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Growth and acid stress response of *Corynebacterium glutamicum* and other ripening bacterial species belonging to soft smear-ripened cheese

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Abbreviations

ATCC	American Type Culture Collection
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ds	double stranded
nt	nucleotides
bp	base pairs
ORF	open reading frame
× *g*	relative centrifugal force
PCR	polymerase chain reaction
CFU	colony forming unit
pH	negative decadic logarithm of the molar concentration of hydrogen ions
RT PCR	reverse transcriptase polymerase chain reaction
FISH	fluorescent *in situ* hybridization
DGGE	denaturing gradient gel electrophoresis

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Summary

Cheese ripening is a complex and long biochemical process mainly influenced by the development of smear microflora whose growth rate depends upon numerous environmental parameters such as pH and temperature. The formation of the smear is an essential step as the bacteria in the smear determine some of the main organoleptic properties of cheese such as color, flavor, and texture. Therefore, the dairy industry is greatly interested in shortening this period. One way to accelerate cheese ripening is using modified microorganism displaying desirable phenotypic characteristics allowing an acceleration of the ripening process. In this work we adapted four cheese ripening species (*C. ammoniagenes*, *C. variabile*, *K. palustris* and *S. equorum*) to low pH. Two species, *C. ammoniagenes* and *S. equorum*, were adapted to pH 5.3. Four mutants were obtained for *C. variabile* which grew at 15°C and 30°C better than the parent strain. For *S. equorum* three mutant strains were obtained which phenotypically surpassed the parent strain at 30°C, however only one strain demonstrated this desirable characteristic at 15°C. *C. ammoniagenes* and *K. palustris* were adapted to pH 5.8 resulting in two and three mutant strains for each of the species, respectively. Both of the *C. ammoniagenes* mutant strains outperformed the parental strain at 15°C and 30°C. Despite demonstrating desirable characteristic at 30°C none of the mutant strains of *K. palustris* showed any improvement over the parent strain at 15°C. Therefore, classical strain improvement methods provide some success in generating mutant strain that could be potentially useful in accelerating cheese ripening.

Quantitative real time PCR was used to monitor species succession of a defined microbial consortium consisting of, one yeast (*Debaryomyces hansenii*) and five ripening bacterial species (*Corynebacterium variabile*, *Corynebacterium ammoniagenes*, *Staphylococcus equorum*, *Kocuria palustris*, and *Staphylococcus warneri*) on a defined cheese agar system. For individual identification, species specific primers were designed based on 16S and 18S rDNA sequences. The agar was inoculated with a mixture containing 10^2 CFU/cm^2 yeast (*D. hansenii*) and 10^5 CFU/cm^2 of the ripening bacteria. Samples taken during the microbial succession over 11 days were quantified for the presence of each of the species by real time PCR as well as culture based methods. The samples demonstrated a good correlation with culture based methods showing a 0.929 correlation in average total aerobic count and a 0.915 correlation in the average yeast count. It was observed that *S. eqourum* was the dominant organism followed by *C. variabile* and *C. ammoniagenes*. The least dominant organism was *K. palustris*. None of the microorganisms were displaced from the consortium which is evidence that the species were

well adapted to their environment. It is concluded that real time PCR is a suitable method to monitor species succession in a microbial consortium of limited complexity.

Microorganisms are constantly exposed to fluctuating environments. In order to survive changing conditions due to temperature, nutrient limitation and other, microorganisms adapt by switching on the synthesis of special proteins which in turn regulate gene expression. Challenges due to an acidic environment are experienced by both, pathogenic and fermentative organisms. In this study, microarray technology was applied to examine the global gene expression profile of acid adapted *Corynebacterium glutamicum*, a biotechnologically important microorganism. A total of 116 up regulated and 90 down regulated genes were identified, representing 10% of the genes in the genome. Most of these genes can be classified in the three categories transcriptional regulators, transport, and metabolism. Four operons belonging to an ABC type cobalamin/Fe^{3+} siderophore transport system were observed among the ORFs showing an upregulation. To characterize the gene expression of the siderophore transport system at low pH iron availability was assessed in minimal and complex medium. These studies resulted in lower gene expression in minimal medium compared to complex medium suggesting an iron limiting conditions present at low pH in complex medium.

To further characterize the function of the up regulated genes 17 disruption mutants were generated which were phenotypically analyzed in respect to low pH. *sigB* and *sigE* mutants showed a significant negative effect to low pH suggesting a function in acid adaptation. No significant effects were observed for the other disruption mutants hinting to a redundancy in the pH adaptation response of *C. glutamicum*.

Zusammenfassung

Die Käsereifung ist ein komplexer und langwieriger biochemischer Prozeß, der hauptsächlich von der Entwicklung der Mikroflora der Käseschmiere beeinflußt wird und von zahlreichen Umgebungsparametern, wie pH und Temperatur abhängig ist. Die Zusammensetzung der Schmiere ist insofern bedeutsam, als sie für die meisten sensorischen und optischen Eigenschaften des Käses, wie Geschmack, Farbe und Textur verantwortlich ist. Aus diesem Grund zeigt die milchwirtschaftliche Industrie ein besonderes Interesse an der Verkürzung der Reifungszeit. Eine mögliche Methode den Reifungsprozess zu beschleunigen ist die Verwendung speziell modifizierter Mikroorganismen, die dennoch die gewünschten phänotypischen Eigenschaften aufweisen. Im Verlauf dieser Arbeit wurden vier an der Käsereifung beteiligten Spezies, *C. variabile, C. ammoniagenes, S. equorum* und *K. palustris*, an niedrige pH-Werte adaptiert. Die Arten *C. ammoniagenes* und *S. equorum* wurden an pH 5,3 adaptiert. Für *C. variabile* ergaben sich vier Mutanten, die bei 15°C und 30°C ein besseres Wachstum aufwiesen als die Ausgangsstämme. Im Fall von *S. equorum* wurden drei Mutanten gefunden, die phänotypisch den Ursprungsstamm bei 30°C übertrafen. Allerdings zeigte nur ein Stamm die gewünschte Eigenschaft bei 15°C. *C. ammoniagenes* und *K. palustris* wurden an pH 5,8 adaptiert, woraus zwei bzw. drei mutierte Stämme resultierten. Beide Stämme von *C. ammoniagenes* übertrafen den Ursprungsstamm bei 15 bzw. 30°C. Die wünschenswerten Eigenschaften der *K. palustris* zeigten sich jedoch nur bei 30°C und ergaben keine nennenswerte Verbesserung gegenüber dem Ausgangsstamm bei 15°C. Aus diesen Ergebnissen folgt, dass die klassische Methode zur Verbesserung der Stämme mit gewissen Einschränkungen für die Entwicklung von Stämmen zur Beschleunigung der Käsereifung nützlich sein kann.

Im zweiten Teil der Arbeit wurde mittels quantitativer Real-Time-PCR die Sukzession eines definierten mikrobiellen Konsortiums in einem definierten Käse-Agar-System bestimmt. Das Konsortium bestand aus der Hefe *Debaryomyces hensenii* und fünf verschiedenen Arten von Reifungsbakterien (*Corynebacterium variabile, C. ammoniagenes, Staphylococcus equorum, S. warneri* und *Kocuria palustris*). Zur individuellen Identifizierung wurden spezies-spezifische Primer auf Basis der 16S und 18S ribosomalen DNA-Sequenzen entwickelt. Der Agar wurde mit einer Mischung aus 10^2 CFU/ml Hefe *D. hansenii* und 10^5 CFU/ml Reifungsbakterien beimpft. Über einen Zeitraum von elf Tagen wurden Proben entnommen. Die Identifizierung und Quantifizierung der Spezies erfolgte sowohl über Real-Time-PCR als auch über Kultivierungsmethoden. Dabei zeigte sich eine gute Übereinstimmung der Ergebnisse aus beiden Methoden, mit einer Korrelation von 0,929 bzw. 0.915 bei der Bestimmung der

Gesamtkeimzahl bzw. Zellzahl der Hefen. *S. equorum* zeigte sich dabei als dominanter Organismus, gefolgt von *C. variabile*, *C. ammoniagenes* und *K. palustris*. Keines der Mikroorganismen wurde aus dem Konsortium verdrängt, was ein Hinweis auf ihre gute Anpassung an die Umgebung ist. Die Ergebnisse lassen darauf schließen, dass Real-Time-PCR eine geeignete Methode für das Monitoring von Spezies in einem Konsortium begrenzter Komplexität ist.

Mikroorganismen sind ständig veränderlichen Umweltbedingungen ausgesetzt. Um z.B. Temperaturschwankungen und Nährstoffmangel zu überdauern, adaptieren sie sich über die Veränderung ihrer Genexpression. Saure Umgebungsbedingungen betreffen sowohl pathogene als auch fermentative Mikroorganismen. In dieser Arbeit wurde mittels Microarray Technologie das Expressionprofil eines säure-adaptierten Stammes von *C. glutamicum* als einem biotechnologisch bedeutsamen Mikroorganismus untersucht. Insgesamt wurden 116 hoch regulierte und 90 herunter regulierte Gene nachgewiesen, die zusammen etwa 10% der Gene im Genom ausmachen. Die meisten Gene konnten in folgende drei Gruppen eingeteilt werden: Transkriptionsregulatoren, Stofftransport und Metabolismus. Unter den hoch regulierten ORFs befanden sich vier Operons Fe^{3+}-abhängiger ABC-Transporter. Um die Expression der Siderophore unter niedrigen pH-Bedingungen zu testen, wurde die Verfügbarkeit von Eisen in Minimal- und Vollmedium bewertet. Die Untersuchungen ergaben eine niedrigere Expressionsrate im Minimalmedium vermutlich aufgrund Eisen-limitierender Bedingungen in Vollmedium mit niedrigem pH.

Für eine weitere Charakterisierung der hoch regulierten Gene wurden 17 Deletions-Mutanten hergestellt und phänotypisch im Bezug auf niedrige pH untersucht. Die *sigB* und *sigE* Mutanten zeigten einen signifikanten negativen Effekt bei niedrigem pH-Wert. Deshalb wird eine Bedeutung dieser Gene bei der Anpassung an sauere Umgebungsbedingungen vermutet. Für alle anderen Deletions-Mutanten ergaben sich keine signifikanten Effekte, was ein Hinweis auf die Redundanz des pH-Homöostase-Systems ist.

1 Introduction

Microorganisms are essential components of all cheese varieties and play an important role in both cheese manufacture and ripening. During cheese ripening complex interactions occur between individual components of the cheese flora. Environmental factors such as pH, temperature and water activity within the cheese also contribute to these interactions. Elucidations in the time dependent changes in the microorganism community provide a better understanding in the ripening process (10). Most of the population analysis methods in studying microbial interactions describe the isolation and identification of the "most predominant" species (48) and provide semiquantitative information by reporting the frequency of isolation of specific organism (58). In this work we describe a novel molecular technique, quantitative real time PCR, which addresses these limitations in analyzing population dynamics.

Cheese manufacture involves the transfer of the developed microflora from mature ripened cheeses to young cheeses by a method of old young smearing. By this process not only the desired microflora but, undesired microorganisms such as *Listeria monocytogenes* are transferred capable of growth at low pH and temperature. Initial acidity of the cheese influences the development of the bacterial flora which contributes to cheese ripening through the production of metabolites that are responsible for the characteristic flavor, color and texture of cheese (101). Therefore, the dairy industry is greatly interested in accelerating the development of cheese flavor characteristics and at the same time producing a biologically safe product. Until recently, most of the efforts concentrated on improving the performance of starter culture. However, there have not been any studies described in accelerating cheese ripening through the adaptation of cheese ripening species.

Bacteria are constantly exposed to changing environmental conditions. Challenges due to an acidic environment are experienced both by fermentative and pathogenic organism. Food fermentative organisms encounter acidic condition during industrial processes. In order to achieve their effect probiotic microorganisms also have to survive the transit through the digestive tract. Bacteria survive acid stress by turning on specific stress regulators which in turn coordinate the expression of genes altering different cell processes (145). In order to identify regulators and regulatory network in acid stress we analyzed the acid adaptation response of *Corynebacterium glutamicum* a biotechnologically important organism using DNA microarray technology.

1.1 Microbial composition of surface smear-ripened cheese

Smear ripened cheeses are produced in large quantities in various European countries (10) and are characterized by the formation of a viscous red-orange colored smear comprised of a complex mixture of yeast and bacteria producing a rich aromatic piquant flavor (48, 124). The traditional method of cheese inoculation is the old-young smearing, where the surface flora of mature ripened cheeses is washed off with a brine solution that is later used to inoculate the surface of young cheeses. In general, smear ripened cheeses are soft or semi-soft in texture as in Limburger, Tilsit and Romadour (13, 124) but, some hard cheeses such as Gruyère, Beaufort and Comté also belong to the family of smear ripened cheeses.

The microbiology of the smear is complex and poorly understood. There have been numerous attempts to determine the composition of the microbial consortium growing on the cheese (21, 45, 46). Yeasts, such as *Debaryomyces hansenii* and *Geotrichum candidum* are the predominant organism identified at the early ripening stages due to their tolerance to low pH. Yeasts are also strong utilizers of lactic acid causing a decrease in acidity and promoting the growth of bacteria whose proteolytic and lipolytic activities are essential for cheese ripening (49). Several studies have shown coryneforms comprised of *Arthrobacter*, *Corynebacterium*, *Microbacterium*, *Rhodococcus*, and *Brevibacterium* as the dominant bacteria found on smear ripened cheeses (21). There is evidence that other genera such as *Staphylococcus* and *Micrococcus* also play a role in ripening (28) and *Staphylococcus* being the major organism found in the early ripening stages being replaced by coryneform bacteria only at later stages (10, 11)

1.1.1 Yeast

The low pH, relative low moisture, high salt content and low temperature promote the growth of yeast on the surface of smear ripened cheeses. Yeast grow early in the ripening stages metabolizing lactate, deaminating amino acids thereby, contributing to the de-acidification of cheese. *D. hansenii* is the dominant yeast isolated from virtually all cheeses such as Tilsit, Romadour Limburger (51). The other important species are *Yarrowia lipolytica*, *Kluyveromyces lactis*, and *G. candidum*. Yeast contribute positively to flavor and texture development through enhanced enzyme activity resulted from an increase in surface pH. The increase in pH modifies the rheological properties of the cheese resulting in a soft body which is a typical characteristic of most type of this cheese (20). Yeast also produce stimulatory growth substances which appear to promote the growth of certain bacterial species (147). According to studies performed on Limburger cheese, yeast produce a substantial amount of panthotenic acid, niacin, and riboflavin which were shown to promote the growth of *B. linens* (20).

1.1.2 Coryneforms

Coryneforms are Gram and catalase positive, non-spore forming and generally nonmotile rods. Exponentially growing cells are pleomorphic, showing the presence of irregularly shaped rods including wedged, club and curve shaped. Coryneform bacteria (*Arthrobacter*, *Brevibacterium*; *Corynebacterium*, *Brachybacterium*, and *Microbacterium*) are comprised of various taxa belonging to the Actinomycete branch of Gram-positive bacteria. Coryneforms are ubiquitous in nature tolerating high salt concentration therefore, numerous species have been isolated from cheese samples particularly from Tilsit (20). As seen by *Brevibacterium linens,* coryneforms cannot grow under pH values of 6 (15, 41). *B. linens* is thought to be one of the most important ripening bacteria found on cheeses due to its proteolytic and lypolytic activities and biochemical characteristics that influence ripening and final characteristics of smear ripened cheeses (123). Other coryneform species have been isolated aside from *B. linens* such as *Corynebacterium ammoniagenes*, *Corynebacterium variabile*, *Corynebacterium casei, Microbacterium gubbeenense*, *Arthrobacter nicotianae*, and *Brachybacterium alimentarium* (13, 21, 136, 144). *Corynebacterium* spp. are the major components on bacterial smear ripened cheeses (13, 21, 100, 112). They are important in cheese ripening due to their production of sulphur containing compounds such as methanethiol which is an important flavoring compound found on Tilsit, Limburger and Appenzeller (19).

1.1.3 Staphylococci and micrococci

There is evidence that beside the coryneforms other genera such as *Staphylococcus* and *Micrococcus* play an important role in ripening (11, 28, 72, 144). *Micrococcus* is a high-GC content organism and is included in the actinomycetes branch of the eubacteria, while staphylococci have a low GC content and are included in the clostridial branch.

Staphylococci are more important than micrococci, with *S. equorum*, *S. xylosus S. saprophyticus*, and *S. caseolyticus* being the dominant species isolated from cheese (20). Among different French and German cheeses, *S. equorum* was the dominant bacterium isolated from the *Staphyloccoccus* spp. (13, 28). Due to their tolerance to high salinity staphylococci are the major organism found early in ripening and are replaced by coryneforms some days later (21). The most dominant micrococci appear to be *Kocuria varians* and *Micrococcus luteus.*

1.2 Natural adaptation of cheese ripening bacterial strains to low pH

Traditionally, cheese production involves the transfer of ripening flora from mature ripened cheeses to young cheeses. This process enables the transfer of desirable

microorganisms but it can also transfer undesirable pathogens such as *L. monocytogenes* which can develop on the cheese surface despite low temperature and pH. Initial acidity of the cheese slow the development of the ripening microbial flora Therefore, there is great interest from dairy industries to accelerate the ripening process and producing a biologically safe product. In the next sections techniques developed to accelerate cheese ripening and classical strain improvement will be presented.

1.2.1 Acceleration of cheese ripening

Cheese ripening involves a complex series of microbiological and biochemical events which result in the development of flavor and texture characteristics of each variety (143). For these reasons cheese ripening is slow and consequently an expensive process.

There have been numerous attempts to accelerate cheese ripening using non recombinant techniques. One of them was elevating ripening temperature. Although ripening temperature is the most important single factor determining flavor intensity, relatively few studies have been conducted on the effect of elevated temperature on cheese ripening. Ripening temperature influences the rate of proteolysis, lypolysis, cheese microflora, texture and quality of cheese. Ripening at elevated temperatures up to 15°C has been recommended for some cheeses like Cheddar. The drawback of this approach is an increasing risk of spoilage and non specific ripening reactions leading to the production of off flavors (157).

Another method is the addition of exogeneous proteolytic enzymes to cheese, increasing the enzyme pool which eventually helps in accelerating the rate of certain reactions in cheese. However, there are certain limits to enzyme addition as a method for accelerating enzyme ripening due to legal restrictions in the use of such enzymes in cheese making. Adding these enzymes to cheesemilk directly is the best stage for their incorporation due to homogeneous mixing of the enzyme with milk. The downside of this method is that most of the enzymes degrade casein to peptides thereby decreasing the casein matrix leading to difficulty in curd handling (89). Addition of micro-encapsulated enzyme in a phospholipids liposome capsule is a better alternative, however there are high cost associated in liposomes production making its application on large scale not feasible (143). The use of commercially available enzyme preparations like Accelase, or Neutrase showed that cheese ripening time is decreased in half. However, in using Neutrase flavor defects were reported due to excessive proteolysis (89).

The use of attenuated starters together with primary starters provides an alternative means of increasing enzyme pool in cheese curd without the drawback of exogenous enzyme

addition. In addition to accelerating ripening, recent work has shown the potential of attenuated starters in modifying flavor (84).

Starter lactococci contain proteinases and peptidases which degrade large peptides derived from casein into small amino acids (52). During ripening this microorganism die and lyse releasing these enzymes. Given to this fact another possible mechanism for acceleration of cheese ripening is the autolysis of starter bacteria owing to the fact that lysis of starter cells releases enzymes into the cheese matrix resulting in high levels of free amino acids and reduction in bitterness due to the breakdown of hydrophobic peptides by peptidases. Autolysis is strain dependent and can be accelerated by environmental factors such as temperature, salt concentration and lactose depletion (40) .

The use of bacteriocins or bacteriocin producing starters in cheesemaking is a novel approach for accelerating lysis (101). This involves the use of adjunct starter cultures containing a plasmid encoding gene for bacteriocins.

In recent years attention has been focused on genetic engineering as a means of producing starters expressing intracellular enzymes such as PepC, PepN, PepO, which play a role in secondary proteolysis in cheese during ripening. Up to now several genetically engineered strains have been created containing different enzymatic genes such as *Lc. lactis*, *Lb. delbruechi* with reported increases in amino acid production during ripening as compared to the wild type strain (30, 31). In the reported studies genetically modified organism seems to be a very promising method for acceleration of ripening through the increase in the uniform distribution of the enzyme pool throughout the cheese matrix. However, legal barriers, consumer concern, and lack of knowledge of the importance of key or limiting lactococcal enzymes in cheese ripening are the stumbling block to the successful adoption of genetically modified starters in industrial settings.

1.2.2 Classical strain improvement

Genetically modified organisms present a reliable approach in cheese production due to technologically high performing bacterial strains, possessing excellent organoleptic characteristics and facilitating product innovation not possible to achieve with classical techniques. However, classical methods can be applied readily in food industry whereas, bioengineered organisms cannot.

Classical strain improvement involves genetic modification through natural adaptation without direct human intervention. There are three ways that genetic modification through natural adaptation can occur; (1) small local changes in the nucleotide sequence of the genome, (2) intragenomic reshuffling of genomic segments of genomic sequences, (3) and the

acquisition of DNA sequences from another organism (3). Most of the strain improvement studies have dealt with the first method of spontaneous mutation due to its ease in selection.

Spontaneous mutation has been extensively performed in lactic acid bacteria such as *Lb. bulgaris*. *L. bulgaris* is important in yogurt production by contributing to the typical yogurt flavor and lowering pH values below 4.2. This acidification leads to the gradual increase in acid and bitter taste of yogurt. In order to control this post acidification step it is necessary to regulate the growth and development of *L. bulgaris* by controlling its energy metabolism. For this reason *L. bulgaris* starter strains were screened for the presence of spontaneous Lac minus mutants exhibiting little or no β-galactosidase activity. Such mutants were unable to grow in milk as single strain without the addition of glucose and peptones. However, when grown in mixed culture with *S. thermophilus*, *L. bulgaris* was able to grow without the addition of glucose. This resulted in a mild and non post acidified yogurt product (57, 104).

Lactic acid bacteria are often used in a concentrated frozen or freeze dried form, but freezing causes a decrease in cell viability. A study has shown that their resistance to freezing can be increased by performing successive cycles of subcultivation of freezing and thawing through natural adaptation (105).

1.2.3 Real Time PCR

Conventional culture based methods are time consuming, offer a poor sensitivity, are open to interpretation errors and mostly depend upon the growth of the organism. Therefore, in the last ten years numerous technologies have been developed to ease and accelerate the enumeration and identification of microbial organisms. Polymerase chain reaction (PCR) has been increasingly used in diagnostic and microbiology laboratories as a standard in detection and identification of microorganisms (97) offering the advantage of being a fast, reliable and reproducible method that is easy to handle. Real time PCR is a kinetic based identification technique with the added advantage of online monitoring of amplicon generation thereby eliminating post-amplification manipulations (26). Real time monitoring is achieved by labeling either, primers, or oligonucleotide probes or the amplicon with fluorescent dye molecules. These labels produce a signal change followed by hybridization to the amplicon. The transmitted signal is correlated to the amount of amplicon present at each cycle and will increase as the amount of amplicon increases.

Amplicon detection involves the use of fluorescent dyes such as SYBR Green I that bind to double stranded DNA. The dye exhibits no fluorescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA (25). An increase in the fluorescence signal can be observed during the polymerization step (Fig. 1.1).

Fluorescence is measured at the end of the elongation step of every PCR cycle to monitor the increase of DNA. The specificity of the reaction is determined solely by the primers.

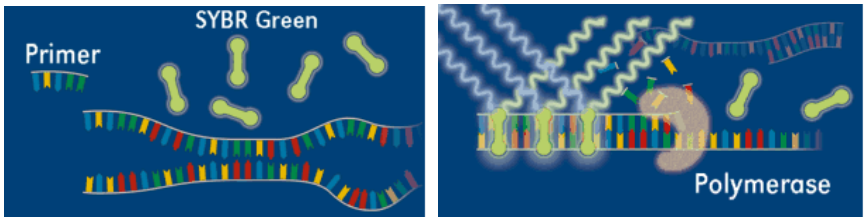


Fig. 1.1: Chemistry behind SYBR Green I detection.

In solution SYBR Green I exhibit no fluorescence. During elongation increasing amount of dye molecules bind to the minor grooves of the double stranded DNA. The increase in signal is observed during polymerization**.**

The production of unspecific products is analyzed through the creation of an amplicon melting curve. In this case the temperature is slowly increased above the melting of the amplicon and fluorescence is measured. As the melting depends upon the nucleotide composition of the amplicon, it is possible to identify the signal obtained from the correct product which will distinguish it from amplification artifacts that melt at other temperatures.

