed based on the results obtained so far.

Determination of the genome sequences of the evolved derivatives and bio-informatics comparison with those of their ancestors is clearly necessary. With the recent advent of next-generation sequencing technologies these analyses are straightforward. Moreover, with further progress in sequencing technologies the detailed analysis not only of the genomes of provisional "endpoints" of the experiment (such as strains $MG1655_{858}$ or $MG1655_{1119}$), but also of interesting intermediates or even of the full "fossil record" of samples stored weekly at -70 °C will become within reach, allowing to define a detailed adaptive path towards halophily at the molecular level. Molecular analysis has to be accompanied by functional studies, which address the mechanism of evolved halotolerance in the output of my work in

more details. For example, the hypothesis of chronically elevated intracellular K^+ in the evolved derivates must be tested directly. Ideally, this should be extended to the functional analysis of selected proteins, which are known to be sensitive to high K^+.

DNA sequence analyses should be extended to comparative transcriptome and proteome analysis. Which genes are up- and downregulated in their expression during growth of the evolved cells in media of various osmolarity? Initial 2-D gel electrophoretic analyses revealed a number of proteins whose expression changed dramatically in evolved MG1655 cells growing under limiting salt concentrations as compared to wild type cells growing in medium with low salinity. These proteins should be identified by mass-spectrometric analysis of the "spots" cut out from the gels. Are these changes constitutive or are they induced by the osmolarity of the growth medium?

Finally, the differences in the adaptive kinetics of mutator-wild type und *mutD* strains deserve attention. It is generally believed that higher variability is beneficial during adaptation in challenging environments. Also, one of the strains cultivated during the Lenski long-term experiment acquired a mutator phenotype (Barrick et al., 2009). Yet for adaptation to high osmolarity lower variability appears preferable, although a contribution of the Δ*def/fmt* mutation in this experiment cannot be formally ruled out. Once mutations in the genome, which are crucial for adaptation, have been found and their order of appearance determined, mathematical simulation of the dynamics of populations with different genetic variability under stressful conditions could shed light on this problem.

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Erklärung

Hiermit versichere ich, dass ich die Dissertation mit dem Titel "Experimental evolution of halotolerance in *Escherichia coli*" selbstständig und ohne unerlaubte Hilfsmittel angefertigt habe.

Berlin, den 17.06.2011

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Mohammed Khlaf