In the last ten years there has been a tremendous increase in the usage of real time PCR as identification tool in molecular diagnostics fields. The detection and enumeration of pathogenic bacterial species in diagnostic samples is performed routinely in clinical fields allowing a rapid report of patient infection status thereby induced a rapid response in treatment. Detection of pathogenic bacteria is also important in the qualitative assessment of food products. There have been numerous studies performed in quantifying and detecting various food pathogens such as *L. monocytogenes* (69, 111), *Staphylococcus aureus* (70), and *Salmonella* species (47, 54), oral pathogens such as *Streptococcus mutans* (160), *Porphyromonas gingivalis* (96), and fermentative organism such as *Bifidobacterium* (27).

1.3 pH adaptation of *Corynebacterium glutamicum*

Bacteria periodically encounter life threatening stresses in a variety of hostile and natural situations. For example in food processing bacteria encounter stresses such as heat treatment during pasteurization, freezing, dehydration, salt treatment and production or addition of acid (23).

One of the most widely encountered environmental factors by microorganism is acid stress. Challenges due to an acidic environment are experienced by both pathogenic and fermentative microorganisms. Pathogenic organisms encounter a low pH environment while passing through the gastrointestinal tract, in the oral cavity as well as in macrophage

phagosomes (32). Fermentative organisms are potentially exposed to low pH, e.g., during food processing or in anoxic environments. Therefore, mechanisms allowing successful adaptation of microorganisms to low pH are essential for survival and proliferation. Acid adaptation is also an important factor in the virulence of pathogenic microorganisms and has significant implication in food safety.

To increase survival chances due to sudden changes in pH, bacteria turn on a programmed mechanism that includes the synthesis of stress inducible proteins which lead to the regulation of gene expression (50). An adaptive acid response generated by moderately low pH also enables bacterial cells to survive extreme acidity. This adaptive process, termed acid tolerance response (ATR), has been extensively studied in a number of pathogenic and gastrointestinal organism, namely *Escherichia coli* (29), *Salmonella typhimurium* (6), *Helicobacter pylori* (151), *Listeria monocytogenes* (44), *Lactococcus lactis* (53), *Propionibacterium freudenreichii* (77), and *Streptococcus mutans* (91). The mechanisms used in acid stress response are well described in gram negative organism. However, there is limited knowledge available about acid stress response mechanism in Gram positive organisms such as *Corynebacterium glutamicum*.

1.3.1 Acid stress response

Acid stress includes the combined effect of low pH caused by inorganic acids and organic acids present in the environment (7). Uncharged organic acids can passively diffuse across the cell membrane and dissociate in the cytoplasm into negatively charged molecules and protons, which can permeate the cell membrane, thereby lowering the pH of the cytoplasm (145). The lower the external pH, the more undissociated organic acid is available to cross the cellular membrane to affect cytoplasmic pH (7). The maintenance of cytoplasmic pH homeostasis under such conditions is crucial for the uninterrupted function of the bacterial cell. Deviations of more than one pH unit from the optimal cytoplasmic pH cause considerable changes in cellular functions, damaging enzymes and DNA integrity (50). Therefore, the cell induces the synthesis of proteins which aid in the maintenance of pH homeostasis as well as in preventing and repairing damages caused by acid stress.

As a result bacteria have evolved adaptive strategies to minimize acid stress by utilizing different as well as overlapping approaches for coping with acid stress (50). Some systems attempt to alkalinize the cytoplasmic pH via F_0F_1 ATPases or specific amino acid antiport system. Another way for bacterial cells to aid in the acid adaptation process is the synthesis of general stress response proteins that control the transcription of numerous stress response genes.

1.3.2 Maintenance of cytoplasmic pH

One of the well described mechanisms in maintaining pH homeostasis is the F_1F_0 ATPase which can either synthesize ATP using the proton gradient or it can expel protons from the cell using the energy obtained from ATP hydrolysis (32). In aerobic organisms, such as in *E. coli* and *B. subtilis*, the F_1F_0 ATPase mainly functions in ATP synthesis (102). In organisms without a respiratory chain such as *L. lactis* (86), *Enterococcus hirae* (85), *S. mutans*, *S. sanquis* (9) *Lactobacillus acidophilus* (88) and *L. monocytogenes* the F_1F_0 ATPase system plays an important role in maintaining cytoplasmic pH homeostasis in acid adaptation via proton extrusion (55, 79).

In addition to the F_1F_0 ATPase, cation transport ATPases such as K^+ ATPases contribute to pH homeostasis through the exchange of K^+ for H^+. In a study performed with *S. mutans* using glucose energized cells it was observed that at an outer pH of 5 the cytoplasmic pH in the absence of K^+ was 5.5. However, by the addition of 25mM K^+ the cytoplasmic pH increased to 6.14 (33). Similar observations were observed in *L. lactis* (78) and *E. hirae* (4).

1.3.3 Glutamate decarboxylase

Amino acid decarboxylases also play an important role in controlling pH homeostasis by consuming hydrogen ions in the process. Examples of these reactions include lysine, arginine and glutamate decarboxylases which function by combining an internalized amino acid (e.g. lysine, arginine, or glutamate) with a proton and exchanging the resulting product (e.g. cadaverine, agmatine, or γ- aminobutyrate i.e. GABA) for a new substrate (32).

The glutamate decarboxylase system is one of the most studied among gram-positive organism due to its presence in *L. monocytogenes* a gastrointestinal pathogen (33) and in fermentative organisms such as *L. lactis* (145). According to studies, the glutamate is taken up by specific transporter into the cell where it undergoes decarboxylation resulting in the consumption of an intracellular proton. The product, GABA (γ- aminobutyrate) is transporter from the cell via an antiporter. The net result is an increase in the pH of the cytoplasm due to the removal of hydrogen ions and a slight increase in the extracellular due to the exchange of extracellular glutamate for the more alkaline GABA.

1.3.4 Repair mechanisms

A number of proteins that play a role in the protection or repair of macromolecules such as DNA and proteins are essential for optimal acid resistance. One such protein, RecA, acts as a mediator of homologous recombination. A decrease in cytoplasmic pH leads to the

loss of purine and pyrimidine from DNA at a greater rate than at neutral or alkaline pH. This involves protonation of the base involved followed of cleavage of the glycosyl bond. The residues left at sites of base loss are referred to as AP (apurinic, apyrimidinic) sites whose repair is initiated by AP endonucleases (120, 150).

Another enzyme that functions in DNA damage repair is UVrA (131). It has been demonstrated in *S. mutans* that UVrA mutants were more sensitive to pH 5 than wild type cells, but were unable to survive at pH 3. It was suggested that exposing the cells to these harsh conditions resulted in more DNA damage being evident in the mutant than in the wild type. Therefore, it is said that AP endonuclease function to repair minor DNA damage, whereas larger DNA lesions caused by acid and other DNA damaging agents is performed by UVrA (64).

Chaperones intervene in numerous stresses for various tasks such as protein folding, renaturation, and protection of denatured proteins. Induction of chaperones due to acid stress has been demonstrated in numerous organisms such as *S. mutans*, *L. lactis* (67, 78). Chaperones such as DnaK and GroEL are induced following acid adaptation in *Lactobacillus delbrueckii* (93), while GroEL is induced in response to acid in *Clostridium perfringes*, *S. mutans* and *L. monocytogenes* (115, 148, 156). In addition to the protective roles of chaperones the HtrA protein is proposed to be involved in proteolysis of abnormal proteins synthesized under stress conditions.

1.3.5 Changes in cell membrane composition

It has been demonstrated in bacteria experiencing acid stress that the cell membrane undergoes changes in fatty acid profile. *S. mutans* grown at pH 5 demonstrated increased levels of monounsaturated and long chain fatty acids compared to cell grown at pH 7. Acid adapted *L. monocytogenes* were more tolerant to nisin and other ionophores than non adapted cells. For this effect has been suggested that the increase in production of straight chain fatty acid of C_{14} and C_{16} as well as the decreased C_{18} levels associated with acid adaptation may be responsible for this enhanced resistance to bacteriocin.

1.3.6 Arginine deiminase

Arginine deiminase pathway (ADI) has been identified in a variety of microorganism such as lactic acid bacteria, Pseudomonads, *Bacillus* spp. catabolizing arginine to ornithine, ammonia and CO_2 (34). The system has three enzymes; ADI, ornithine transcarbamylase and carbamate kinase encoded by arcA, arcB and arc, respectively. The three enzymes appear to be inherently acid tolerant, displaying activity at pH 3.1 and even lower in some species. It was

demonstrated that in bacteria such as *Lactobacillus sanfranciscensis* enhanced tolerance to acid stress assisted cell survival during storage at 7°C and favored production of ornithine, an important precursor of crust aroma compounds (36). The ADI system also acts as a virulence factor in the human pathogen *S. pyogenes*. A protein originally designated streptococcal acid glycoprotein and originally characterized as being an inhibitor of stimulated human peripheral blood mononuclear cell proliferation was ultimately identified as an ADI (37). Its role in acid resistance was demonstrated using mutants for the protein where a nine log survival was observed in the wild type cells after 6 h at pH 4. This acid sensitivity is likely to be responsible for the observation that this mutant demonstrated a reduced ability to enter and survive in epithelial cells.

1.3.7 Regulators

Regulation of gene expression in response to the extracellular environment is an adaptive response that is required for bacterial replication and survival. Alternative sigma factors have been demonstrated to play an important role in coordinating gene expression in bacteria undergoing cell stress.

sigB is an alternative sigma stress factor that has been identified in *L. monocytogenes*, *B. subtilis* and *Staphylococcus aureus*. It was initially discovered in *B. subtilis* as controlling the transcription of about 100 genes in response to environmental stresses (62, 158). A *B. subtilis* sigma B mutant exhibited 50-to 100-fold reduced ability to survive heat, ethanol, salt or acid shock as well as freezing, dessication, and exhaustion of glucose or phosphate (149).

In *L. monocytogenes*, a function of *sigB* has been demonstrated in response to several stresses such as oxidative stress, osmotic stress, carbon starvation and growth at low temperatures: e.g., a *sigB* null mutant exhibited a 1000 to 5000-fold decrease in survival when exposed to pH 2.5 (155).

Studies in *Brevibacterium flavum*, a closely related strain of *C. glutamicum*, showed an effect of *sigB* on growth and viability of cells under acid, salt, alcohol, heat and cold (61). *SigB* is therefore a general stress response protein and controls transcription of various stress related proteins in Gram positive bacteria.

sigE is an extracytoplasmic function sigma factor which regulates gene expression in response to the extracellular environment. The role of *sigE*'s influence on survival has been demonstrated in a number of mycobacteria such as *M. smegmatis*. A *sigE* mutant of *M. smegmatis* was more susceptible to killing at pH 4 (159).

Bacteria can also sense and respond to environmental changes through the use of two component signal transduction systems. Two components typically consist of a membrane

associated histidine kinase sensor and a cytoplasmic regulator. Two component systems function in bacterial adaptation, survival, and virulence by sensing environmental parameters and giving bacteria a mechanism through which they can respond. An increase in the expression of histidine kinase regulators due to stress has been shown in other gram-positive organisms, namely in *L. monocytogenes,* but a disruption mutant in the regulatory components of histidine kinase did not reveal differences in growth in presence of stress compared to the wild type strain (80). In *H. pylori* a similar effect was observed in studying the histidine kinase sensory component which demonstrated that deletion of the sensory component has no effect on the cells' function and its role can be overtaken by other genes (151). Similarly, in this study no significant effect was observed at low pH condition on the growth rate of the histidine kinase disruption mutant.

1.3.8 Corynebacterium glutamicum

Corynebacterium glutamicum is a gram positive biotechnologically important microorganism for its fermentative production of amino acids (92). It was first isolated in 1957 by Kinoshita as a natural L-glutamate producer (128). Due to its biotechnological importance high-producing strains for the production of L-glutamate and L-lysine have been generated through classical mutation and selection (129). With the advent of molecular biology methods for this organism targeted metabolic pathway engineering became possible which led to the rational improvement of *C. glutamicum* strain for the production of D-pantothenate, L-isoleucine, L-valine and L-threonine (128).

Recently, the sequencing of the *C. glutamicum* genome allows genome-wide expression analysis with DNA microarrays to unravel global regulatory mechanisms (139). Detailed information about the physiological state of the cell, the metabolic pathways and their regulation is indispensable for further increase of productivity and yields of already highly productive strains. During fermentative processes *C. glutamicum* is exposed to various stressors such as temperature, and oxygen supply which can affect fermentative production yields. Up to now there is very limited information available about the gene expression under heat and oxidative stress conditions (107) . The similarities between *C. glutamicum* and *M. tuberculosis* (also belonging to *Corynebacterianeae* genus) take into account that global regulatory circuits controlling general functions such as acid response are likely to be conserved and progress made for either of the different members of this genus could be easily transferred to another (152).

1.4 Microarray

In bacterial cells gene regulation occurs at the transcriptional level. The transcriptome is highly dynamic, it responds rapidly and dynamically to sudden perturbations in the environment. Initial gene expression studies were performed using conventional methods such as reporter fusions and in vivo expression technology which are limited in their usage and are labor intensive (71). Recently, an increase in the availability of genomic sequences paved the way for the development of new transcriptomic analysis techniques.

DNA microarray is one of the high throughput technologies allowing the monitoring of gene expression changes by screening thousand of genes at once in a single experiment. Microarrays also allow the detection of subtle changes in gene expression which is not possible with other molecular subtraction methods. The resulting gene expression patterns resulting from abrupt shift in the bacterial environment, analyzed through microarray, provide important information of potential biochemical pathway that occur in the cell (94).

A microarray is a slide made of glass or silicon to which DNA or oligonucleotide are attached (66). There are two basic types of microarrays; spotted microarray and high density oligonucleotide array. In spotted arrays genes are represented by DNA fragments of several hundred nucleotides in length and are printed onto glass slides. In oligo arrays, genes are represented by 15-20 different 25-mer oligonucleotides that are used as sequence detectors.

Microarrays use the same hybridization principle of nucleic acids as Southern and Northern blotting do, but two different samples are used, control and experimental, and are independently labeled with Cy3 or Cy5 fluorescent dyes. In gene expression studies mRNA samples are transcribed to cDNA and are fluorescently labeled during transcription. Labeling can also be performed after reverse transcription. The labeled samples are mixed and co-hybridized to the microarray slide contained the immobilized DNA target. The slide is scanned and the obtained red and green intensities are proportional to the amount of Cy5 and Cy3 labeled cDNA, respectively, hybridized to DNA at that spot (24). The quantitative ratio of the two dye intensities gives comparative information of gene expression in the experimental sample.

Microarrays have been successfully applied in various fields for numerous purposes. They have been used to study gene expression analysis in bacterial and eukaryotic species in response to stressors like starvation, acid and temperature (151). It has been used in the medical field to study interactions between host and parasitic organisms, identifying pathogenic virulence factors and in the discovery of new pathogens. They have been

successfully implemented in the therapeutic fields for cancer classification, determining therapeutic responses and in exploring signal transduction pathways and many other uses.

1.5 Aims of this work

The organoleptic properties of cheese are largely depended upon the development of the surface flora. Therefore it is essential to monitor it using appropriate methods. For long the ripening flora has been assessed using traditional cultivation methods. However, these methods provide biased information by identifying only the culturable fraction of the microflora. Quantitative real time PCR is a novel molecular method allowing the simultaneous detection of a variety of microbial species in one sample. Therefore, we used real time PCR to monitor the microbial succession of six microorganisms on a defined cheese agar.

The growth rate of the smear microflora on the cheese surface depends upon various environmental factors such as pH and temperature which in turn affects ripening rate. Numerous attempts were made to accelerate cheese ripening, however, to our knowledge, there have been no attempts made to adapt ripening strains to low pH which can accelerate ripening. Therefore, the second objective was to adapt the ripening bacterial species to low pH and to monitor their progression during ripening on cheese agar using real time PCR.

Fermentative microorganisms are exposed to acid stress under industrial conditions. However, there is very little information available about the regulatory networks expressed under acidic conditions. Therefore, the third task was to analyze the global gene expression profile of acid adapted *C. glutamicum* cells using DNA microarray.

2 Materials and methods

2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. The cheese ripening strains were obtained from a dairy company. *Escherichia coli* strain TOP10 (Invitrogen) in combination with plasmid pCR®2.1-TOPO® was used for cloning. Plasmid pWLQ2 was used in the transformation experiments involving *Corynebacterium glutamicum*.

Strain/plasmids	Relevant characteristics	Source/reference
Debaromyces hansenii	Cheese ripening strain	Dairy company
Corynebacterium ammoniagenes[a]	Cheese ripening strain	Dairy company
Staphylococcus equorum[a]	Cheese ripening strain	Dairy company
Kokuria palustris[a]	Cheese ripening strain	Dairy company
Staphylococcus warneri	Cheese ripening strain	Dairy company
Corynebacterium variabile[a]	Cheese ripening strain	Dairy company
Corynebacterium glutamicum		ATCC 13022
Escherichia coli TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80 *lac*ZΔ.M15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
pWLQ2	Ap^R, Km^R; *E.coli-C glutamicum* shuttle expressions vector; lac repressor regulated tac promoter	Liebl *et al.* (1992)
pCR®2.1-TOPO®	Ap^R, Km^R; cloning vector for PCR products with 3`-A-overhangs	Invitrogen

[a] denotes strains used in the pH adaptation experiment

Tab. 2.1: Summary of strains and the plasmids used in this work

Oligo-nucleotide	DNA sequence	Function/Feature	T_m °C[a]
16S F	AGAGTTTGATCCCTCA	Amplification 16S	54.5
16S R	CGGCTACCTTGTTACGAC	Amplification 16S	56
NS1 F	GTAGTCATATGCTTGTCTC	Sequencing/amplification 18S	52.4
SR 4 R	AAACCAACAAAATAGAA	Sequencing/amplification 18S	40.7
609V F	TTAGATACCCT(AG)GTAGT	Sequencing 16S	46.7
699R	GGGTTG(AGT)GCTCGTT	Sequencing 16S	44.7
YS 1-5F	TCTGGTGCCAGCAGCC	Strain specific primer *D. hansenii*	56.9
YS 2-5R	AAGTCCTGGTTCGCCAAA	Strain specific primer *D. hansenii*	53.7
BS 6-9F	GGTGGGGCGCTAGGTGTA	Strain specific primer *C. ammoniagenes*	57.6
BS 6-9R	TCTAGCCCRATCCGGTAC	Strain specific primer *C. ammoniagenes*	57.1
BS 12-16F	GTGTGGGGGTCTTCCACGA	Strain specific primer *C. variabile*	61
BS 12-16R	GGGAAAACGCATCTCTGC	Strain specific primer *C. variabile*	56
BS11F	GGCACTAGGTGTGGGGGA	Strain specific primer *K. palustris*	57.6
BS11R	TTCCAGAGATGGTTCTTCCC	Strain specific primer *K. palustris*	57.3
BS10F	TGTTAGGGGTTTCCGCC	Strain specific primer *S. equorum*	55.2
BS10R	AACTAAATGCTGGCAACTAAGT	Strain specific primer *S. equorum*	54.7
BS13F	TAAACGATGAGTGCTAAGTGTTA	Strain specific primer *S. warneri*	57.6
BS13R	AGACTCTATCTCTAGAGCG	Strain specific primer *S. warneri*	54.5
ORF1169F	GCCTCCTTAAACCTAGCGC	Primer used for gene mutagenesis	57.1
ORF1169R	GGCAAGTCCCAATTCGTG	Primer used for gene mutagenesis	56.5
ORF1170F	TTGGCGTCGCCGGATAT	Primer used for gene mutagenesis	60.2
ORF1170R	CGGGTGCCGAGGAAGTA	Primer used for gene mutagenesis	57
ORF1346F	GCAACGGCTTAGTCCC	Primer used for gene mutagenesis	50.9
ORF1346R	AGAAGTCGGATGATAGGTGA	Primer used for gene mutagenesis	51.6
ORF2920F	CACTGGATGCCGAACC	Primer used for gene mutagenesis	51.6

Oligo-nucleotide	DNA sequence	Function/Feature	T_m°C[a]
ORF2920R	TCCCTTGTTGCCTTCG	Primer used for gene mutagenesis	52
ORF3347F	TCAGCGACAATGAAGATG	Primer used for gene mutagenesis	49.3
ORF3347R	ACCCGACCAAACAGC	Primer used for gene mutagenesis	47.8
ORF3452F	TGCGGGTTTGGTTGTTT	Primer used for gene mutagenesis	54.8
ORF3452R	GCCATAAGGATAAGGGAAGTA	Primer used for gene mutagenesis	53.6
ORF3544F	CGCAAATGGTAGTGCTTAT	Primer used for gene mutagenesis	51.4
ORF3544R	CCCTGCCGTGTAGAAAC	Primer used for gene mutagenesis	50.9
ORF3549F	AACGGCTGCGGCAAAT	Primer used for gene mutagenesis	57.7
ORF3549R	GGGCGGCTTGGTTGAGA	Primer used for gene mutagenesis	59.6
ORF3550F	GCGATTACCCACTGTCTTTG	Primer used for gene mutagenesis	55.6
ORF3550R	GCGGGTGGAGTTTGTTTT	Primer used for gene mutagenesis	55.5
ORF3551F	AATCGTCGTGGCACTGGG	Primer used for gene mutagenesis	59.4
ORF3551R	CAGGGATGCAGCAAGGTC	Primer used for gene mutagenesis	56.4
ORF518F	GCGGTGGCGTAAATGAGC	Primer used for gene mutagenesis	59.7
ORF518R	GAATGTGAATCAACAGTTGGGAG	Primer used for gene mutagenesis	58.8
ORF857F	CAGGGTGCGAGCAGTCA	Primer used for gene mutagenesis	55.8
ORF857R	TCAACAGAACCGGAGGC	Primer used for gene mutagenesis	53.8
ORF927F	GCAGACAGTGCCACAGAGC	Primer used for gene mutagenesis	56.7
ORF927R	TGTTCACGGTTGACCAAAT	Primer used for gene mutagenesis	53.8
ORF1632F	CCAATGCGTGTTGCTGA	Primer used for gene mutagenesis	54
ORF1632R	ATCTTGCCGCCGAGGAC	Primer used for gene mutagenesis	58.7
ORF854F	GGCAACTTCGCATTCACC	Primer used for gene mutagenesis	56.7
ORF854R	CGCAGATGCTTGGGTCA	Primer used for gene mutagenesis	55.9
ORF2779 RT_F1	AGCCCTTCGTAAAGTCCC	Primer used for real time PCR	56

Oligo-nucleotide	DNA sequence	Function/Feature	T_m°C[a]
ORF2779 RT_R1	GATCCAACACGCCACAAC	Primer used for real time PCR	56
ORF854 RT_F	GAAACTTGCACCTCGTATGC	Primer used for real time PCR	57.3
ORF854 RT_R	TTCGATACTCGGTTTGAGCT	Primer used for real time PCR	55.3
ORF3549 RT_F1	ACATCGCCCACCAATACG	Primer used for real time PCR	56
ORF3549 RT_R1	TTGAACCATCTCGGCAGTT	Primer used for real time PCR	54.5
ORF1168 RT_F1	ACGGTGAATTGTTGATGGAA	Primer used for real time PCR	53.2
ORF1168 RT_R1	CCTGAACGTGGGTTGGAT	Primer used for real time PCR	56
ORF857 RT_F1	ATCAGCCACCAAGAACAAAC	Primer used for real time PCR	55.3
ORF857 RT_R1	GGTAAATTCGCCTCAGAACG	Primer used for real time PCR	57.3
sigB F	CGCAGGATCTCGCAACGA	Primer used for real time PCR	63.4
sigB R	GCCGATGCCGTTGAGGTAAA	Primer used for real time PCR	63.3
sigE_F	GTCCCGAGATGACGCACCCG	Primer used for real time PCR	67.9
sigE_R	GGCATGTCTGCCTGTCCAGC	Primer used for PCR	63.5
16S_F	GTAGGGTGCGAGCGTTGTCC	Primer used for real time PCR	63.5
16S_R	CGCCATTGGTGTTCCTCCTG	Primer used for real time PCR	61.4
ORF 2920	CCCGAAGCCGTAGAGCA	Insertion analysis	57.4
ORF 3452	TTTGATTTCCCTAACCC	Insertion analysis	49.7
ORF 518	CTGCGTGGGCAATGGG	Insertion analysis	58.9
ORF 3551	GACGGCGGTGGCGGTAGA	Insertion analysis	64.8
ORF 3550	CGCAGTTTCAGTGCAGAGG	Insertion analysis	57.2
ORF 857	CCTCGACAACGTCACCG	Insertion analysis	55.1
ORF 854	GCCCTCGCCTCACTTGCTT	Insertion analysis	62.5
ORF 3544	TATTAGCAGCCTCTGTCGC	Insertion analysis	53.9
ORF 1346	CCGATCAGCTCAAGTGG	Insertion analysis	51.4
ORF 1170	AGCCTTAGCCATCAACTCA	Insertion analysis	54.5
ORF1169	ATCAACTATCCGCCGAAGAA	Insertion analysis	55.3
ORF3347	GAGACTATCTGCGACTACATT	Insertion analysis	55.9
ORF3549	GAATAACCGCCTGACCATG	Insertion analysis	56.7
ORF1632	CACATCTACCGACCTTTGCG	Insertion analysis	59.4

[a] Annealing temperature calculated according to manufacturer MWG (Ebersberg)

Tab. 2.2: Summary of the oligonucleotides used in this work

2.2 Media and growth conditions

The cheese ripening strains as well as *C. glutamicum* were cultivated at 30°C in tryptic soy broth (TSB, BD, Heidelberg, Germany). For the acid adaptation experiments the pH of the medium was adjusted with the addition of 90% lactic acid (Merck, Ismaning, Germany) and the salt content was modified to a final concentration of 1.95% (Oxoid, Wesel, Germany) when necessary. Luria-Bertani (LB) medium was used for the incubation of *E. coli* cells at 37°C. All media were prepared using demineralized water, all buffers or solutions were prepared using MilliQ water.

LB broth (per l)

Trypton	10 g
Yeast extract	5 g
NaCl	8 g

pH 7.4

For plates add agarose 1.5 % (w/v)

Cheese agar plates (per 100 ml)

grated green cheese	30 g
NaCl	1.3 g
agar	1.5 g

pH 5 using 90% lactic acid

2.3 Natural adaptation of the bacterial species

The adaptation of bacterial species was performed using pH gradient plates. For the acid adaptation experiments the pH of the TSA^+/TSB^+ medium was adjusted by the addition of 90% lactic acid (Merck, Ismaning, Germany) and the salt content was modified to a final concentration of 1.95% NaCl (Oxoid, Wesel, Germany). In the adaptation process pH gradient plates were prepared using two separate layers in achieving the desired pH range. Three types of pH gradient plates were made: pH6-pH7, pH5.5-pH6 and pH5-pH5.5. In making the pH gradient plates 30 ml of TSA agar was poured in a quadratic Falcon® plate (BD, Heidelberg, Germany) raised at one end by a 2 ml glass pipette. After the bottom layer has solidified the plate was leveled and a second layer of 30 ml agar was poured. 5 ml of the overnight culture was 10,000-fold diluted and 100 µl were streaked onto pH6-pH7 gradient plates. The plates were incubated at 30°C for a period of 48h. Colonies growing at the lowest

pH were inoculated in 5 ml of TSB^+ medium acidified to the pH where growth was observed on the gradient plates. Cultures for which growth was observed were reinoculated onto a pH 6-7 gradient plate until growth was obtained at the lowest pH of the plate. At this point, the colonies showing growth in broth at the same pH were streaked onto the next gradient plate of pH 5.5-6. The same procedure was repeated until the final gradient plate concentration of pH 5-5.5 was reached.

2.3.1 Determination of phenotypic characteristics of the adapted cheese ripening mutants

The growth characteristics of the adapted strains were determined using the honeycomb well reader Bioscreen C (Thermo Labsystems, Dreieich, Germany) at two temperatures, 30°C and 15°C, and stability of the mutants was determined at 30°C.

The potential mutants were inoculated in 5 ml TSB^+ medium adjusted to the pH that they were adapted to, and allowed to grow at 30°C for 48h. The cell suspension was 100 fold diluted and 10 µl were used to inoculate the honeycomb well plates which contained in each well 240 µl of neutral medium or medium acidified to the mutants' pH in 5 replicates. The wild type strain of the corresponding mutant species was used as control which was grown at neutral TSB^+ at 30°C for 24h. One ml of cell suspension was centrifuged for 2 min at 13,200 rpm (Eppendorf). The pellet was resuspended at the acidic pH of the corresponding mutant and the above mentioned amount was used to inoculate the honeycomb well plates. The plates were incubated with continuous shaking at 30°C and OD_{600} measurements were obtained every 30 min for 24 h and at 15°C every hour for 48h.

The stability of the mutants was tested by incubating the mutants and the wild type strains in neutral TSB^+ medium at 30°C for 24h as above.

2.4 Monitoring of cheese ripening consortium on model cheese by real time PCR

2.4.1 Sequencing of cheese ripening strains

Strains were grown on TSA supplemented with 2% glycin (Oxoid, Wesel, Germany), to weaken the cell wall, for 24h at 30°C. Cells were washed off from the agar plate in 1 ml sterile water and transferred to a 2 ml Eppendorf tube. DNA was released from cells suspended in 1 ml sterile water (Millipore distilled, deionized) through a freeze/thaw procedure by cycling the suspension between rapid freezing at –20°C and rapid thawing at 65°C 4 times. For amplification of rDNA of the bacterial species, universal primers were chosen to amplify 1500bp of the 16S rDNA. The 5' forward primer is located at positions 8-

26 in the *Escherichia coli* numbering system, and the 3' reverse primer of position 1511-1493 in the *E. coli* numbering system (22). For the yeast, previously published primers by White, et al. (154) were used to amplify 800bp of the 18S rDNA. With these primers, 50 pmol/µl, 50 µl PCR reactions were performed using 7.5 µl of the cell lysates. Twenty nine µl MilliQ water, 4.5 µl 10X buffer (15 mM $MgCl_2$, 500 mM KCl, 1 mg/ml gelatine, 100 mM Tris/HCl pH 9) and 2 µl dNTP (5 mM, Promega, Mannheim, Germany), 2 µl 60% sucrose (Sigma, Osterode, Germany), 3 µl Kreosol red (1.5 mg/ml), 0.4 µl Taq polymerase dilution buffer (Promega, Mannheim, Germany) and 0.1 µl Taq polymerase (5 U/µl, Promega, Mannheim, Germany). PCR was performed under the following conditions; denaturation at 95°C for 3 minutes followed by a period of 35 cycles with a 15 seconds denaturation at 95°C, annealing for 30 seconds at 55°C for bacteria and 45°C for yeast, elongation at 72°C for 2 minutes and final extension at 72°C for 5 minutes. The products were verified by 1% agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen Hilden, Germany) according to manufacturer's recommendation. Sequencing primers were chosen to sequence 800-1000 bp of the 16S rDNA: 609V forward *E. coli* numbering 788-804 and 699R reverse, *E. coli* numbering 1114-1100 (95). For sequencing the yeast 18S rDNA, the same primers were used as for amplification. Sequencing was performed by Sequiserve (Vaterstetten, Germany). Based on the results specific primers for each individual strain were designed.

2.4.2 Separation of DNA fragments by agarose gel electrophoresis (AGE)

To prepare a 1 % gel, 0.5 g agarose (SeaKem LE, FMC BioProducts) were dissolved in 50 ml 1 x TAE buffer using a microwave. The hot agarose solution was poured into a gel tray with slot-formers. Five µl of DNA containing samples were mixed with 1 µl 6x gel loading buffer (GLB) Blue/orange 6x loading dye (Promega, Madison, USA) and pipetted into the gel slots. Additionally an appropriate molecular weight marker was loaded. After electrophoresis, the gel was stained with ethidium bromide (EtBr, 0.5 µg/ml) for 30-45 min and visualized by UV-translumination using the ImageMaster VDS (Pharmacia Biotech, Freiburg, Germany). Electrophoresis was performed using the following equipment: GNA-100 submarine electrophoresis unit (Pharmacia Biotech, Freiburg, Germany); Easy-CastTM Electrophoresis system, Model #B1A (Owl Scientific, Inc.); Electrophoresis Power Supply EPS 600 (Pharmacia Biotech, Freiburg, Germany).

TAE (Tris/acetate/EDTA) electrophoresis buffer 50 x stock solution:

2 M Tris-base

1 M NaAc

50 mM EDTA

(pH 8.0)

Gel loading buffer: (GLB 6x)

0.10 % Xylen-Cyanol FF

0.25 % Bromophenolblue

0.20 % SDS

5 mM EDTA

50% (v/v) Glycerol

(pH 8.0)

2.4.3 Inoculation of model cheese with the ripening strains.

0.5 ml of overnight cultures of individually inoculated ripening bacterial strains (adapted and wild type) were spread onto TSA plates and incubated at 30°C for 24h, only the yeast strain *D. hansenii* was incubated for 48 h. The cells were scraped off from the plate, resuspended in 3 ml 1.26 % sterile NaCl, 10-fold diluted, and OD_{600} was measured using the Biotech Ultrascope 2000 (Pharmacia, Freiburg, Germany). Based on this information, a mixture was produced containing 10^8 CFU/ml for 5 of the bacteria and 10^4 CFU/ml of *D. hansenii*. 500 µl of this mixture was used to inoculate the cheese agar plates. After inoculation, the plates were placed in a desiccator (containing 1l of saturated sterile KCl solution (Sigma, Osterode, Germany) to provide 90% relative humidity at 15°C and incubated for 11 days. The plates were sampled seven times during the ripening process. The surface of a 3 cm diameter area of the agar plate was scraped off using a sterile inoculation loop. The removal of the surface samples was followed by DNA isolation and by real-time PCR analysis.

2.4.4 Isolation of genomic DNA from cheese ripening consortium

The isolation of genomic DNA was performed using the Nucleobond Tissue kit (Macherey Nagel, Düren, Germany) with modifications. Shortly, 5 ml of the overnight cultures were harvested by centrifugation at 13,000 rpm for 3 minutes. The pellet was resuspended in 400 µl T1 buffer and cells were lysed in a Ribolyser (Hybaid, Heidelberg, Germany) using a 1:1 mixture of 0.1mm and 0.5mm diameter zirconia/silica beads (Roth, Karlsruhe, Germany) for 45 s at 6.5 m/s three times. The cells were cooled on ice between the runs. Finally, tubes were centrifuged for 2 min at full speed and supernatant was removed into a fresh tube. At this point the protocol was followed according manufacturer's recommendation. The same method was implemented for isolating DNA from the cheese samples.

2.4.5 Generation of a cell count standard curve

Overnight cultures of the ripening strains were 10-fold serially diluted in TSB. Five ml were centrifuged at 5000 x g for 10 min. DNA was isolated as previously described and cell counts on plate have been compared to the threshold cycle of the real time PCR.

2.4.6 Real-time PCR monitoring of the microbial consortium

All reactions were performed using the iCycler (Bio-Rad, Germany). For a 25 µl reaction 12.5 µl ABsolute™ QPCR SYBR® Green Fluorescein mix, (ABgene, UK) 0.5 µM of each primer and 2 µl of DNA were used. The cycling conditions were 40 cycles of 95°C for 15 s, 57°C for 15 s, and 72°C for 20 s preceded with a 15 min enzyme activation at 95°C. Data collection was performed at the elongation step of the reaction. Data were analyzed using the Bio-Rad iCycler software version 3.0.

2.4.7 Inoculation of model cheese with the acid adapted species

The inoculation of the model cheese with the adapted species was performed as described in section 2.4.3 followed by real time PCR monitoring as described in section 2.4.6.

2.5 Microarray analysis of acid adapted *Corynebacterium glutamicum*

2.5.1 Isolation of genomic DNA from *C. glutamicum*

The DNA from *C. glutamicum* was isolated using a previously published protocol by Tauch *et al.* (140). Ten ml of overnight culture was harvested by centrifugation. The pellet was resuspended in 3 ml B1 solution containing 20 mg/ml lysozyme (Promega, Mannheim, Germany) and incubated at 37°C for 2 h. The cells were lysed with the addition of 200 µl 20 % (w/v) SDS. The lysate was further incubated at 37°C with the addition of RNAse A and 5 mg Pronase (Serva, Heidelberg, Germany). DNA was extracted 2x with phenol/chloroform (1:1) [v/v]. DNA in the sample was precipitated by the addition of 0.3 M sodium acetate (pH 5.2, 0.1 volume of 3 M) along with 2 to 2.5 volumes of ethanol (-20°C) and incubated for 30 min at -20°C. The sample was then centrifuged at 4°C (maximum speed) in a microcentrifuge (Sigma) and washed once with 70 % ethanol. After a second centrifugation the pellet was left to dry and finally resuspended in 300 µl MilliQ water.

B1 solution

25 mM Tris-HCl

10 mM EDTA

50 mM glucose

2.5.2 Measurement of cytoplasmic pH

Internal pH was determined with the fluorescent pH indicator 2`, 7`-bis- (2-carboxyethyl)-5[and-6]-carboxyfluorescein (BCECF) as described by Negulescu *et. al.* (109) with some modificatons.

Six ml of *C. glutamicum* suspension grown in TSB at pH 7.5 was harvested at exponentional phase (OD_{600} = 0.5) by centrifugation (5000 rpm, 22°C, 3 min). The cells were washed two times with 50 mM potassium phosphate buffer at pH 7.5 and resuspended in 3 ml of the same buffer. Thirty µl of lipophylic acethoxymethyl ester of BCECF (BCECF-AM) dissolved in DMSO (0.1mM) was added to the cell suspension (in 1µM end conc.), which was then shaken at 30°C, 200 rpm in the dark for 30 minutes. The cells were harvested by centrifugation, washed two times with phosphate buffer and resuspended in phosphate buffer energized by 50 mM glucose at appropriate pH value.

A fluorescence excitation of intracellular BCECF was recorded using VICTOR™ multilabel counter (EG&G Wallac, Turku, Finland). The dual-excitations wavelengths for BCECF were at 450 nm (pH independent isobestic point) and at 490 nm and the emission wavelengths were at 535 nm measured over time intervals of 1 s. The normalization with the fluorescent intensity ratio at the isosbestic point eliminated the fluorescent measurement artefacts including photobleaching, leakage and non-uniform loading of the pH-indicator. *In situ* calibration was performed by using a mixture of ionophores: nigericin, gramicidin and valinomycin to the final concentration of 10, 20, and 10 µM, respectively, to equilibrate the intracellular pH with the controlled extracellular medium between pH 3.5 and pH 7.5. The cytoplasmic pH was estimated using a calibration curve obtained from the normalized emission values correlated to the outer pH values.

2.5.3 Fermentation

Continuous cultures of *C. glutamicum* ATCC 13032 were grown in a 2 l turbidostat fermentor (Biostat B; B. Braun Biotech, Melsungen) at 30°C. The oxygen concentration was kept at 50% of saturation by continuous aeration (2 l/min) with a mixture of air and nitrogen. The fermentor was inoculated with 2% overnight culture. Once the optical density at 600 nm reached 0.5 it was kept constant by continuous addition of TSB. During the fermentation the pH was corrected by addition of 1 M NaOH and 1 M lactic acid, respectively. Samples (50 ml) were drawn after five generations of growth at pH 7.5 and pH 5.7; respectively. The generation times were calculated from the consumption of medium. Cells were harvested by

centrifugation with 5000 rpm for 5 minutes at 4°C. The pellets were frozen in liquid nitrogen and stored at -70°C until RNA extraction.

2.5.4 Generation of *C. glutamicum* DNA microarray

DNA microarrays based on PCR products of *C. glutamicum* were generated for use in global gene expression. The genes were amplified in 96-well plates with genomic DNA of *C. glutamicum* ATCC 13032 as the template and gene-specific primers purchased from degussa (Frankfurt, Germany). The identities and quality of the PCR products were checked by gel electrophoresis, and the PCR products were precipitated with isopropanol, resuspended in 3x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and transferred to 384-well plates as described previously (75). The PCR products were printed onto poly-L-lysine-coated glass slides by using an arraying robot. The DNA microarrays were rehydrated in a 1x SSC atmosphere, UV cross-linked (650 μJ), and blocked in 230 ml of methyl pyrrolidinone containing 15 ml of 1 M boric acid (titrated to pH 8.0 with sodium hydroxide) and 4.4 g of succinic anhydride. The *C. glutamicum* whole-genome DNA microarray contained 3,673 PCR products covering 2,860 of the 2,994 genes (506 genes in duplicate) described for the genome according to the National Center for Biotechnology Information (NCBI) and 284 additional putative coding sequences (23 sequences in duplicate). In general, the PCR products were 500 ± 50 bp long and represented regions of the genes which facilitate specific hybridization. Additionally, 100 spots of *C. glutamicum* genomic DNA were used as normalization controls, and 16 spots of λDNA, 16 spots of *E. coli* DNA, and one spot of the *E. coli aceK* gene were used as negative controls.

2.5.5 Total RNA preparation and cDNA synthesis

Aliquots (50ml) of exponentially growing (OD_{600} ~ 0.5) *C. glutamicum* cultures were harvested by centrifugation (5 min; 3,500 x g; at RT). The cells were resuspended in 350 μl of RNeasy RLT buffer (Qiagen, Hilden, Germany) and mechanically disrupted for 30 s of bead beating with 0.5 g of 0.1-mm-diameter zirconium-silica beads (Roth, Karlsruhe, Germany) using a Silamat S5 (Vivadent, Ellwangen, Germany). After centrifugation (2 min; 14,500 x g), the supernatant was processed using the RNeasy system (Qiagen, Hilden, Germany) with on-column DNase treatment according to the manufacturer's instructions for RNA extraction. The quantity and quality of the extracted total RNA were determined by UV spectroscopy (at 260, 280, and 230 nm) and visually by denaturing formaldehyde agarose gel electrophoresis (116).

Identical amounts (20 to 25 μg) of total RNA were used for random hexamer-primed synthesis of fluorescently labeled cDNA by reverse transcription with Superscript II (GibcoBRL-Life Technologies, Gaithersburg, Md.) using the fluorescent nucleotide analogue FluoroLink Cy3-dUTP (green) or Cy5-dUTP (red) (Amersham Pharmacia, Little Chalfont, United Kingdom) as described before (83, 116, 153). The labeled cDNA probes were purified and concentrated using Microcon YM-30 filter units (Millipore, Bedford, Mass.).

2.5.6 DNA microarray hybridization and washing

Combined Cy5- and Cy3-labeled cDNA probes containing 1.2 μg of poly(A) (Sigma, Taufkirchen, Germany)/μl as a competitor, 30 mM HEPES, and 0.3% sodium dodecyl sulfate (SDS) in 3x SSC, were hybridized to the arrays for 5 to 16 h at 65°C. After hybridization, the arrays were washed in 1x SSC containing 0.03% SDS and finally in 0.05x SSC (83). The DNA microarrays were dried by brief centrifugation (5 min; 45 x g).

2.5.7 Data normalization and gene expression analysis

Immediately after stringent washing of the arrays, fluorescence intensities at 635 and 532 nm were acquired using a GenePix 4000 laser scanner (Axon Inc., Union City, Calif.) and processed as TIFF images. Raw fluorescence data were analyzed quantitatively using GenePix version 3.0 software (Axon Inc.). Data were normalized to the average ratio of *C. glutamicum* genomic DNA. The normalized ratio of the median (GenePix) was taken to reflect the relative RNA abundance for spots whose green or red fluorescence signal was at least threefold above the background fluorescence. When both fluorescence signals were less than threefold above background, the signals were considered too weak to be analyzed quantitatively. For statistical analysis, P values from independent replicate experiments were calculated based on Student's t test using log-transformed gene ratios and genomic DNA ratios which were normalized to zero. Only genes showing significantly changed RNA levels (P values of <0.05) were considered for further analysis. Analysis of gene expression data was performed by selecting genes showing at least twofold increase or decreased in average RNA levels.

2.5.8 Construction of gene disruption mutants

Primers were designed to amplify a ~300 bp fragment of the desired ORF. The obtained fragments were ligated in pCR®2.1-TOPO® (Invitrogen, Karlsruhe, Germany) and transformed into chemically competent TOP10 *E. coli* cells (Invitrogen, Karlsruhe, Germany) according to manufacturers recommendation. Transformants were selected by plating the cells on LB plates containing X-GAL and 50μg/ml kanamycin (Invitrogen, Karlsruhe, Germany)

after 1 h incubation at 37°C. The plasmids containing the PCR fragment were isolated using Nucleospin plasmid isolation kit (Macherey Nagel, Düren, Germany) and the inserts were verified by PCR. Plasmids was transformed in *C. glutamicum* as described by van der Rest *et al.* (146). Since the plasmid cannot replicate in *C. glutamicum*, it undergoes a homologous crossover thereby creating a disruption in the reading frame of the ORF (Fig. 2.1). The integration of the plasmid was confirmed by PCR.

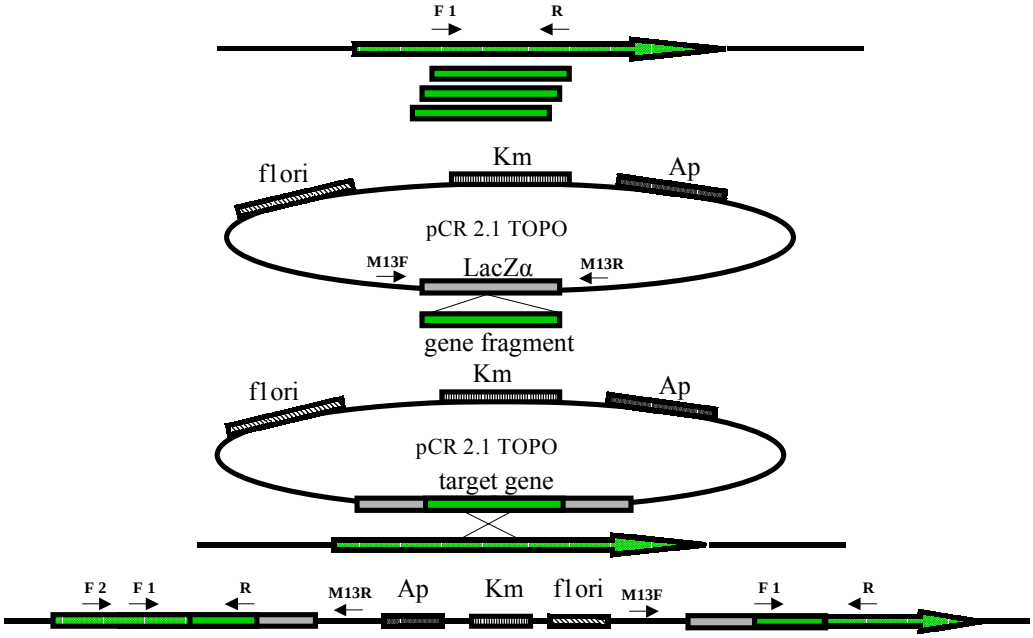


Fig. 2.1 Construction of gene disruption mutants

Outlines the cloning of PCR fragments in pCR 2.1 TOPO which upon transformation into *C. glutamicum,* underwent homologous recombination.

2.5.9 Preparation of *C. glutamicum* competent cells

Competent *C. glutamicum* cells were prepared according to van der Rest *et al.* (146). In this case, one colony was inoculated in 10ml LB medium containing 2% (w/v) filter sterilized glucose and cultivated for 16 h on a shaker at 200rpm at 30°C. 800 ml of Epo medium was inoculated with the overnight culture to an OD_{600} of 0.3. Cells were allowed to grow for 28h at 18°C and 120 rpm to and OD_{600} of 1. The cells were chilled on ice for 10 min followed by centrifugation at 4000 rpm for 10 min. The cell pellet was resuspended and washed 4 times with 100 ml 10 % glycerol precooled to 4°C. Finally the pellet was resuspended in 1 ml 10% glycerol (4°C) and stored in 200 µl aliquots at -70°C.

Epo medium (per 100 ml)

LB 80 ml

Dissolved in 20 ml MilliQ water, filter sterilized and added to LB medium.

isoniazid 400 mg

glycine 2.5 g

Tween 80 0.1 ml

2.5.10 Electroporation

One to two µl of plasmid were electoporated in 2-mm electroporation cuvettes into 40 µl of *C. glutamicum* competent cells on ice using the Gene-Pulser (BioRad, Munich, Germany) with the following conditions:

Resistance: 600Ω

Capacitance: 25 µFD

Volt: 2.5 kV (12.5 kV/cm)

Time constant: 10-12 ms

Following electroporation, 1 ml BHIS-medium (Brain heart infusion broth, Oxoid; Wesel, Germany) was added directly to the electroporation cuvettes and the suspension was transferred to a 2ml Eppendorf tube. The tube was incubated 6 min at 46°C. For regeneration of the host cells, the mixture was incubated at 30°C for 1 h. 500 µl aliquots were plated on LBHIS plates supplemented with 25 µg kanamycin ml $^{-1}$. Plates were incubated for 4 days at 30°C.

LBHIS (LB, Brain heart infusion, sorbitol per L)

Trypton 5 g

Yeast extract 2.5 g

NaCl 5 g

Brain heart infusion powder 18.5 g

Agar 15 g

Separately autoclaved sorbitol 91 g

2.5.11 Polymerase Chain Reaction (PCR)

Oligonucleotides used for PCR were obtained from MWG Biotech (Ebersberg, Germany). PCR amplification was performed in an automated thermocycler in 0.2-ml thin-walled PCR tubes (Advanced Biotechnologies, Germany). Reactions were carried out in 50-µl volumes containing 25 µl 2x ReddyMix PCR Mastermix (ABGene, Hamburg, Germany), 50 pmol of each oligonucleotide primer. After an initial denaturation step (95°C, 5 min)

amplification was carried out with 25-35 cycles (denaturation at 95°C for 15s, annealing at 50°C for 20s and extension at 72°C for 30s) followed by a final extension step at 72°C for 10 min. The annealing temperature was adjusted to the melting temperature of the primers and the extension time to the size of the expected fragment.

2.5.12 RNA isolation

Hundred ml TSB pH 7.5 and 5.7 were 1:200 inoculated with 5 ml overnight culture. At an OD_{600} of 0.5, 10 ml cells were harvested by centrifugation at 5000 *x g* for 5 min and pellets were immediately frozen in liquid nitrogen. Total RNA was isolated using RNeasy Tissue kit (Qiagen, Hilden, Germany) according to manufacturer recommendation with the following modifications: the frozen cells were resuspended in 700 µl RTL buffer containing 7 µl mercaptoethanol. The cells were disrupted in a Ribolyzer (Hybaid, Heidelberg, Germany) using Lysing Matrix B beads (QBiogene, Heidelberg, Germany) for 45 s at 6.5 m/s 3 times. The cells were cooled on ice between runs. Finally, tubes were centrifuged for 2 min at full speed and supernatant was removed into a column. On column DNAse digestion was performed for 15 min at 30°C and the RNA was eluted in 50 µl DNAse and RNAse free water. Despite optimization of the DNase treatment some DNA contamination remained which was removed by a separate DNAse digestion using Ambion's DNA-free™ (Ambion, Cambridgeshire, UK) according to manufacturer's instruction.

2.5.13 Reverse transcriptase PCR

One µg of total RNA was transcribed to cDNA by Superscript™ III (Invitrogen, Karlsruhe, Germany) in a 20 µl reaction which included 20 mM dNTPs, 100 ng/µl random primers. The mixture was heated at 65°C for 5 min and placed on ice for 5 min. Next, 4 µl of 5x First strand synthesis buffer, 1 µl of 0.1M dDTT, 1 µl RNAse out Inhibitor and 200 U/µl of Superscript III RT was added. cDNA synthesis was performed for 1.5 h at 50°C followed by an enzyme inactivation step for 15 min at 70°C. The cDNA was 10 fold serially diluted and real time PCR was performed.

2.5.14 Real-time PCR

All reactions were performed using the iCycler (Bio-Rad, Munich, Germany). For a 25 µl reaction 12.5 µl ABsolute™ QPCR SYBR® Green Fluorescein mix, (ABgene, Hamburg, Germany) 0.5 µM of each primer and 2 µl of cDNA were used. The cycling conditions were 40 cycles of 95°C for 15 s, 55°C 15 s and 72°C for 20 s preceded with a 15 min enzyme activation at 95°C. For each amplification run, the calculated threshold cycle (C_t) of the 16S

rRNA was used for normalization. The formation of secondary products was analyzed using the melting curve function of the iCycler software version 3.0.

2.5.15 Iron availability assay

An iron availability assay was performed to assess the solubility of iron at low pH in undefined complex growth media such as TSB and BHI compared to the defined minimal medium CGXII. Four growth media were prepared; TSB, TSB +Fe containing 10 mg/L of $FeSO_4$·7H2O and protocatechuic acid to final concentration of 0.1mM, BHI, and CGXII. CGXII was prepared according to Keilhauer et al. (82) except, protocatechuic acid was added to a final concentration of 0.1mM. Protocatechuic acid acts as a chelator, facilitating the bioavailability of iron in the growth medium. For all of the media pH was adjusted to 7.5 or 5.7. Hundred ml of medium at pH 7.5 and 5.7 were inoculated with 1 ml of overnight culture. At mid-exponential growth phase (OD_{600} of 0.5), 10 ml cells were harvested by centrifugation at 5000 *x g* for 5 min and pellets were immediately frozen in liquid nitrogen. The RNA isolated from the cells was subjected to real time PCR analysis as described above.

2.5.16 Determination of growth characteristics of the deletion mutants

The growth characteristics of the mutants compared to the wild type strains were determined using the honeycomb well plate reader Bioscreen C (Thermo Labsystems, Dreieich, Germany). A 5 ml overnight culture was 50 fold diluted in TSB (pH 7.2) and 10 µl were inoculated in honeycomb plates containing 240 µl of TSB at pH 7.5 or pH 5.7. The plates were incubated (continuous shaking, 30°C) and OD_{600} measurements were obtained every 15 minutes for a period of 24 h. The growth rates of the cultures were determined by plotting and analyzing the change in OD_{600} over time using the Bioscreen C Growth Curve software.

2.5.17 Acid shock assays

Acid shock was performed to determine the role of the disrupted gene on the survival capabilities under acidic conditions. Hundred ml TSB pH 7.5 or 5.7 were inoculated with 1 ml and 1.5 ml of overnight culture, respectively. At an OD_{600} of 0.5, 50 ml cells were harvested by centrifugation at 5000 *x g* for 5 min. The cells were suspended in 50 ml TSB pH 4 and incubated at 30°C for 30 min. 100 µl samples were removed before and after acid shock, serially diluted in TSB pH 7.5 and plated on TSA plates. The percent survival was calculated

by comparing the cell counts obtained following acid shock to those in the original pH 7.5 cell suspension prior to acid shock.

2.6 Bioinformatics

The Blast algorithms (1) were used for similarity searches in the database available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were performed using ClustalW from the European bioinformatics institute (http://www.ebi.ac.uk/clustalw).

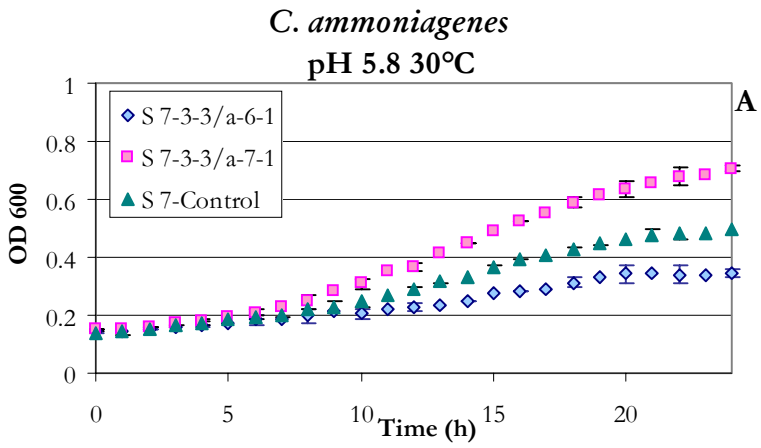
3 Results

3.1 pH adaptation of smear cheese ripening species

Adaptation of the four ripening strains to low acid condition was performed using agar pH gradient plates. The method used to adapt the strains to low pH is described in section 2.4 (see material and methods). To provide a better overview of the results each of the ripening strains are going to be discussed individually.

3.1.1 Corynebacterium ammoniagenes

The adaptation of *C. ammoniagenes* occurred in 5 selections steps. In the first step the strain was exposed to pH 7-pH 6 gradient plates from which colonies were picked and inoculated in pH6. In the next step the colonies were exposed to pH 6-5.5 gradient plates. The passage of the strain to pH 6-5.5 was repeated three times and a total of 52 potential mutant colonies adapted to pH 5.5 were obtained. From these colonies 12 potential mutants were selected that were analyzed with the honeycomb well reader Bioscreen C. From the 12 mutants 2 mutants were isolated. One of the mutants was able to grow at 30°C and pH 5.8 better than the wild type strain. Both mutants were able to grow somewhat better at 15°C and pH 5.8 than the wild type strain. Only one mutant S7-3-3/a-6-1 was able to maintain its ability to grow at pH 5.8 after being exposed to pH 7 for 24 h (Fig. 3.3).



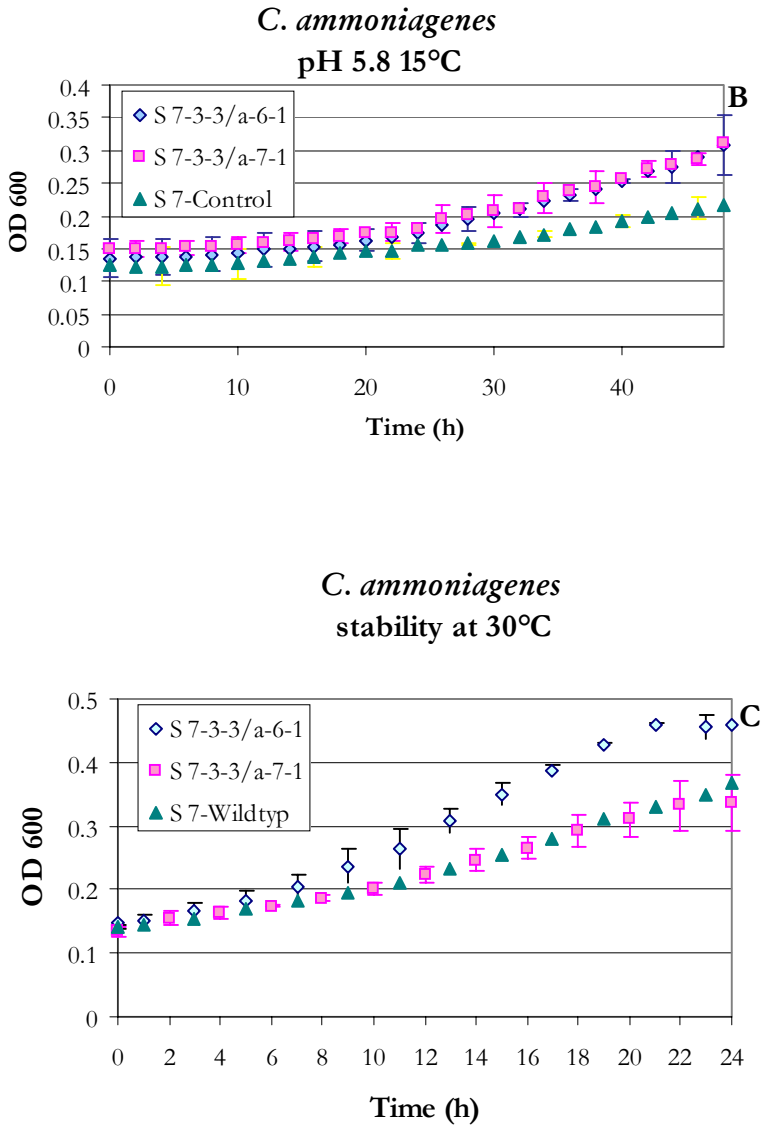


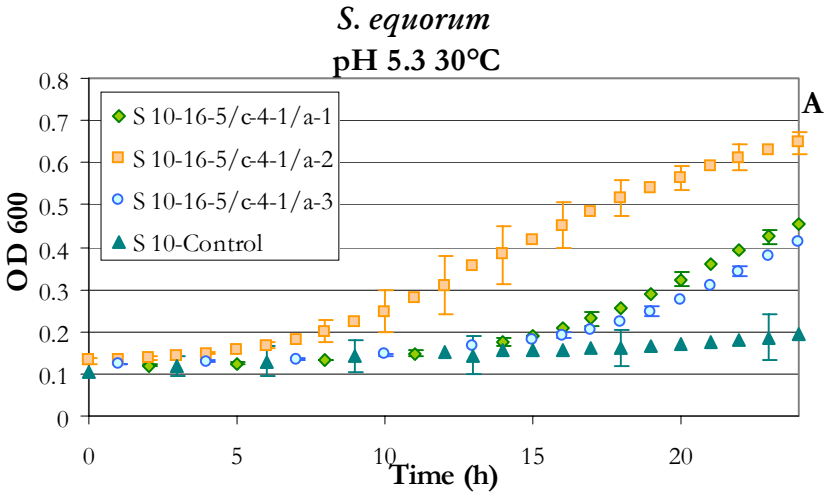
Fig. 3.1 *C. ammoniagenes* acid adapted mutants

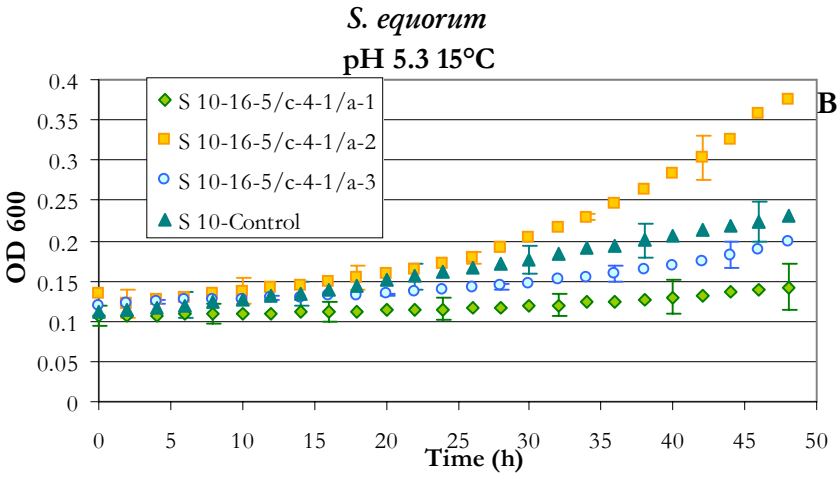
Figures represents growth **A** at pH 5.8 30°C, **B** at pH 5.8 15°C, and **C** stability test performed at 30°C.

3.1.2 Staphylococcus equorum

The pH adaptation of *S. equorum* was performed in seven selection processes. First, the strain was streaked on pH7-pH6 gradient plate. From the 16 mutants that were inoculated in pH 6 medium only 2 have grown that were streaked onto pH 6-pH 5.5 gradient plates resulting in a total of 45 pH 5.5 mutants. The adaptation of these mutants to pH 5 was performed in three steps which resulted in 50 perspective pH 5 mutants. When analyzing these mutants at pH 5 none of them exhibited growth at this pH. Therefore, the pH was increased in 0.1 increments to pH 5.3 where the mutants were able to grow. From here 13 prospective mutants were selected for further testing using Bioscreen C which resulted in

three stable mutants. All the three mutants were able to grow better at 30°C and pH 5.3, but only one of them, *S10-16-5/c-4-1/a-2,* exhibited a better growth characteristic at 15°C and pH 5.3. All three mutants were able to maintain their ability to grow at pH 5.8 at 30°C after being exposed to pH 7 for 24 h (Fig. 3.4).





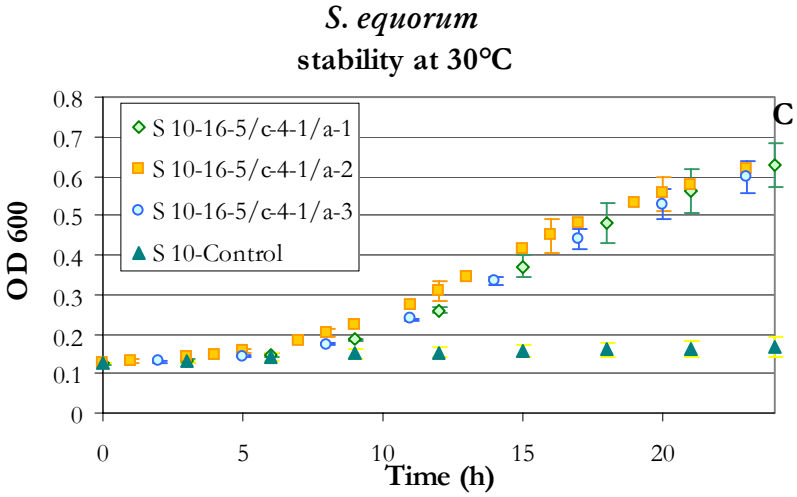
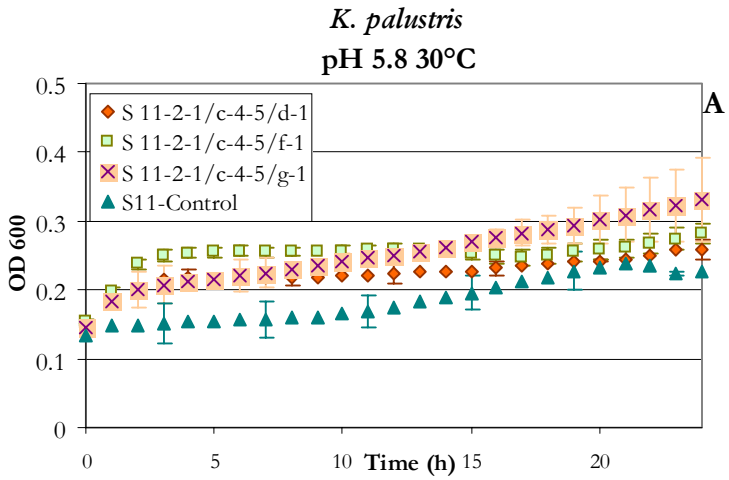


Fig. 3.2 *S. equorum* acid adapted mutants

Figures represent growth **A** at pH 5.3 30°C **B** at pH 5.3 15°C and **C** stability test performed at 30°C

3.1.3 Kocuria palustris

For *K. palustris* the adaptation also occurred in seven steps. In the first step the strain was streaked onto pH7-6 gradient plates 10 colonies were obtained which were inoculated in pH 6 TSB^{+}. These were streaked onto pH 6-5.5 gradient plates. This step was repeated three times and a total of 72 colonies were obtained from this step. Based on these mutants growth characteristic 12 colonies were tested in Bioscreen C. From the 12 colonies tested, three mutants showing the best growth characteristics at 30°C and pH 5.8 were further analyzed. At 30°C only one of the mutants demonstrated the best growth characteristic, whereas at 15°C and pH 5.8 none of the mutants grew better than the wild type strain. All the mutants retained their growth characteristics after exposed to pH 7 for 24 h except for mutant *S11-2-1/c-4-5/f-1* (Fig. 3.5).



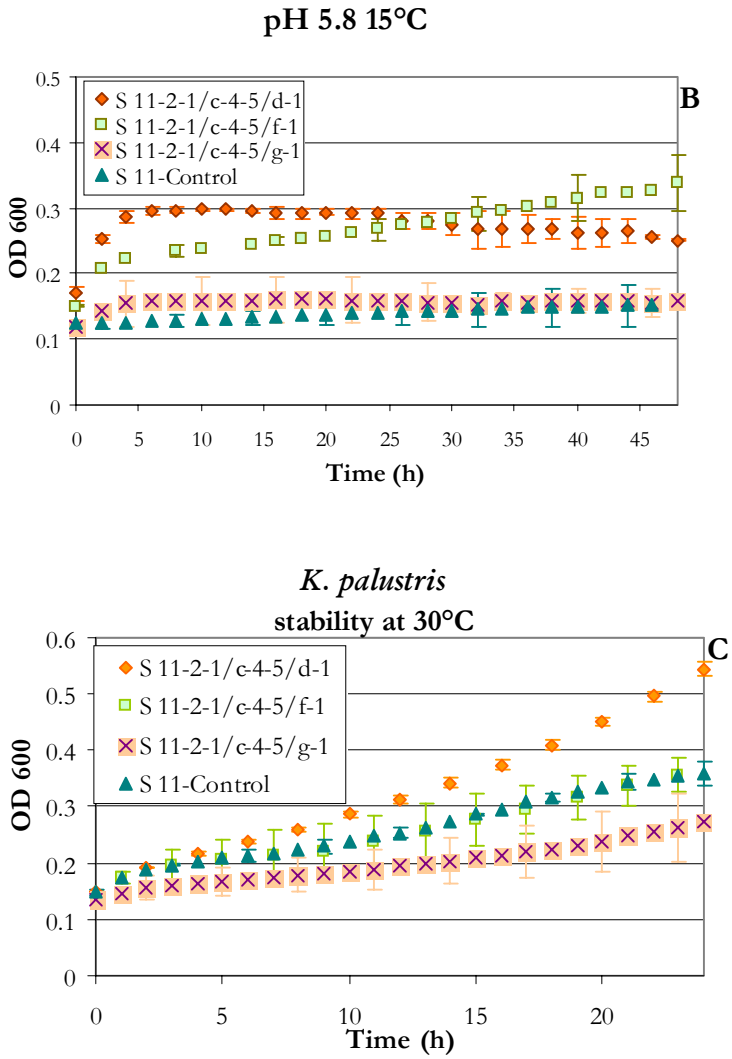


Fig. 3.3 *K. palustris* pH adapted mutants

Figures represent growth **A** at pH 5.8 30°C **B** at pH 5.8 15°C and **C** stability test performed at 30°C.

3.1.4 Corynebacterium variabile

From all of the strains being adapted to low pH the adaptation of *C. variabile* to pH 5 preceded most rapidly requiring only 4 adaptation steps. An aliquot taken from the wild type strain was immediately streaked out on pH6-5.5 gradient plates. Ten colonies obtained were inoculated in pH 5.5 and an aliquot from the cultures was streaked onto the same gradient plate one more time. Next the adaptation to pH 5 began. Fifteen pH 5 colonies were isolated from 39 colonies of potential pH 5.5 mutants which were isolated after a two time passage on the pH5.5-5 gradient plates. Based on Bioscreen C analysis 4 mutants were isolated which were able to grow in pH 5.3 at 30°C. All of the mutants grew better than the wild type at 15°C

at pH 5.3. In testing the stability of the three mutants all of them maintained their accumulated mutation after a passage in pH 7 for 24 h (Fig. 3.6).

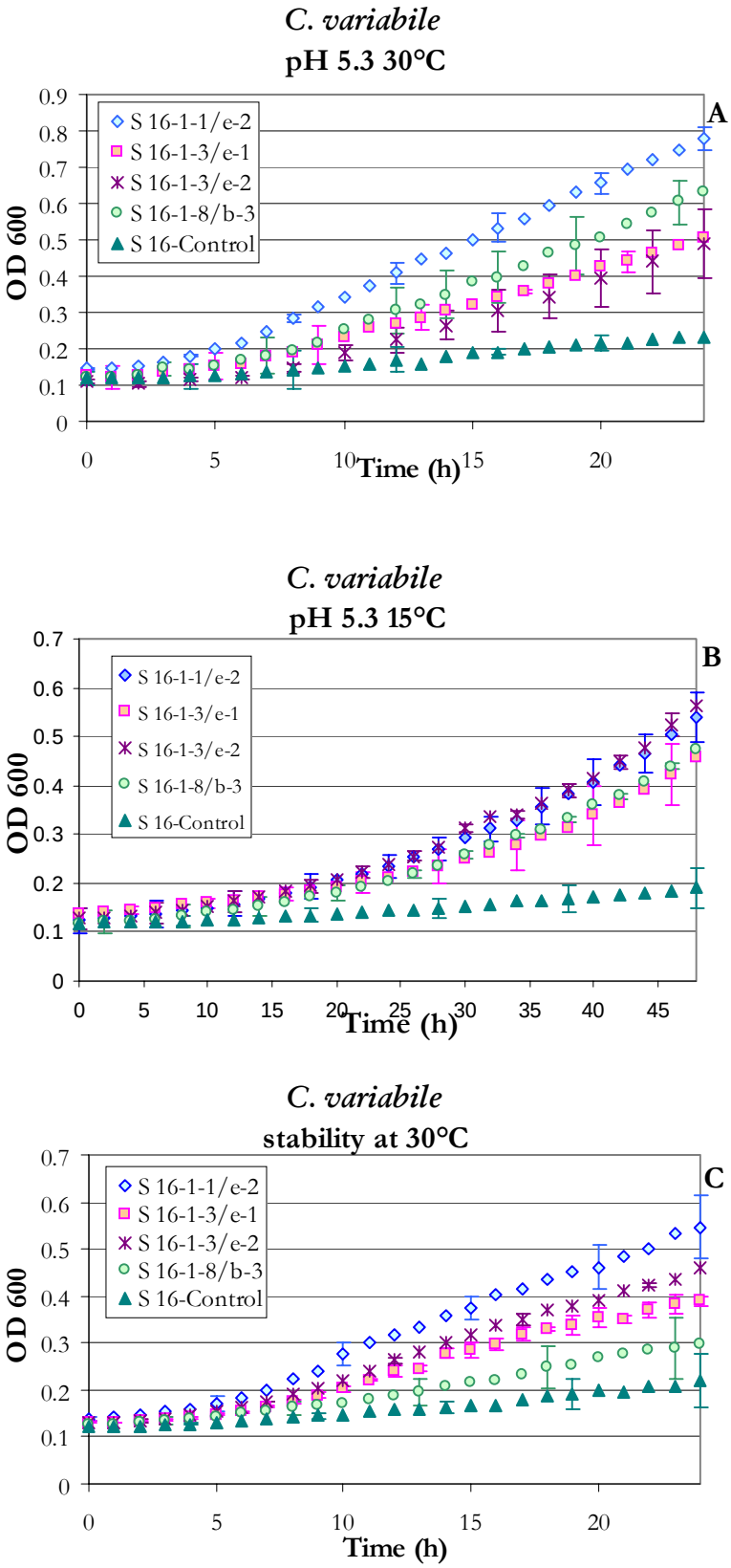


Fig. 3.4 ***C. variabile* pH adapted mutants** Figures represents growth **A** at pH 5.3 30°C **B** at pH 5.3 15°C and **C** stability test performed at 30°C.

3.1.5 Mutation frequency

The mutation frequency at pH 6 was estimated for each of the bacterial species during the adaptation experiment to estimate the inhibitory effect obtained from lactic acid (Tab. 3.1). 100 µl of an overnight culture was 1000 fold diluted and plated on three pH 7/pH 6 gradient plates. The frequency estimates reflect the ratio of colonies present at pH 6 compared to the amount of cells present at that dilution. Throughout the adaptation experiment, depending on species, between 51 to 104 colonies were screened obtaining two mutant strains for *C. ammoniagenes* at pH 5.8, four mutant strain for *C. variabile* at pH 5.3, three mutants for *S. equorum* at pH 5.3 and three mutant strain for *K. palustris* at pH 5.8 (Tab 3.1).

Strain	Average cells plated on pH7/pH 6 plates	Average no. of colonies obtained at pH 6	Mutation frequency at pH 6[a]	No. of colonies screened[b]	No. of pH mutant strains[c]	Mutation frequency at the mutants adapted pH[d]
C. ammoniagenes	1.05×10^3	104.5	9.9×10^{-2}	77	2	2.6×10^{-3}
C. variabile	4.95×10^4	153	3.6×10^{-3}	51	4	2.8×10^{-4}
S. equorum	1.97×10^3	86.5	4.39×10^{-2}	104	3	1.3×10^{-3}
K. palustris	2.54×10^2	98.5	3.83×10^{-1}	83	3	1.4×10^{-2}

Tab. 3.1 Summary of mutation frequencies and mutants obtained.

[a] shows the mutation frequency obtained for each of the species at pH 6

[b] represents the number of colonies screened in the entire experiment through the passages from pH 7/pH 6 and pH 6/pH 5.5 gradient plates

[c] represents the number of pH adapted mutant strains obtained at the end of the experiment for each of the bacterial species *C. ammoniagenes* and *K. palustris* pH 5.8 for *C. variabile* and *S. equorum* pH 5.3

[d] represents the mutation frequency of the bacterial species at the their adapted pH

3.2 Monitoring of ripening consortium using quantitative real time PCR on model cheese

3.2.1 Primer design for the ripening microbial consortium

In order to design strain specific primers for the real time PCR analyses of the microbial consortium on the model cheese surface, the 16S and the 18S region were sequenced using the sequencing primers described in Table 2.1. The obtained 1500 bp sequences from the bacterial 16S region were aligned using the ClustalW software. The aligned sequences exhibited large conserved areas with some variable regions.

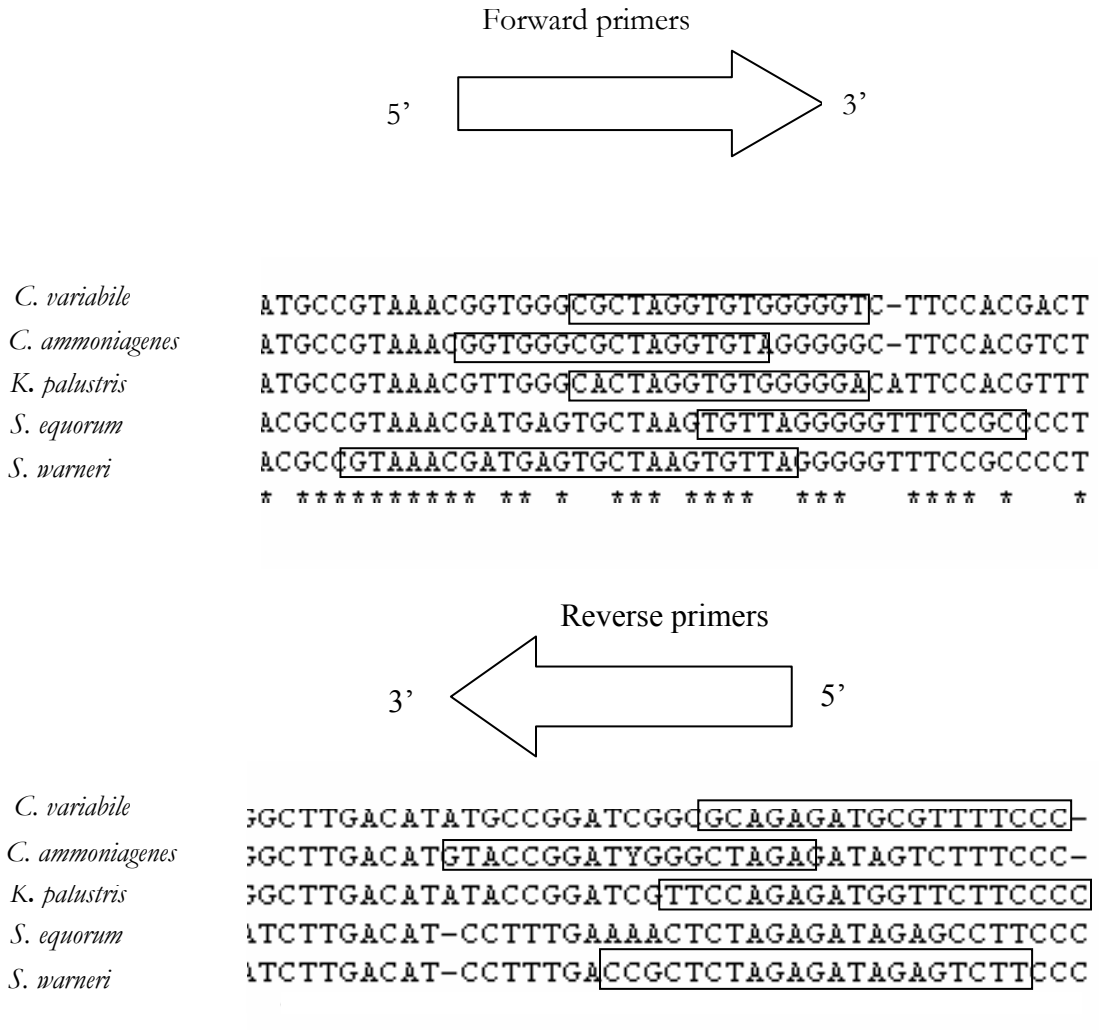


Fig. 3.5 Bacteria forward and reverse primers.

The boxed areas show the designed primers based on 16 S sequencing.

The primers for the bacterial strains used for real time PCR were designed in such a way that the 3' ends of the primers were positioned in the non-conserved region, providing strain specificity. The amplicon size was kept constant at 200 bp for each of the strains except in the *Staphylococcus* species. Due to difficulties in differentiation among the *Staphylococcus* species separate reverse primers were designed which resulted in an increase in the amplicon size of the *S. equorum* to 300 bp. The same principle was used in designing the 18S yeast strain specific primers.

Before the primers were used in real time PCR, they were tested under standard PCR conditions to confirm specificity and to ensure that they don't exhibit cross reactivity with the other strains used in the ripening experiment.

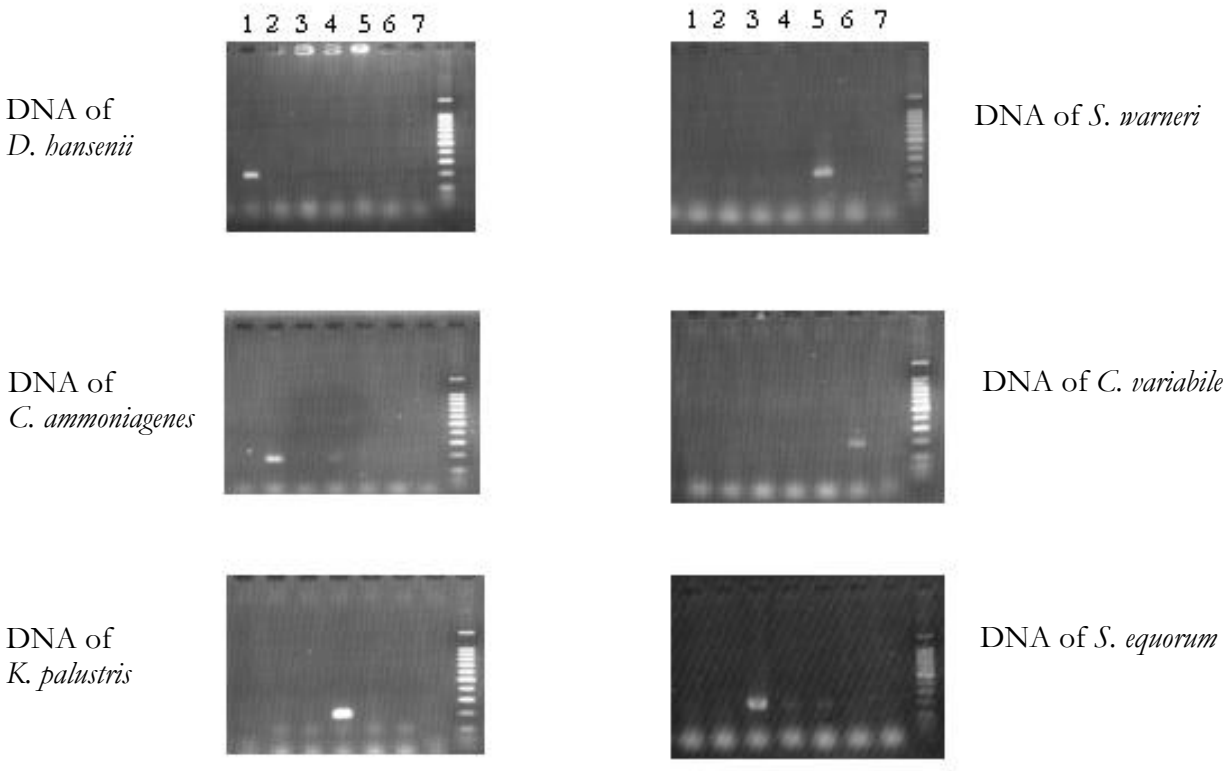


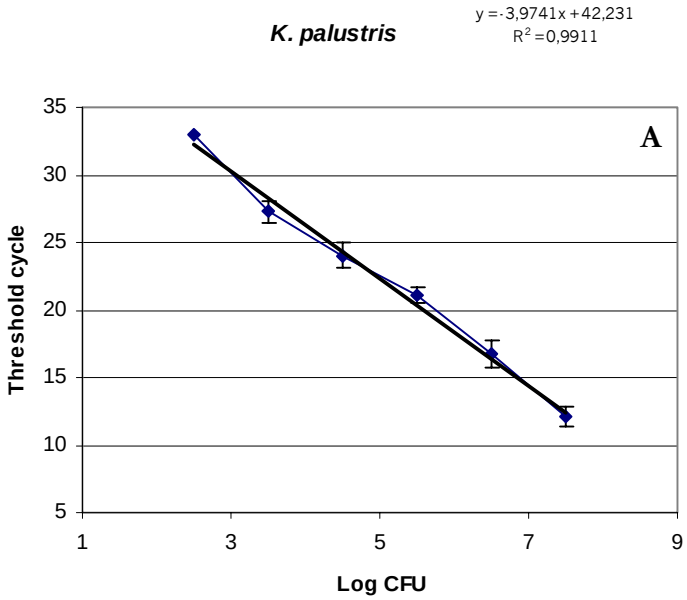
Fig. 3.6 Primer specificity.

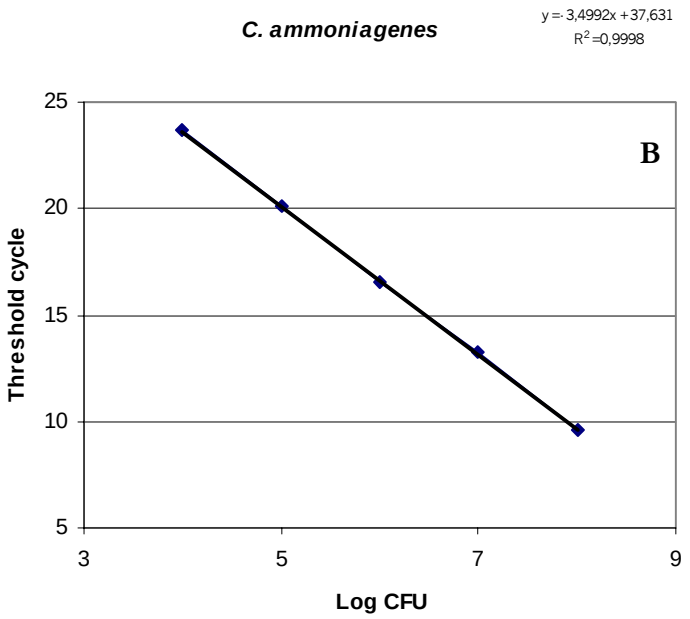
The name represents the primers pair used for that strain and the other blank lanes contain the other strain as a negative control. In the last lane the 100bp marker is shown. As can be seen the primers were very specfic for the strain that they were designed for and they did not amplify the other species. Lanes 1 through 7 are *D. hansenii*, *C. ammoniagenes*, *S. equorum*, *K. palustris*, *S. warneri*, *C. variabile*, and negative control.

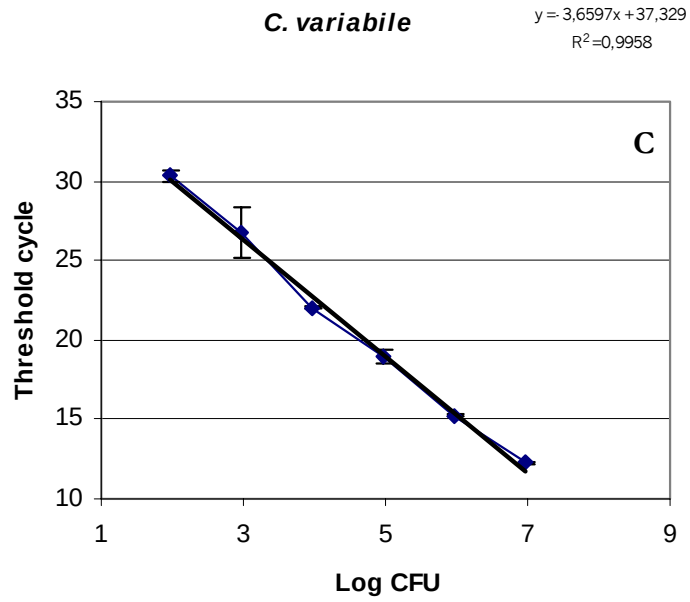
As can be seen in Fig. 3.2, the primers are specific and do not amplify DNA of the other strains. Another experiment was performed using a DNA mixture imitating conditions existing on the surface of the model cheese to observe any inhibition in the reaction due to the presence of large amounts of DNA in the reaction as a result of some dominant species during ripening. No inhibition was observed (data not shown).

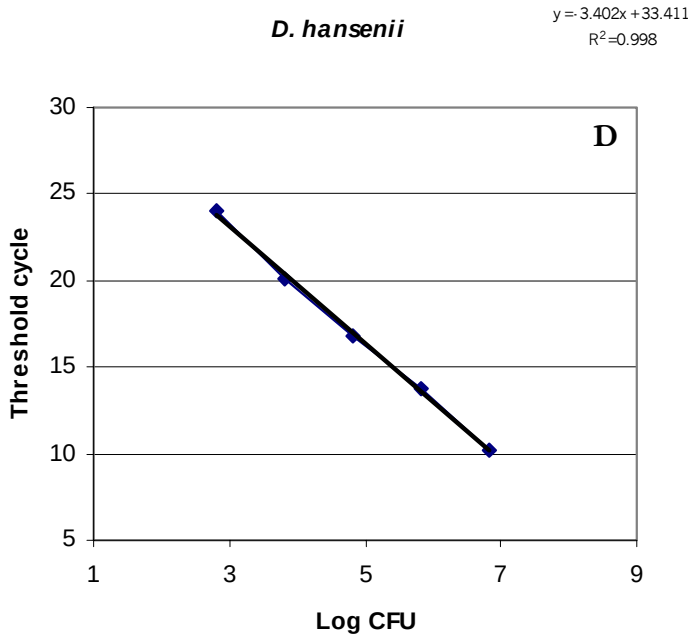
3.2.2 Construction of a cell count standard curve

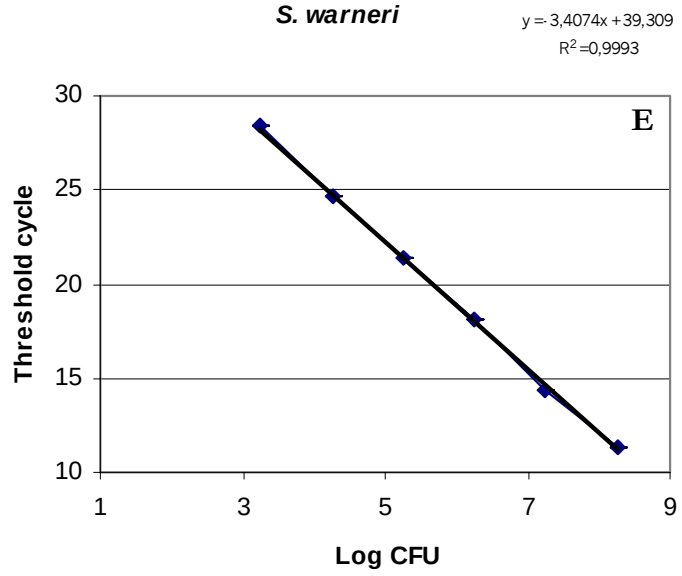
To properly quantify the microbial consortium growing on the surface of the model cheese construction of a cell count standard curve was necessary. A cell standard curve was constructed using DNA isolated from independently inoculated overnight cultures. The cells were 10-fold serially diluted in TSB, DNA was isolated and used in the real time PCR assay. The standard curve based on cell dilution showed a linear relationship between log input cell concentration and threshold cycle. The correlation coefficients of the standard curves ranged from 0.991 to 0.999 and the slope of the curves ranged from –3.4 to –3.9 (Fig.3.7)




C. ammoniagenes
y = -3,4992x + 37,631
R² = 0,9998
B
Threshold cycle
25
20
15
10
5
3
5
7
9
Log CFU


C. variabile
y = -3,6597x + 37,329
R² = 0,9958
C
Threshold cycle
35
30
25
20
15
10
1
3
5
7
9
Log CFU


D. hansenii
y = -3.402x + 33.411
R2 = 0.998
D
Threshold cycle
30
25
20
15
10
5
1
3
5
7
9
Log CFU


S. warneri
y = -3,4074x + 39,309
R2 = 0,9993
E
Threshold cycle
30
25
20
15
10
1
3
5
7
9
Log CFU

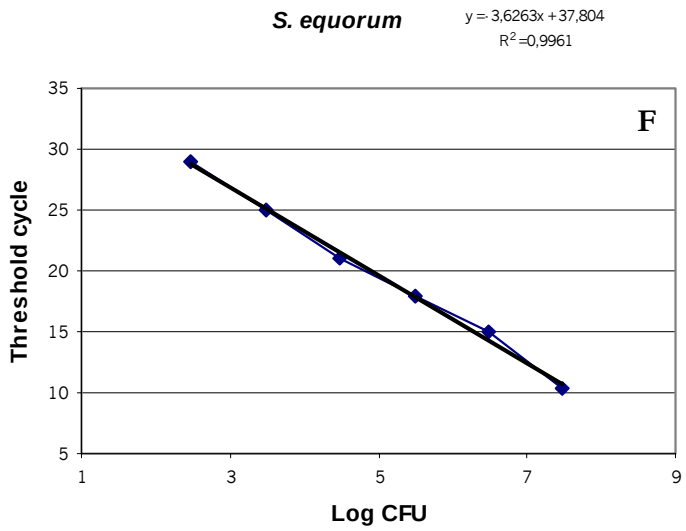


Fig. 3.7 Cell standard curves

A *K. palustris*, **B** *C. ammoniagenes*, **C** *C. variabile*, **D** *D. hansenii*, **E**, *S. equorum* and, **F** *S. equorum*

Detection limits varied between species, from low of 10^2 CFU (*C. variabile*) to a high of 10^3 (*C. ammoniagenes*). The generation of non specific product was analyzed using the melt curve function of the iCycler software. Due to the non specificity of the SYBR Green dye it was expected that primer dimers would form during PCR reaction. For most of the strains in the no template controls the development of primer dimers were observed between threshold cycles 32 and 40 however, no unspecific products formed in the reaction.

3.2.3 Analysis of microbial succession on model cheese

Samples were analyzed at seven time points during the experiment including the sample that was used to inoculate the agar plate. The DNA was isolated diluted to 10 ng/µl and the threshold cycle was determined. Colony forming units (CFU) were determined based on the cell count standard curve results and calculated per cm^2.

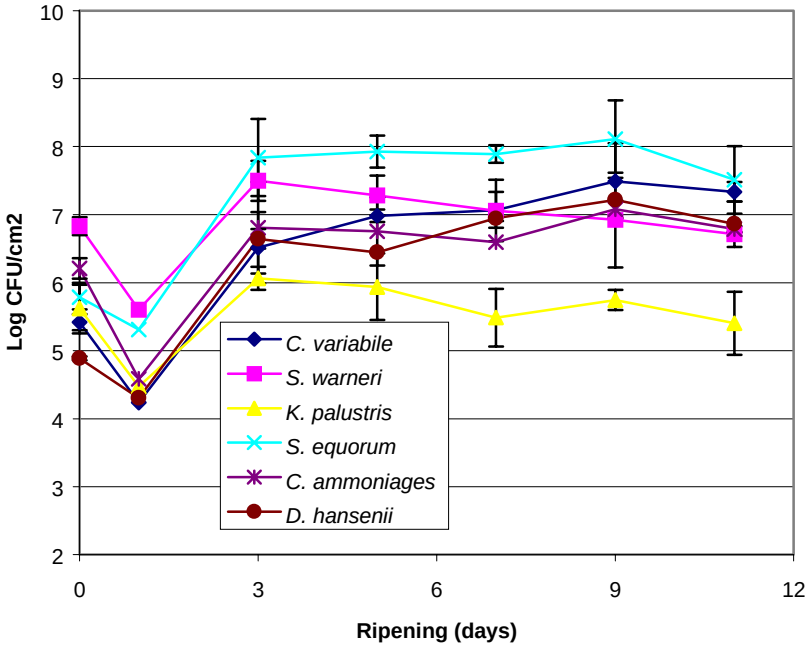


Fig. 3.8 Cell count vs. ripening

Log CFU/cm^2 changes on model cheese during 11 days of ripening.

On the model cheese containing wild type ripening species the dominant bacterial species was *S. equorum* (Fig. 3.8). *S. equorum* reached its maximum count of 10^7/cm^2 on day 3 staying the same until day 9 and declining in count on day 11. The cell counts of *C. ammoniagenes* progressively declined after reaching maximum counts on fifth day, whereas *C. variabile* increased continuously throughout the process reaching maximum count of 10^7 on day 9.

The least dominant species was *K. palustris*. *K. palustris* reached peak cell count of 105 on day 5 declining to a half log unit on day 11. The cell count of the yeast, *D. hansenii* increased from 10^4 to 10^7 CFU/cm^2. None of the species added to the experimental consortium was displaced during the experiment. This indicates that all six species were well adapted to grow together under these conditions.

3.2.4 Comparison of quantitative real time PCR to cell culture method

The total CFU/cm^2 counts were used to compare the results for culture and real time PCR methods. As shown in table 3.1 there is a maximum of 0.8 log unit difference between culture and PCR CFU counts. The Pearson linear correlation test provided a result of 0.929 demonstrating a good correlation between culture and real time PCR methods.

Ripening (days)	Log $_{10}$ CFU/cm^2 as determined by;	
	Culture	Real time PCR
0	6,20	7,00
1	6,64	6,50
3	8,92	8,19
5	8,92	8,12
7	8,63	8,08
9	8,79	8,44
11	8,75	7,92

Tab. 3.2 Comparison of average plate count with quantitative real time PCR

Average yeast counts obtained by serial plating on yeast selective agar and by real time PCR were compared to each other. As shown in Table 3.2 in some cases the results obtained by real time PCR were 2 magnitudes higher than culture obtained counts. Despite these results the Pearson linear correlation test ($r = 0.914$) showed a good correlation between plate count and real time PCR results comparable to total count results.

Ripening (days)	Log $_{10}$ CFU/cm^2 of *D. hansenii* as determined by;	
	Culture	Real time PCR
0	2,71	4,88
1	3,10	4,30
3	6,78	6,64
5	7,10	6,45
7	6,98	6,94
9	6,23	7,22
11	6,32	6,86

Tab. 3.3 Comparison of average yeast plate counts with quantitative real time PCR

3.2.5 Comparison of microbial succession of non-adapted and adapted species

The microbial succession between control and pH adapted species were analyzed using real time PCR. At the end of the ripening period the dominant ripening flora was *C. variabile* followed by *S. equorum* (Fig. 3.9).

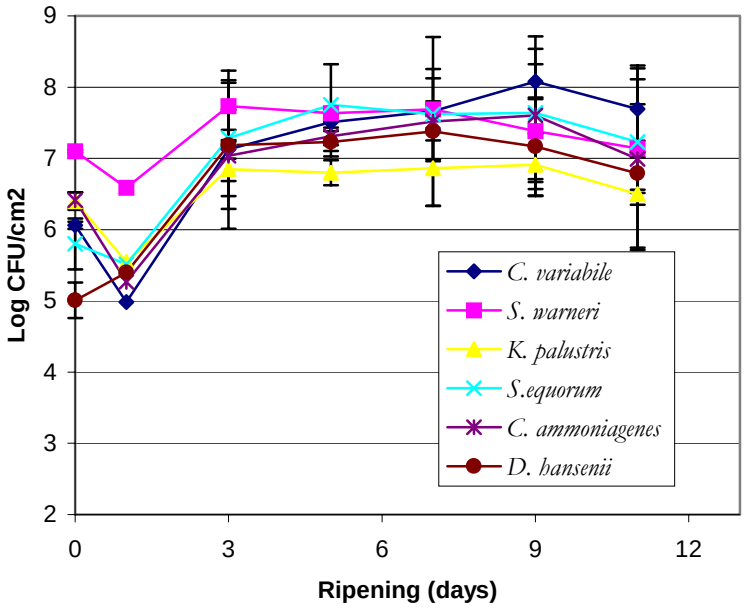


Fig. 3.9 Development of the mutant consortium on cheese agar

Log CFU/cm^2 changes of mutant species during model cheese ripening

In comparing the growth characteristics under the same conditions one species *K. palustris*, exhibited subtle differences in the acid adapted and non-adapted strains (Fig. 3.10). The trends observed during the ripening of the two strains were the same reaching maximum cell counts on day three. There were no significant differences observed in the other species' adapted and control strains

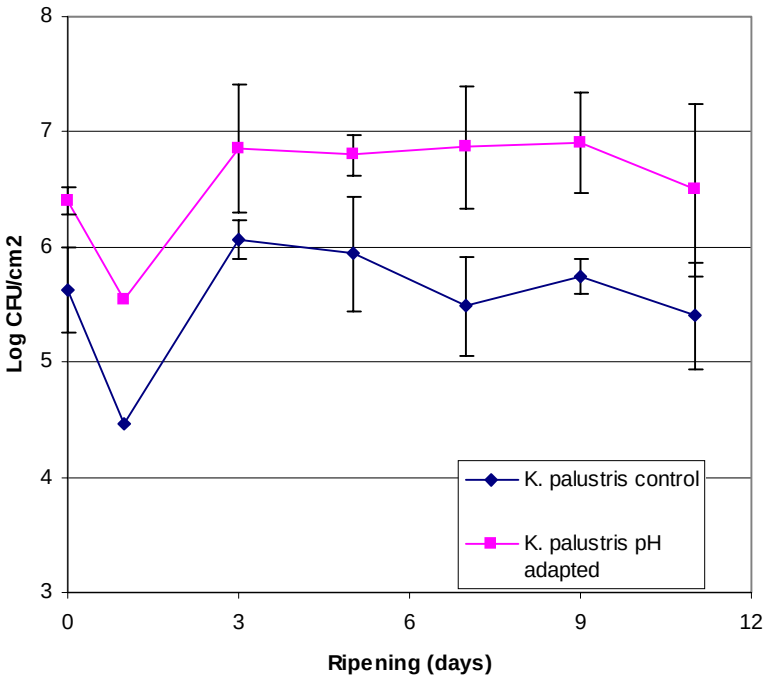


Fig. 3.10 Comparison of pH mutant *K. palustris* with wild type

Comparison of growth characteristics on model cheese of *K. palustris* acid adapted and control strains

3.3 ATR of *C. glutamicum*

Initially the ATR of *C. glutamicum* was investigated under different pH conditions. Exponentially growing cells in control pH (pH 7) and adaptive pH (pH 6 and 5.7) were exposed to pH 3.5 for 90 minutes. Survival percentage was determined by serial plating samples obtained at 30 and 90 minutes after exposure to pH 3.5. In the non-adapted culture no viable cells were detected after 30 and 90 minutes exposure whereas in the adapted cultures 10% viability at pH 6 and 80% viability at pH 5.7 were observed after 30 minutes exposure (Fig. 3.11). Therefore, cells adapted to pH 5.7 demonstrate an increase in the viability when shocked at pH 3.5 which clearly shows the ATR of *C. glutamicum*.

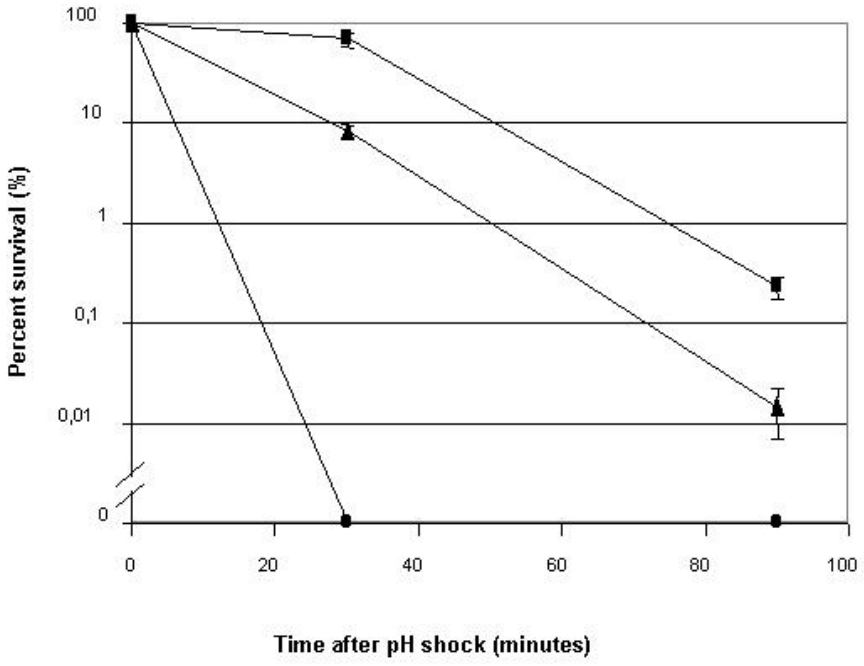


Fig. 3.11 Acid tolerance response of *C. glutamicum*

Adapted and non-adapted cells of *C. glutamicum* were investigated in acid shock experiment. Exponential phase cells were exposed to pH 3.5 after adaptation at pH 7.0 (●), pH 6.0 (▲) and pH 5.7 (■). Survivors after 30 and 90 minutes were determined by serial plating.

3.3.1 Measurement of cytoplasmic pH

Changes in cytoplasmic pH were determined using BCECF, pH dye indicator, in comparison to alterations in outer pH (Fig. 3.12). Based on the results, the cytoplasmic pH remained relatively constant up to mildly acidic outer pH conditions. From an outer pH of 5.5 a dramatic decrease in cytoplasmic pH was observed suggesting a collapse in the internal pH

homeostasis of the bacterial cell. This data correlates well with the fact that the observed pH minimum of growth of *C. glutamicum* is at pH 5.5.

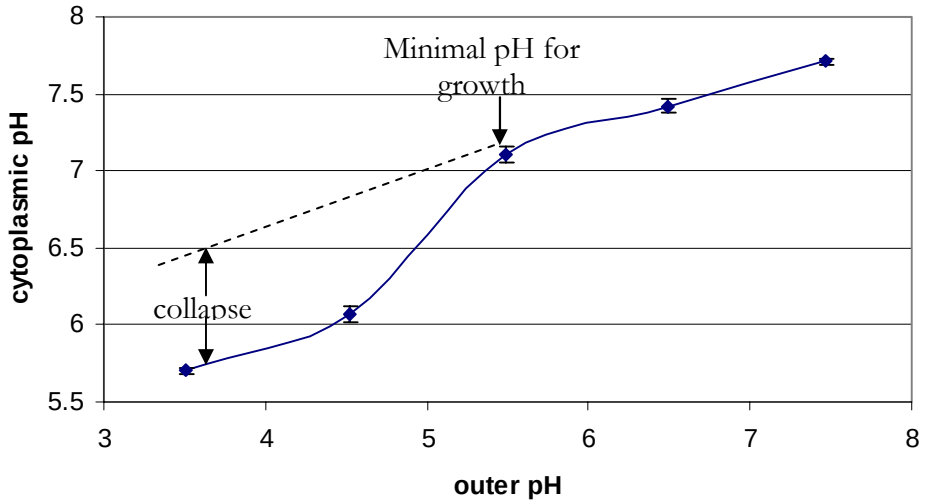


Fig. 3.12 Changes in cytoplasmic pH compared to outer pH alterations.

Cytoplasmic pH changes were measured in *C. glutamicum* ATCC 13032 which were grown in pH 7.5 to an OD_{600} of 0.5 using a fluorescent dye BCECF. The cytoplasmic pH remains up to an outer pH value of 5.5. Beyond this point there is a sudden decrease in the internal pH hinting to a collapse of the cytoplasmic pH homeostasis.

3.3.2 Comparison of gene expression at acidic and neutral pH conditions

To identify differentially expressed genes in response to acid adaptation, the global gene expression patterns of *C. glutamicum*, fermented in a continuous turbidostat fermentor at neutral and acidic conditions, were analyzed using DNA microarray (75). Throughout the experiment an OD_{600} of 0.5 was maintained. From two independent fermentation experiments four DNA microarray analyses were carried out. Genes whose transcript ratios showed ≥3 fold intensity increase above the background and were differentially expressed in at least three of the experiments were examined. A total of 116 up regulated and 90 down regulated genes have been identified representing 10% of the affected genes in the genome (Tab. 7.1 and 7.2). Based on their function the up regulated genes can be classified in three main categories: transporters, transcriptional regulators/proteins and cell metabolism proteins. Hypothetical proteins of unknown function comprised 38% of the up regulated ORFs.

Thirty genes encode proteins belonging to different transport system 20 of which are organized in 5 putative operons. Four of these operons showed similarity to ABC type cobalamin/Fe^{3+} siderophore transport system and one operon (ORF 1516 and 1517) codes for a predicted ABC type multidrug transport system. Another group comprises proteins

involved in the transport of ionic compounds as in cation transport ATPase, Co/Zn/Cd efflux component and divalent heavy metal cation transporter.

Six putative transcript regulators were recognized among the up regulated ORFs. Two transcriptional sigma factors, *sigB* and *sigE,* were previously identified in other microorganisms to be involved in general stress responses (68, 121). The sensory component of histidine kinase (ORF 1518), Mn dependent transcriptional regulator (ORF 3470) as well as two putative transcriptional regulators (ORF 927, 1642) completes the list.

The remainder of the up regulated ORFs represents proteins involved in numerous cell metabolic pathways as in carbohydrate metabolism as well as components for oxidative fermentation.

Subunits of F_0F_1 ATP synthase, (ORF 1807-1813) which play a role in the proton motive force, and the heat shock proteins GroES and GroEL (ORF 1088 and 1092, respectively), whose role in temperature stress is well documented showed a 2-fold down regulation. Other proteins showing a down regulation were 25 subunits of ribosomal proteins and several transport system involved in amino acid and cation transport.

3.3.3 Validation of expression profile of fermentation via real time RT-PCR

Changes in pH causes sudden alterations in the solubility of some metal ions, among them iron, which influences their accessibility to the microbial cell. These changes in the expression patterns are also observed at the mRNA level of numerous genes in the course of the adaptation experiment.

The fermentor harbors numerous metallic parts and during the extended fermentation process trace amounts of particles could have dissolved in the medium. To refute the possible occurrence of this phenomenon the expression profile of some of these genes were analyzed by real time PCR in cells grown in batch culture in glass vessel. An overnight culture of *C. glutamicum* was inoculated in fresh medium at pH 7.5 and 5.7. RNA isolated from exponentially growing cells was reverse transcribed and subjected to real time PCR analysis using the iCycler. For each amplification run the calculated threshold cycle (C_t) of the 16S rRNA was used for normalization. The analyzed genes included the two sigma factors (s*igB* and *sigE*), ORFs from putative iron transport operons and a cation efflux transporter. The genes examined by real time PCR (Fig. 3.12) show the same up regulated trend as in the microarray expression profile, thereby disproving any suspicion generated from the microarray data.

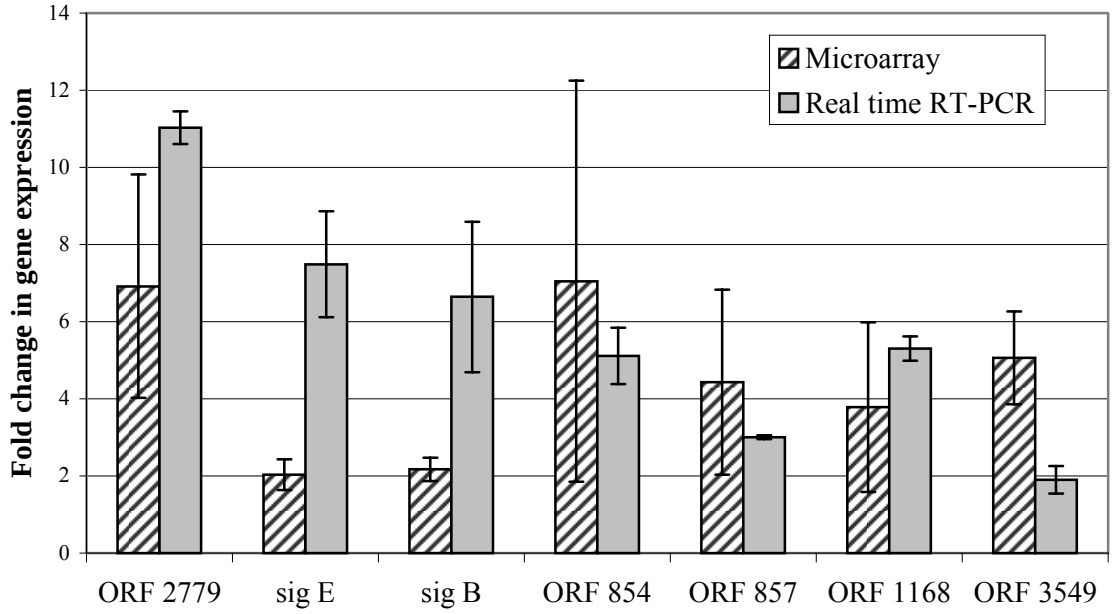


Fig. 3.13 Comparison between microarray analysis and real time PCR results

Fold changes of genes expressed in cells adapted to pH 5.7 in a fermentor as analysed by microarray were compared to real time RT PCR of *C. glutamicum* cells grown in batch culture

3.3.4 Iron bioavailability in undefined complex and defined minimal medium

To further investigate the up regulation of ABC type cobalamin/Fe^{3+} siderophore tranport operons an experiment was performed using three different complex media (TSB, BHI, and TSB+Fe) and one defined minimal medium (CGXII). ORF 855, one of the genes in the iron siderophore operons, was analyzed by real time PCR for expression level changes in cells grown at neutral and acidic pH in all of the media. First, we calculated expression level changes of ORF 855 in each media separately by comparing mRNA level at pH 5.7 to pH 7.5. The mRNA level increased in TSB, TSB+Fe and BHI whereas it decreased in CGXII (Fig. 3.13).

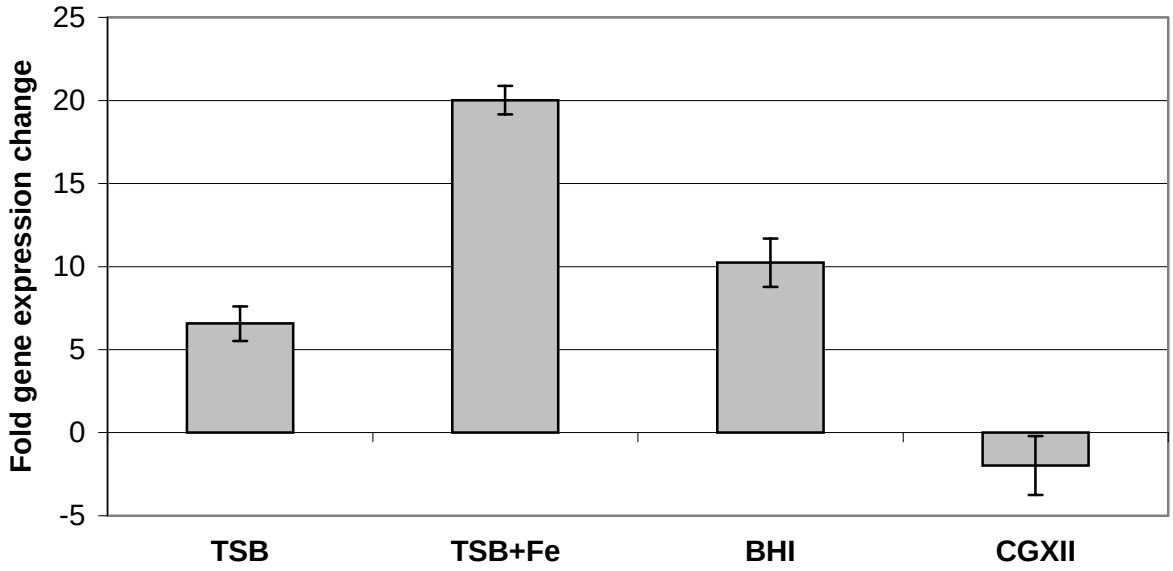


Fig. 3.14 Expression of iron siderophore in complex and minimal media.

Expression level changes of ORF 855 analyzed by real time PCR in three complex (TSB, TSB+Fe, BHI) media and one minimal medium (CGXII). Expression levels were determined for each medium separately which showed a considerable increase in TSB+Fe and only a moderate increase in BHI. Expression decreased in CGXII. Error represent standard deviations obtained from two biologically independent experiments.

Next, we compared expression level of ORF 855 at pH 5.7 in TSB+Fe, CGXII and BHI with TSB at pH 5.7. At acidic pH the mRNA level decreased in all three media compared to TSB. In TSB+Fe and BHI decrease in mRNA expression was modest, but at drastic decrease was observed in CGXII compared to TSB. The same comparison was performed at neutral pH. At pH 7.5 we also observed a decrease in mRNA level in all three media with a considerable decrease in mRNA level in CGXII and TSB+Fe, but only a slight decrease in BHI compared to the expression level detected in TSB at neutral pH (Fig. 3.14).

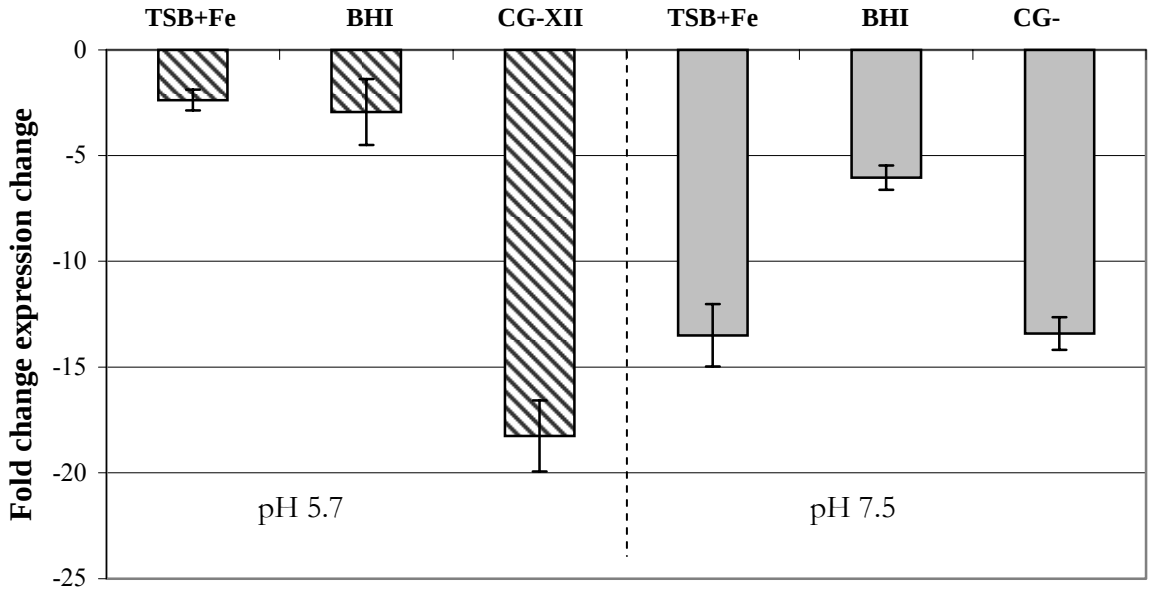


Fig. 3.15 Bioavailability of iron in complex and minimal medium

mRNA level of ORF 855 in three media (TSB+Fe, BHI, CGXII pH 7.5 and 5.7) were compared to mRNA levels in TSB pH 7.5 and 5.7. At pH 7.5 a considerable decrease in expression level in CGXII and TSB+Fe was noticed. At pH 5.7 expression level further decreased in CGXII, however it increased dramatically in TSB+Fe.

3.3.5 Influence of acidic pH on the growth of the disruption mutants

To investigate the function of the up regulated genes in acid adaptation, single crossover mutants of 17 significantly up regulated ORF were generated (Table 3.3).

ORF	NCBI no.	Annotation	Ratio[a]
854	NCgl0377	hypothetical membrane protein	7.05
857	NCgl0380	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe3+-siderophores transport systems, ATPase components	4.43
927	NCgl0430	bacterial regulatory protein, predicted arsR family transcriptional regulator	4.87
3347	NCgl1646	hypothetical protein	4.29
3549	NCgl0482	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe3+-siderophore transport system	5.06

ORF	NCBI no.	Annotation	Ratio[a]
3550	NCgl0483	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe3+-siderophore transport system	4.36
3551	NCgl0484	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe3+-siderophore transport system	4.32
3452	NCgl2965	similar to permeases of the major facilitator superfamily	5.42
2920	NCgl2450	involved in propionate catabolism	35.16
1169	NCgl0636	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe3+-siderophores transport systems	4.99
1170	NCgl0637	ABC-type transporter, permease component, similar to ABC-type cobalamin/Fe3+-siderophores transport systems	5.58
3544	NCgl0382	hypothetical membrane protein	6.23
518	Ncgl0123	hypothetical protein	6.99
1346	NCgl0773	siderophore-interacting protein	7.40
1703	NCgl1075	DNA-directed RNA polymerase specialized sigma subunit sigB similar to sigma 70 / sigma 32	2.03
2000	NCgl1844	DNA-directed RNA polymerase specialized sigma subunit, similar to sigma 70factor (ECF subfamily) and sigma 24	2.17
1632	NCgl1012	Mg-chelatase subunit ChlI	4.12

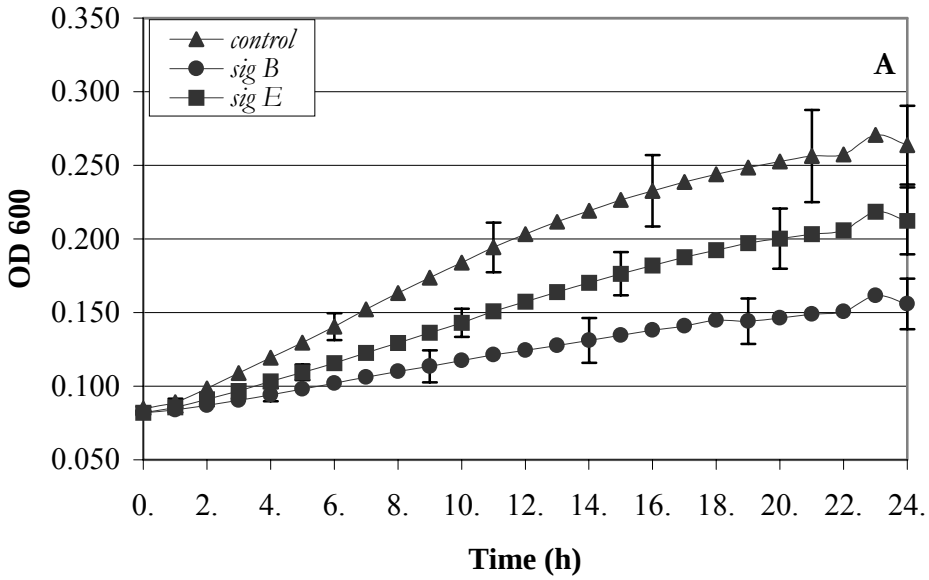
Tab. 3.4 ORFs showing at least a two fold increase in transcription levels in response to acid adaptation at pH 5.7.

[a] ratios are the average of four microarray experiments.

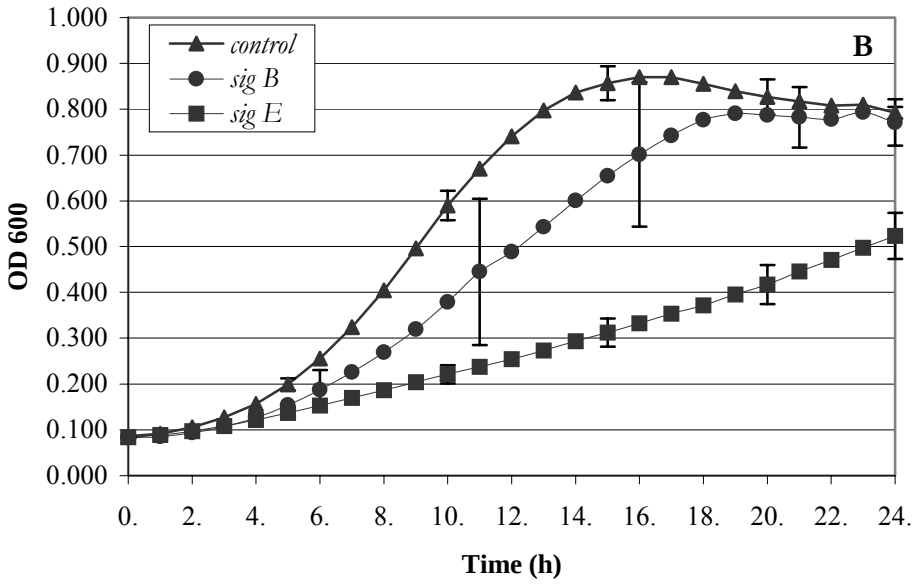
To test for the mutants' pH sensitivity growth experiments were performed at pH 7.5 and 5.7 in a honeycomb microplate reader Bioscreen C. The wild type strain contained plasmid pWLQ2 to exclude the effect of the kanamycin resistance genes used in creating the disruption mutants.

Only two mutants, *sigB* and *sigE*, showed a decreased growth when grown in pH 5.7 compared to the wild type strain (Fig. 3.15 A, B). As shown in figure 3.15 A, the *sigB* mutant was more susceptible to pH 5.7 compared to *sigE* mutant, whereas at pH 7.5 sigE's (Fig. 3.15 C) doubling time was about three fold higher compared to the other two strains. This suggests that *sigE* has an effect under normal condition but it is not required growth under stressed conditions whereas, *sigB* is primarily responsible for the acid tolerance response in the acid adapted *C. glutamicum* cells.

***C. glutamicum* pH 5.7**



***C. glutamicum* pH 7.5**



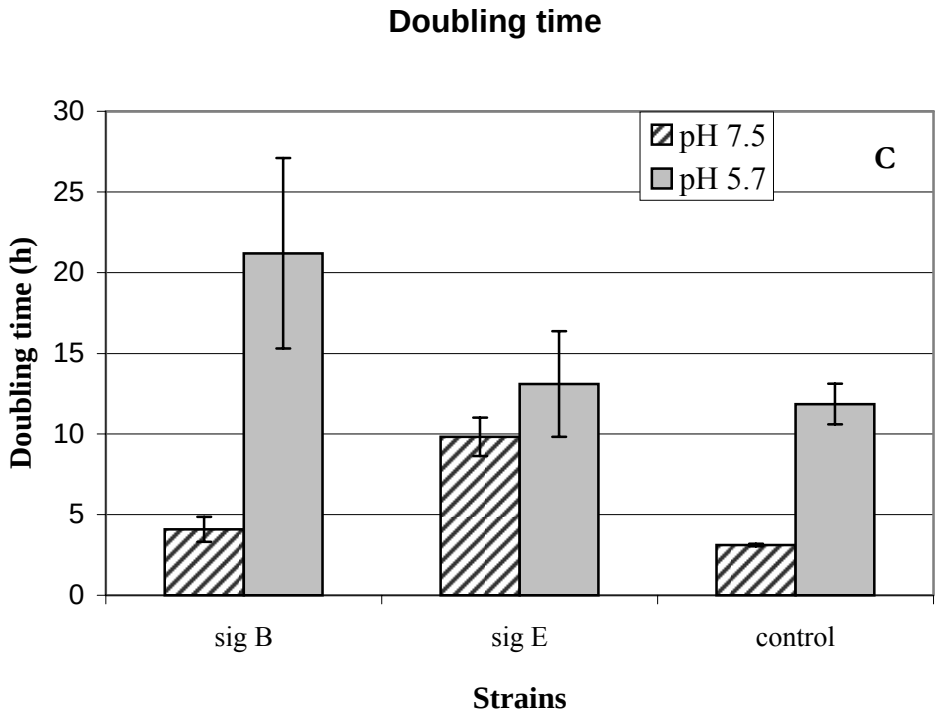


Fig. 3.16 Growth curves and doubling times of *C. glutamicum*, *sigB* and *sigE* mutants

Cells were precultured overnight in TSB pH 7.5, 50 fold diluted and inoculated in **A** pH 5.7 TSB and **B** pH 7.5 TSB. Panel **C** shows the doubling times of the three strains at pH 5.7 and 7.5.

To further analyze the response of the mutants to acidic pH, acid shock experiments were performed. Exponentially growing cells were harvested, resuspended in pH 4 TSB and shocked for 30 minutes. Samples were taken before and after acid shock and survival percentage was determined by serial plating. Aside from *sigE* and *sigB* mutants there were no significant differences observed in the acid shock response and pH sensitivity of the disruption mutants showing a possible redundancy in the acid stress response of *C. glutamicum*. The *sigE* mutant exhibited a 10 fold and the *sigB* mutant 1000 fold reduction in survival compared to the wild type strain (Fig. 3.16). Therefore, the obtained results strongly confirm our hypothesis that *sigB* plays a major role in the regulation of pH adaptation response.

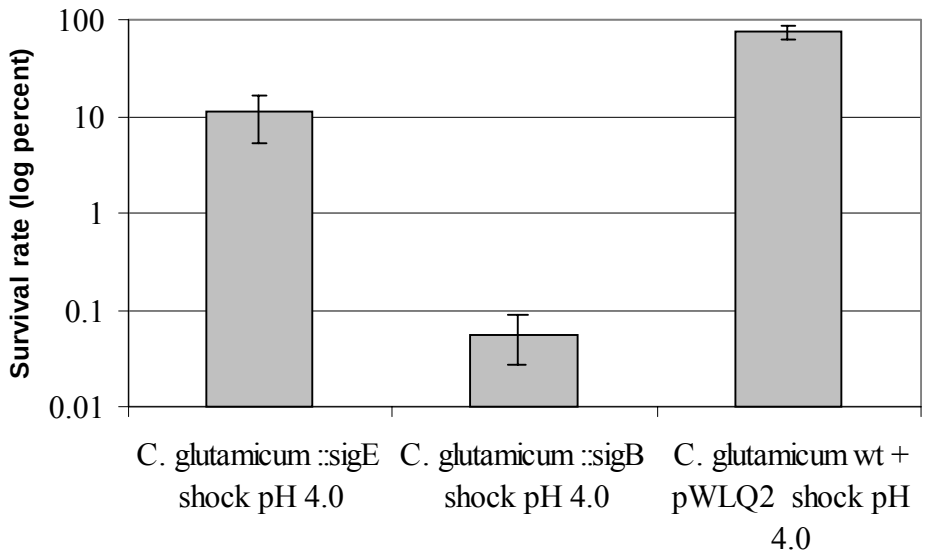


Fig. 3.17 Acid shock of *C. glutamicum*, *sigB* and *sigE* mutants

Overnight cultures were inoculated in pH 7.5 TSB and were allowed to grow to mid logarithmic phase (OD_{600} of 0.5). Cells were harvested at 5000 *x g* and suspended in pH 4 TSB. Survival percentage was calculated by comparing the cell counts obtained following acid shock to those in the original pH 7.5 cell suspension prior to acid shock.

4 Discussion

Given that cheese ripening is a complex, long biochemical and at the same time an expensive process, acceleration of cheese ripening has received considerable attention in the scientific community. One way of accelerating this process is through the selection of better adapted species to the cheese environment. Strain improvement through the use of natural selection is one of the most effective ways in obtaining microorganism adapted to specific environmental conditions. This method is also plausible for the food industry since the mutant strains obtained by this method are widely accepted as less significant process changes and are allowed for consume. Up to now, studies performed in accelerating cheese ripening concentrated on modifying the starter culture added during cheese production. To our knowledge modification of ripening bacterial species existing in the smear flora were not performed.

4.1 Adaptation of cheese ripening bacterial species to low pH

Before application of the secondary flora, depending on cheese variety, acidity of the cheese ranges between pH 4.5 and 5.3 (10). This low pH has an effect on the growth of acid sensitive bacterial species found in the secondary flora. The acidity of the cheese is due to the accumulation of organic acids such as lactic, acetic and propionic acid where lactic acid is the least and propionic acid is the most effective inhibitor. However, it has been shown that lactate in cheese curds is present at much higher concentration than either of the two other acids (10). Therefore in this work, we used lactic acid to adapt four ripening bacterial species (*C. ammoniagenes*, *C. variabile*, *S. equorum* and *K. palustris*) to low pH.

We used natural selection in adapting the species to low pH by gradually exposing the species to low acidity. It has been shown that constant exposure of microorganisms to acid with time will lead to acid resistant strains through the induction of gene synthesis. This effect has been demonstrated in numerous organisms such as *S. mutans* and *E. hirae* (8) where the adaptation of the above named organisms in a continuous culture had given rise to strains that were acid adapted after being exposed to acidic conditions for only one generation (18). It has been also shown that prolonged exposure to stress leads to changes in genome structure ranging from simple alteration in DNA sequences, such as point mutation, to dynamic events in the genome structure (161). This is demonstrated by a study performed in *E. coli* using a semi-continuous culture propagated for 10,000 generations. In examining the genetic structure of the obtained mutants genomic rearrangements including transpositions, rather than point mutations were detected (113).

In adapting the species, we cycled the mutants between solid and liquid culture imitating conditions present during cheese production. The species were adapted to acidic condition at 30°C.

Strain/pH	No of pH adapted mutant strains obtained	No. of mutant strains growing at 30°C	No. of mutant strains growing at 15°C	No. of mutant strains showing stability at 30°C
C. ammoniagenes/pH 5.8	2	1	2	0
K. palustris/pH 5.8	3	3	0	1
S. equorum/pH 5.3	3	3	1	3
C. variabile/ pH 5.3	4	4	4	3

Tab. 4.1 Summary of pH adaptation results describing the effective number of mutant strains obtained for each bacterial species growing at their adapted pH and at different temperatures (30°C and 15°C) at the end of the adaptation experiment.

C. ammoniagenes and *K. palustris* were adapted to pH 5.8 whereas, *S. equorum* and *C. variabile* were adapted to pH 5.3. Repeated attempts to adapt the obtained mutant strains to lower pH were not successful.

Based on the growth characteristics of the mutant species it is noticeable that there are phenotypic differences between the mutant strains obtained from the same species. For *C. ammoniagenes* we obtained two mutant strains of which one mutant strain S7-3-3/a-6-1 was able to grow better at 30°C as the parent cell whereas the second mutant was not. However, both mutant strains were able to grow at 15°C better than the parent strain.

For *S. equorum* three mutant strains were obtained. Two of the mutant strains grew moderately better than the parent strain at 30°C and pH 5.3 however, at 15°C they grew worse at pH 5.3 than the parent strain. There were also differences observed in three *C. variabile* mutant strains which exhibited minor differences compared to mutant strain 16-1-1/e-2. Based on these results we can assume that the mutations accumulated in the strains imparted different phenotypic characteristics to them. Differences in phenotypic characteristics among three parallel culture of one species were also made in a study improving the cryotolerance of *L. delbrueckii* by natural selection (105).

Although the selection process took place at 30°C it is observed for some species that acid adaptation has also improved their growth characteristic at 15°C. It is also interesting to

observe that some of the mutant strains exhibiting substantially positive phenotype compared to the parent cell at 30°C also demonstrate the same phenomenon at 15°C. These results would suggest that mutations that improved acidity have also improved growth at lower temperatures. It is also described in literature that exposure of a organisms to one stress leads to improve resistance to other stresses (145).

4.1.1 Mutation frequencies

Mutations may be associated with the change of a single nucleotide through substitution deletion or rearrangement of one or more nucleotides in the chromosome. Most mutations occur at a low frequency, 10^{-5}-10^{-10} per generation, at any point along the gene but certain mutation lead to a organism that is better adapted to its environmental and improve its biocatalytic performance (114).

The mutation frequency at pH 6 has been estimated for each of the species by comparing the ratios of the mutants in the total cell population. As observed in Table 3.1 the mutation frequency ranged from 10^{-3} to 10^{-1} representing higher mutation frequency as the one documented in the literature. Most of the documented frequencies were obtained in strain improvement experiment using chemical mutagenic agents such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG). MNNG induces mutations by the transition pathways 99% of the time and 95% of these are GC to AT transitions thereby maximizing the frequency of desired mutations (5). Under these conditions the mutation frequency typically ranged from 10^{-3} to 10^{-4} assessed through the use of antibiotics, e.g. rifampicin known to cause mutations in the *rpoB* gene encoding the beta subunit of RNA polymerase, whose mutation rates have been estimated in a number of species (74). Using chemical mutagenic agents several strain were obtained with increasing production for bacterial metabolites such as erythromycin analogues, penicillin, and amino acids (38, 130). However, there are disadvantages in using chemical mutagenic agents namely the increase of undesirable mutations caused by the accumulation of numerous random mutagenic events due to its non-targeted effect.

In using lactic acid the mutation frequency is higher compared to chemical mutagens, but at the same time it acts as a specific agent in the selection process by imparting a target oriented mutagenesis. It can be assumed that spontaneous mutations, using specific stressor, have a tendency to create mutations in a certain area of the genome affecting genes under selection. This phenomenon has been previously demonstrated in a screening experiment generating spontaneous β-gal mutants of *L. bulgaricus*. The mutants obtained under the X-gal selection pressure demonstrated DNA rearrangements giving rise to different deletions located predominantly within the β-gal gene, and in one case, causing the deletion of the entire

gene (104). Therefore, the higher mutation frequency is probably due to the extreme redundancy resulting from numerous changing producing a pH resistant phenotype.

4.1.2 Disadvantages of natural selection

There are numerous disadvantages associated with natural selection which lies not only on the process but also on the selection method. It is documented that during natural selection numerous uncharacterized spontaneous mutations occur in the organisms' genome. It has been estimated by Chao & Cox that the rate of advantageous mutation is $5x10^{-9}$ or five advantageous mutation equivalents per generation (39). It has also been demonstrated in *E. coli* cells that 0.1% of mutations taking place during generations are advantageous and the rest have a neutral effect on the organism (39). Repeated exposure can also result in the accumulation of deleterious mutation off-setting the effect of desired mutation. This can be observed in our study where some of the mutant strains exhibited worse phenotypic characteristics compared to the parent cell. The appearance of the deleterious mutations can be explained the following way. Due to prolonged stress conditions a new mutator allele arises termed a stress induced mutagenic allele. These stress induced mutator allele produces more mutation than a constitutive allele therefore, the beneficial mutations accumulated in the constitutive allele have a greater chance of being lost in genomes loaded by deleterious mutations (141). For this reason a large number of strains need to be screened in order to find a mutant strain with the desired phenotype which requires a vast amount of time and resources. In generating our mutants we faced the same difficulties as generation of mutants was species depended. The easiest to adapt was *C. variabile* and the most difficult was *K. palustris*. This effect is also demonstrated by the number of mutant that we needed to screen for each of the bacterial species.

The next challenge of natural selection is the stability of the mutations. Most genetic changes are relatively stable and are detected as a mutation through mutant phenotype. However, it has been estimated that one in 10^3 cells in a population may carry a duplication of a chromosome segment. The amplification of this region is not a stable genetic change and reversion of mutation occurs within one generation when cells are grown under non-selective conditions (18). As shown in Table 4.1 some of the mutants have lost their beneficial characteristics at growing at low pH after cultivated at neutral pH for 24 h which could be due to the above mentioned reversion. Therefore, in order to sustain the desired phenotype the organisms must be always under the selection pressure that was used to generate the mutant species. It was also noticed that reinoculation of the mutant species from one plate to another has also caused the loss of the phenotype although the mutant species was always under

selection pressure. This phenomenon has been described in literature and to counteract this phenomenon the mutant species once generated need to be frequently reisolated. This has been described for a number of industrial production organisms such as *Ashbya gossypii* that overproduces riboflavin and *Penicillium chrysogenum* a penicillin hyperproducer (161). In case of *Ashbya* after prolonged reculturing the fungus has reduced its riboflavin production although it has improved its growth rate. When the selection pressure on penicillin production was decreased or interrupted, *Penicillium chrysogenum* reduced the copy number of its plasmid and as a consequence lost its high penicillin productivity.

Since classical strain selection creates unspecified mutations it would be interesting to analyze the mutants obtained from each of the strains to determine which genes have been affected by this process. It would be also interesting to establish if there are variations among the mutants in the affected genes. This information could provide a better overview as to what genes are affected under acid adaptive conditions thereby, paving the way for further studies in genetic manipulation of these organisms.

4.2 Real time analysis of ripening consortium

The microbial evolution of the species in the smear of soft ripened cheeses has not been fully investigated due to the inadequateness of methods that have been applied. In order to provide a better overview of species succession on the cheese surface new techniques needed to be developed. One of these techniques is quantitative real time PCR. In using real time PCR to monitor species succession proper primer design is one of its crucial steps. The primers not only have to meet the required guidelines described for real time PCR but they also have to distinguish between closely related species. Furthermore, the method also needs to be correlated to other conventional methods in order to be acceptable.

4.2.1 Identification based on 16S and 18S sequencing

Enumeration of dairy microorganism was previously based on bacterial cultivation followed by identification of the dominant microorganism through phenotypical methods. These methods are tedious, restricted to the cultivation of viable population and thereby providing erroneous information about population dynamics (58). Furthermore, nucleotide base sequences provide tangible evidence of the identity of a bacterial isolate compared to sometime subjective interpretation of phenotypic observations (125). For these reasons molecular approaches based on 16S and 18S rRNA genes have facilitated a culture independent approach for analysis of complex ecosystems. In sequencing the 16S and 18S rRNA genes of the ripening consortium there was a high degree of similarity observed in the sequences of the ripening bacterial species e.g. between *C. ammoniagenes* and *C. variabile* and between *S. equorum* and *S. warneri*. There were nucleotide base differences observed in the sequences, therefore the primers were positioned in these areas. The primers were designed in such a manner so that the non conservative sites were located at the 3' end of the primers. As shown in Fig. 3.1 the primers designed were specific and amplified only the desired ripening species. Therefore, we successfully designed primers that were able to distinguish between closely related species.

4.2.2 Detection limits using real time PCR

Specificity, a key factor for accurate quantification of the bacteria of interest, was determined in performing the reaction with a mixture of genomic DNA obtained from the six species used in the study and analyzed for unspecific products. Nonspecific signals were not detected in the reaction. The reaction was also tested for inhibition due to competitive binding

of a nontarget template in the PCR reaction. In this case, there was also no inhibition observed with increasing non template concentration in the PCR reaction.

Detection limits varied from species to species ranging from 10^2 CFU to 10^3 CFU, corresponding to 8 to 80 CFU per PCR reaction, respectively. The variability in the detection limit is probably due to the primer binding region or to the secondary structure occurring in the template. However, our detection limits correspond to the detection limits reported in other studies (98, 111).

The real time quantitative PCR results correlated adequately with enumeration obtained by culture dependent method. In comparing the total aerobic count obtained by real time PCR and serial plating results there was an insignificant difference of 0.5 log units between the two methods. Compared to quantification by determination of viable counts, the quantification of *D. hansenii* by real time PCR yielded approximately 100 fold higher numbers at the first 2 sample points. This is probably due to the yeast cells entering a growth phase on the first day of ripening and the possibility of detecting dead cells via PCR.

4.2.3 Monitoring of ripening consortium by quantitative real time PCR

We used real time PCR to monitor and quantify the progression of six ripening consortia on a model cheese. The species chosen were representative of microorganism occurring on smear ripened cheeses. In monitoring the progression of the species at 15°C at a RH of 90% for 11 days it was noticed that *S. equorum* was the dominant species, followed by *C. variabile* and *C. ammoniagenes*. The least dominant species in the microbial consortium was *K. palustris*. *D. hansenii*, counts decreased on day 1 increasing on day three after which remained constant during ripening (Fig. 3.8).

The trends observed under this condition are similar to trends observed under other model cheese studies (11, 14). There have been some studies performed on ripening using a model cheese smeared with a defined microflora consisting of 5 species. Under these conditions, it was observed that *S. equorum* was the dominant species for the first two weeks of ripening. The coryneforms species included in the smear dominated only in the later stages of cheese production at which points a decrease in *S. equorum* cell counts were observed (11, 14). The results of these studies confirm the results obtained in our study since the cheese agar plates were "ripened" only for 11 days. There has been a slight increase in *C. variabile* cell count therefore we presume that after prolonged ripening conditions *C. variabile* would have reached levels reported in literature (15).

It is interesting to notice the decline in cell count of *K. palustris*. It has been previously shown that micrococci represent the smallest percentage among the microbial flora isolated

from surface smeared cheeses. This result demonstrates the possibility that the isolation of this organism is overlooked due to its low cell count present on the cheese. Up to now the largest percentage (20 %) of micrococci have been reported in a French smear ripened soft cheese, Chaumes (12).

4.2.4 Comparison of alternative methods to real time PCR used to study microbial succession

Up to now there have been few studies performed reporting species succession during cheese ripening (21, 45, 46, 100). These studies reported that coryneforms (~80%) are the dominant species and coagulase negative staphylococci comprise about 5% of the species detected in the ripening periods (13). The statistical information is dependent upon the cheeses examined in the studies. However, most of these studies in enumerating microbial communities were performed using culture based techniques (FT-IR macrospectroscopy) or molecular techniques such as single strand conformation polymorphism (SSPC), denaturing gradient gel electrophoresis (DGGE) and random amplified polymorphic DNA (RAPD) (21, 45). Some of these techniques such as RAPD, DGGE and FTIR require isolation and cultivation of single components in order to obtain valid statistical analysis. Therefore, they still provide biased information. SSCP can detect various dominant species in a consortium, however it provides semiquantitative information about the development of species in the microflora. Real time PCR makes possible the monitoring of various microbial species in a single reaction, therefore providing information about the development of individual species in the microbial community. However, it requires prior knowledge about possible presence of microorganisms in the microflora in order to design primers for the detection.

There have been some culture independent methods that have been used to monitor and study the spatial distribution of individual species in microbial ecosystems under various environmental conditions. One of the well known methods is fluorescent in situ hybridization (FISH). FISH uses fluorescently labelled oligonucleotide probes targeting 16S rRNA and allows in situ identification of individual microbial cells (2). This method like real time PCR has been implemented under several environmental conditions (42, 43). The accuracy of FISH compared to culture based methods is at least 1 magnitude (2) and it depends on several factors namely probe penetration, and physiological status of the cell. Probe penetration possesses a problem in gram positive organisms despite of available optimized protocols (106). The accuracy in quantification in FISH is also depended upon the abundance of 16S rRNA whose availability is growth phase depended. A slow growing organism can be

overlooked due to the low copy level of its rDNA content leading to false negative results. This effect can also pose challenges in real time PCR however, there have been numerous published studies using the 16S rDNA in quantification of organisms (125) demonstrating good correlation to culture based methods. The same effect can be also observed in our study which has yielded a Pearson linear correlation test of 0.929 demonstrating a positive correlation between culture based counts and real time PCR. FISH cannot compete with the speed of real time PCR analysis. However there is only one documented study implementing this method in cheese.

Another method studying population dynamics is length heterogeneity PCR (LH-PCR). It enables the detection of dominant species in microbial community. This method has a fairly high detection limit of 10^5 CFU/ml according to some studies (90). According to other studies an extensive analysis of the complete diversity of soil microbial community was not possible by LH-PCR (127).

Microarray is a new technique used in studying population dynamics in mixed cultures (162). The development of community genome arrays allows the identification of microorganisms in microbial communities at species and in some cases at species level. Up to this point there are very few applications studies are available but preliminary data performed with mixed DNA cultures from different bacteria suggest a strong correlation observed between hybridization signals and from target DNA in pure culture and target DNA in mixed culture suggesting a possibility to quantitate microorganism in environmental samples. However, stringent conditions must be used in detecting bacterial organism in mixed microbial community to minimize potential cross hybridizations.

4.2.5 Implementation in the dairy industry

Production of smear ripened cheeses generally involves the practice of old young smearing where the developed microflora of ripened cheeses is transferred to young unripened cheeses. This process not only transfers desirable microorganism but it also enables transfer of harmful pathogens such as *Listeria monocytogenes*. In modern industrial production this step has been replaced by inoculation with controlled ripening cultures to meet sanitary requirements and to maintain reproducible organoleptic properties of the cheese along time. However the development of a defined smear is still in progress. Real time PCR is a fast molecular technique that can help in understanding species development under controlled condition and assisting in the development of an optimal smear flora.

4.2.6 Comparison of pH adapted and control ripening species on model cheese

Wild and mutant strains were inoculated onto the model cheese and monitored for their development during the ripening process using real time PCR. For this study mutant species were selected based on the growth characteristics observed under 15°C and 30°C. In comparing the ripening process of the control to the mutant species the trends observed between the two ripening processes were different. The dominant species at the end of the ripening period was *C. variabile* followed by *S. equorum*. Based on this result one can assume that the accumulated mutations improving the acid tolerance in *S. equrom* have decreased its capabilities under biofilm condition.

One of the mutant species *K. palustris* exhibited significant differences compared to parent cell (Fig3.10). There were no significant differences observed by the other ripening species is could be the result of a number of factors. One of them is that the studies were performed on a model cheese where the behavior of the species can deviate from normal behavior observed on cheese. These differences can also be attributed to the components of the model cheese. Another reason lies in the usage of the selected mutants. We only selected the mutants showing a robust phenotypic characteristic for this experiment. However, this cannot exclude that the other mutants behavior would be worse in cheese production. Up to this point the mutant species were not tested under industry settings therefore, it is unknown whether the mutations have a selective advantage/disadvantage concerning important technological properties such as aroma production.

4.3 Acid adaptation in *Corynebacterium glutamicum*

Acid stress is the combined effect of low pH caused by inorganic acids and organic acids present in the environment (7). *C. glutamicum* encounters a wide range of external pHs in its natural habitat and during industrial processes. Growth at a pH substantially lower than the cytoplasmic pH induces protective responses with two fundamentals aims: to maintain internal pH homeostasis and to initiate the expression of regulatory proteins preventing and repairing damages caused by acid stress. *C. glutamicum* is a neutrophile and the effects of acid stress on its cell physiology have not been studied in detail. In this study we used DNA microarray to study the genome-wide expression of pH adapted *C. glutamicum*. We found a total of 116 up and 90 down regulated ORFs. In some cases, it is possible to deduce or suggest the functions of the up regulated genes, and in other cases, the rationale for change of expression is at present obscure. In this study we have shown that *C. glutamicum* can maintain its cytoplasmic pH in a relatively broad range of external pH. We have also found the expression of a number of genes relevant to prevention and repair mechanism under acid stress. In the following sections their relevance in acid adaptation is discussed.

4.3.1 Induction of cation and multidrug transport systems

Bacteria utilize various mechanisms to maintain pH homeostasis within the cell. One of the well-described mechanisms is the F_1F_0 ATPase which either produces ATP using the transmembrane proton gradient through proton influx (acidification of the cytoplasms) or expels protons from the cell using the energy provided by ATP hydrolysis (32). In aerobic organisms such as in *E. coli* and *B. subtilis*, the F_1F_0 ATPase mainly functions in ATP synthesis (102). In organisms without a respiratory chain such as *L. lactis* (86), *E. hirae* (85), *S. mutans*, *S. sanquis* (9) and *L. acidophilus* (88) the F_1F_0 ATPase system plays an important role in maintaining cytoplasmic pH homeostasis in acid adaptation via proton extrusion (55, 79). However, there are disadvantages in using this system for internal pH homeostasis due to its high energy cost and there is evidence suggesting the presence of other systems in bacterial species lacking a respiratory chain (16). While the down regulation of the F_1F_0 ATPase system in *C. glutamicum* may result in a decrease of acidification of the cytoplasm, it will also decrease ATP synthesis. We therefore believe that its down regulation does not play a major role in cytoplasmic pH homeostasis and may rather be due to a decreased need in ATP synthesis in acid adapted cells which slows down their growth rate.

Among the transport proteins induced through acid adaptation, several cation transport ATPase genes (ORF 851, 2433 and 2905) were observed in our study. While it is unknown which cations are transported by the proteins encoded, cation transport ATPases such as K^+ -or Na^+ - ATPases have been described in various organisms such as in *L. lactis* (81) and *S. mutans* (35) as constituting one of the systems responsible for pH homeostasis by acidifying or alkalinizing cytoplasmic pH through a cation – proton antiport mechanism (17).

Increased expression of a putative multidrug transport system was also observed. Being a soil bacterium *C. glutamicum* encounters a wide variety of cytotoxic compounds in its natural habitat which lead to the development of various protective mechanisms that can export a wide range of toxic components before these molecules have a chance to damage cellular processes. It is suggested that multidrug efflux system (MDR) are part of the natural defense mechanism of bacteria against toxic compounds, e.g. lipophilic inhibitors, existing in the natural environment (110). The broad substrate specificity of these efflux pumps makes them suitable for this defensive role as the bacteria cannot predict the nature of inhibitors it will be confronted with (117). Due to this broad substrate specificity of the MDR efflux pumps we suggest that this system could also play a role in pH resistance of *C. glutamicum* by expelling the accumulated anionic form of the dissociated organic acid from the cytoplasm.

4.3.2 Iron transport is induced at low pH

Numerous iron transport genes were induced by acidic pH. These ORFs are organized in 4 putative operons encoding components for the iron Fe^{3+} ABC siderophore transporters. Iron siderophores are extracellular iron chelators secreted by bacteria under iron limiting conditions to help solubilize iron prior to transport (87). According to literature, at aerobic conditions and neutral pH, iron is present in the insoluble form of Fe^{3+}, whereas low pH increases solubility of iron by reducing it to Fe^{2+} thereby, boosting bioavailability of iron in the medium (65). Our study in complex media (tryptic soy broth, TSB) assessing the iron availability at low pH proved contradictory to the expected theoretical effect. Expression level of ORF 855 encoding a periplasmic component of an ABC-type cobalamin/Fe^{3+}-siderophores transporter, at pH 5.7 substantially increased in different complex media (BHI, TSB, TSB+Fe) compared to expression levels at neutral pH (Figure 4). On the other hand, in defined minimal medium (CGXII) at acidic pH, ORF 855 showed decreased expression level, verifying the theory on pH-dependence of iron bioavailability in the biological experiment. These results suggest an iron arrestive effect of the complex media at acidic pH.

In the second experiment we compared the mRNA level of ORF 855 measured in BHI, CGXII and TSB+Fe to the mRNA level in TSB. In this experiment we observed a

decrease in expression level at neutral pH in three media (BHI, CGXII, TSB+Fe) compared to TSB. The low mRNA level of ORF 855 in CGXII and TSB+Fe compared to TSB was considerable due to the relatively high amount of added iron. In BHI the expression level was also moderately lower compared to TSB, presumably due to the higher iron content of BHI resulting from the ingredients present in this medium. In summary, at neutral pH the iron supplement in TSB media (TSB+Fe) repressed the expression of the iron siderophore transporter gene (ORF 855).

At acidic pH in CGXII the expression level of ORF 855 was substantially lower compared to that in TSB. This result is in accord to the iron excess in CGXII medium at acidic pH. In TSB+Fe medium, despite iron supplement, we observed only a slightly lower mRNA level of ORF 855 compared to TSB medium. In BHI medium we found the same effect as in the iron supplemented TSB medium (TSB+Fe), indicating the possibility, that this effect is presumably coupled generally to the complex components of the rich media. This led us to suggest that the theoretical facts about the pH effect on the bioavailability of iron are demonstrated in a defined minimal media, but not in complex media. Therefore, the up regulations of the iron Fe^{3+} ABC siderophore transporters observed in our microarray experiment are presumably the result of the complex medium which effects iron availability at low pH, and renders inadequate level of available iron for uptake at acidic pH.

4.3.3 DNA repair in acid adaptation

It is documented that acid stress causes damage to DNA (7). In our array experiment we observed an elevated expression of an ATPase involved in DNA repair (ORF 2607). At the same time, we also observed the upregulation of ribonucleotide reductase genes *nrdE*, *nrdI*, and *nrdH* (ORF 3467, 2930, 2929 respectively). In *C. ammoniagenes* Nrd's are arranged in an operon following the pattern of *nrdH-nrdI-nrdE* and they function in dNTP synthesis in DNA replication and repair, however their concrete function are unclear (142). Attempts to create disruption mutants for *nrdI* and *nrdE* were not successful, suggesting an essential role in cellular function proving previously published attempts (142). Previous studies demonstrated that expression of the *nrd* genes is cell cycle dependent reaching highest expression at exponential phase (61, 76). In acid adaptation experiment exponentially growing control cells at neutral pH grew faster than adapted cells at acidic condition however, there was a clear difference in expression levels observed in acid adapted and non-adapted cell. Therefore, the enhanced expressions of the genes in the *nrd* operon additionally to the DNA repair protein, suggest a cellular response in combating DNA damage.

4.3.4 Role of regulatory proteins in acid adaptation

Regulatory proteins play an important role in stress response by directing protein expression in response to environmental fluctuations. We observed induction of six regulatory proteins (ORF 927, 1518, 1642, 1703, 2000 and 3470) during acid adaptation.

SigB (ORF 2000) is strongly induced in *C. glutamicum*. Its role as a general stress protein has been extensively studied in gram positive organisms under different stress conditions. Null mutations in *B. subtilis' sigB* lead to an increased sensitivity towards low pH, heat and oxidative stress (68). In *Listeria monocytogenes*, a function of *sigB* has been demonstrated in response to several stresses such as oxidative stress, osmotic stress, carbon starvation and growth at low temperatures: e.g., a *sigB* null mutant exhibited a 1000 to 5000-fold decrease in survival when exposed to pH 2.5 (155). Studies in *Brevibacterium flavum*, a closely related strain of *C. glutamicum*, showed an effect of *sigB* on growth and viability of cells under acid, salt, alcohol, heat and cold (63). SigB is therefore a general stress response protein and controls transcription of various stress related proteins in Gram positive bacteria.

ORF 1703 encodes a protein belonging to the extracytoplasmic function family (ECF), showing homology to SigE. SigE's role as an alternative sigma factor regulator was demonstrated, e.g., in *M. tuberculosis, B. subtilis* and *P. aeruginosa* under heat shock and oxidative stress conditions (121). In *M. tuberculosis* a *sigE* disruption mutant exhibited higher sensitivity to various environmental stresses compared to the wild type, but not to acidic pH (99, 122). Our phenotypic studies using the *sigE* disruption mutant also did not support its role in acid stress. We speculate that it is upregulated by stress factors originating as a consequence of pH stress.

ORF 3470 codes for a protein homologous to a magnesium dependent transcriptional regulator (MntR). MntR, first characterized in *B. subtilis*, is a member of the DtxR family of metalloregulatory proteins regulating the expression of metal ion transport systems in response to manganese (119). In vitro studies have shown that DtxR and its relatives respond to various divalent metals such as Fe^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Mn^{2+} which activate the DNA binding function of DtxR and MntR (133-135, 138). DtxR characterized in *C. diphtheriae* is a global iron regulator which regulates the expression of iron uptake genes as well as toxin production under iron limited conditions (118). The function of MntR has been studied in various microorganisms demonstrating a wide variety of effects on gene expression. In *B. subtilis* it has been shown that elevated Mn^{2+} levels disturb iron homeostasis leading to an increase in iron availability in the cell. This causes the activation of *sigB* stress response which is depended on the environmental stress sensor RsuB requiring Mn^{2+} as a cofactor (60). In *S. aureus* it has been shown that MntR has an effect on oxidative stress by controlling Mn^{2+}

uptake (73). DtxR and MntR differ in their metal ion selectivity as shown by protein sequence comparisons (59). Recently, an MntR homologue has been identified in *C. diphtheriae* (132). The role of DtxR like proteins in pH stress is unknown but may relate to the regulation of ion transporters of *C. glutamicum*, which are also induced under acid stress

Another regulatory gene that was up regulated is ORF 1518 encoding the sensory component of the two component histidine kinase system. Two component systems are relatively widespread in bacteria and are reported to assist bacterial cells in the adaptation to various environmental alterations (137). Two component systems are often involved in stress response by directing the synthesis of alternative sigma factors which are required for the synthesis of proteins involved in stress response (103, 108). An increase in the expression of histidine kinase regulators due to stress has been shown in other gram-positive organisms namely in *L. monocytogenes,* but a disruption mutant in the regulatory components of histidine kinase did not reveal differences in growth in presence of stress compared to the wild type strain (80). In *H. pylori* a similar effect was observed in studying the histidine kinase sensory component which demonstrated that deletion of the sensory component has no effect on the cells' function and its role can be overtaken by other genes (151). Similarly, in this study no significant effect was observed at low pH condition on the growth rate of the histidine kinase disruption mutant.

4.3.5 Redundancy of the pH adaptation response of *C. glutamicum*

In all of the 15 disruption mutants, no significant phenotypical effect at low pH conditions was observed except of the knock-outs of two regulatory proteins *sigB* and *sigE* (ORF 2000 and ORF 1703, respectively). This data suggest a considerable redundancy in stress response and, at the same time, demonstrates the complexity of the acid stress response in *C. glutamicum* where the function of the disrupted gene obviously can be overtaken by another gene product. Similar data have been reported for other bacteria, where effects of single insertion mutagenesis on acid stress have been studied. In studying the acid tolerance response of *S. typhimurium* inactivation of single genes known to contribute to acid stress exhibited only a marginal affect on acid tolerance (126). Other studies of *S. typhimurium* showed that inactivation of two or more genes was needed to eliminate acid resistance (56). Similar observations were also reported for *B. cereus* where one acid sensitive single knockout mutant was obtained despite screening 1.7 x 10^8 cells (23). This leads us to conclude that the genes responsible for acid adaptation have widely overlapping functions. Therefore, to obtain acid sensitive mutants, either the disruption of the regulatory protein controlling the

expression of multiple genes in the ATR is needed or more than one gene must be deleted in the genome.

5 Concluding remarks

Due to the long process of cheese production acceleration of cheese ripening is a very important issue for the dairy industry. In this study four ripening species were adapted to low pH. Different phenotypic characteristics were observed among the various mutants obtained from the same parent cell suggesting that mutation effected different genes. Except for one species (*K. palustris*), all other mutants obtained from the other species (*C. ammoniagenes*, *C. variabile*, and *S. equorum*) were able to grow at 15°C even though selection process occurred at 30°C. Identification of the affected genes contributing to the enhanced phenotypic characteristic of the mutants will provide beneficial information that could be later used as a target in genetic manipulation of the organisms. Presently, natural strain selection provides an acceptable alternative method of mutant generation that can be used by the dairy industry in food processing due to the tight regulation in use of genetically modified organisms.

In order to meet sanitary requirements cheese manufacturers are replacing traditional smearing practices with the inoculation of standard and controlled ripening cultures. In order to produce the desired organoleptic properties this defined smear cultures need to be individually designed for each cheese type. Therefore there is a requirement for a fast and accurate method providing information regarding microbial succession. In this study we have shown that under controlled condition the monitoring of microbial population is possible and it correlated well with conventional techniques. Under this condition real time PCR provides an ideal tool for the dairy industry to monitor microbial succession of individual species and help in the development of a defined smear ripening flora adjusted to the technological properties of a particular cheese.

Microarrays provide a global overview of transcriptomic changes in the acid adaptation of *C. glutamicum*. The data obtained in this study suggest a complex and redundant response of *C. glutamicum* since we identified 116 up and 90 down regulated genes. Only a minority of these genes have been described to play a role in other Gram positive bacteria which makes it difficult to delineate a general acid stress response for *C. glutamicum*. Apparently a variety of strategies can exist which gram positive bacteria use to cope with pH stress.

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7 Appendix

	REACTION STEPS	NOTES
DNA-MICROARRAY PREPARATION	**ACID ADAPTATION EXPERIMENT**	two independent fermentation
	EXPERIMENT CONTROL	two replicate RNA isolation
	total RNA — total RNA	
	Cy5-labeled cDNA — Cy3-labeled cDNA	random hexamer primed synthesis of fluorescently labeled cDNA
	competitive hybridization to DNA-microarray	DNA-microarray hybridization and washing
IMAGING	Cy5 signal intensity — Cy3 signal intensity	Fluorescent images at 635nm $I_{Cy5(i=x)}$ and 532nm $I_{Cy3(i=x)}$ using an GenePix 4000 laser scanner
	quantitative analysis — quantitative analysis	raw fluorescent data and analysis using softwareGenePix 3.0
NORMALIZATION	Cy5/Cy3 **RATIO** calculation, log_2 transformation	$\mathbf{R}_{Cy5/Cy3(i=x)} = I_{Cy5(i=x)}/I_{Cy3(i=x)}$ $\mathbf{R_{SN}}_{Cy5/Cy3(i=x)}$ - Cy5/Cy3 ratio of x-th spot on the slide N signal intensity median value
	total intensity **NORMALIZATION** between slides and dyes (Cy5 and Cy3)	Data were normalized to the average ratio of *C. glutamicum* genomic DNA
FILTER	**FILTER** low signal intensity, bad spot quality	**IF** ($I_{Cy3(i=x)}$ **OR** $I_{Cy5(i=x)} \geq 3*I_{Bkg(i=x)}$) **RIGHT** x-th spot were used in the further analysis **WRONG** x-th spot too weak, omitted $I_{Bkg(i=x)}$ - local background intensity value
	FILTER spots with nonsignificant changes	P values were calculated from replicated ratio values based on Student's t test using log transformed gene ratios and genomic DNAratios, which were normalized to zero **IF** ($P_{(i=x)} \leq 0{,}05$) **RIGHT** x-th spot were used in the further analysis **WRONG** nonsignificant, omitted $P_{(i=x)}$ - P values of the x-th spot on the four slides
	ANALYSIS	Analysis of gene expression data was performed by selecting genes showing at least twofold changed average mRNA level.

Tab. 7.1 DNA microarray analysis of acid adapted *C. glutamicum*

Group	ORF	NCBI no.	Annotation	Ratio
Transport protein	1382	NCgl0799	Na^+/proline, Na^+/panthothenate symporter or related permease	0.45
	1011	NCgl0510	ABC-type cobalt transport system, ATPase component	0.32
	1012	NCgl0511	ABC-type cobalt transport system, permease Component, similar to CbiQ and related transporters	0.33
	2042	NCgl1877	glutamate ABC-type transporter, permease component	0.42
	2092	NCgl1915	similar to ABC-type dipeptide/oligopeptide/nickel transport systems	0.31
	2093	NCgl1915	similar to ABC-type dipeptide/oligopeptide/nickel transport systems	0.33
	2094	NCgl1916	similar to ABC-type dipeptide/oligopeptide/nickel transport systems	0.41
	3744	NCgl2562	ABC-type transporter, periplasmic component, similar to ABC-type dipeptide/oligopeptide/nickel transport systems	0.44
	67061	NCgl2562	ABC-type transporter, periplasmic component, similar to ABC-type dipeptide/oligopeptide/nickel transport systems	0.47
	2095		strong similarity to oligopeptide ABC transporter (permease) oppC - Bacillus subtilis	0.40
Regulatory protein	1044	NCgl0536	translation initiation factor IF-1	0.50
	2648	NCgl1324	translation initiation factor IF3	0.45
	1562	NCgl0946	transcription elongation factor	0.42
Metabolic proteins	671	NCgl0251	Catalase	0.30
	670	NCgl0251	Catalase	0.38

Group	ORF	NCBI no.	Annotation	Ratio
	831	NCgl0360	succinate dehydrogenase/fumarate reductase, flavoprotein subunit	0.28
	832	NCgl0361	succinate dehydrogenase/fumarate reductase Fe-S protein	0.29
	976	NCgl0468	ribosomal protein L10	0.49
	978	NCgl0469	ribosomal protein L7/L12	0.50
	988	NCgl0476	ribosomal protein S12	0.46
	989	NCgl0477	ribosomal protein S7	0.40
	3553	NCgl0487	ribosomal protein L3	0.37
	3554	NCgl0488	ribosomal protein L4	0.41
	3555	NCgl0490	ribosomal protein L2	0.36
	3777	NCgl0491	ribosomal protein S19	0.40
	3778	NCgl0492	ribosomal protein L22	0.40
	992	NCgl0493	ribosomal protein S3	0.39
	993	NCgl0494	ribosomal protein L16/L10E	0.40
	994	NCgl0495	ribosomal protein L29	0.42
	999	NCgl0499	ribosomal protein L14	0.48
	1000	NCgl0500	ribosomal protein L24	0.41
	1020	NCgl0517	ribosomal protein L18	0.46
	1022	NCgl0518	ribosomal protein S5	0.41
	1023	NCgl0519	ribosomal protein L30/L7E	0.49
	1044	NCgl0536	translation initiation factor IF-1	0.50
	1046	NCgl0537	ribosomal protein S13	0.48
	1047	NCgl0538	ribosomal protein S11	0.49
	1048	NCgl0539	ribosomal protein S4	0.48
	1050	NCgl0541	ribosomal protein L17	0.48
	1069	NCgl0556	ribosomal protein L13	0.41
	1088	NCgl0572	co-chaperonin GroES, HSP10; chaperonin 10 Kd subunit	0.43
	1092	NCgl0573	chaperonin GroEL, member of the HSP60 family	0.47
	1377	NCgl0795	citrate synthase	0.48
	1421	NCgl0834	ribosomal protein L28	0.48
	1508	NCgl0902	general stress protein Ctc	0.47
	1807	NCgl1159	F0F1-type ATP synthase a subunit	0.25
	1808	NCgl1160	F0F1-type ATP synthase c subunit	0.25
	1809	NCgl1161	F0F1-type ATP synthase b subunit	0.33
	1810	NCgl1162	F0F1-type ATP synthase delta subunit	0.27

Group	ORF	NCBI no.	Annotation	Ratio
	1811	NCgl1163	F_0F_1-type ATP synthase alpha subunit	0.27
	1812	NCgl1164	F_0F_1-type ATP synthase gamma subunit	0.31
	1813	NCgl1165	F_0F_1-type ATP synthase beta subunit	0.31
	2768	NCgl1241	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase	0.41
	2675	NCgl1304	ribosomal protein S1	0.48
	2647	NCgl1325	ribosomal protein L35	0.46
	2425	NCgl1482	aconitase A	0.20
	2511	NCgl1546	orotidine-5'-phosphate decarboxylase	0.44
	2512	NCgl1547	carbamoylphosphate synthase large subunit	0.40
	2513	NCgl1548	carbamoylphosphate synthase small subunit	0.30
	2515	NCgl1549	similar to related cyclic amidohydrolases	0.37
	2516	NCgl1550	aspartate carbamoyltransferase, catalytic chain	0.46
	3792	NCgl2112	similar to heme/copper-type cytochrome/quinol oxidase, subunit 3	0.48
	3478	NCgl2436	phosphoserine phosphatase, contains ACT domain	0.44
	65297	NCgl2555	glucosamine-6-phosphate isomerase	0.41
	3737	NCgl2555	glucosamine-6-phosphate isomerase	0.45
	2095		strong similarity to oligopeptide ABC transporter (permease) oppC - Bacillus subtilis	0.40
Hypothetical proteins	787	NCgl0334	hypothetical protein	0.41
	830	NCgl0359	hypothetical membrane protein	0.29
	1013	NCgl0512	hypothetical membrane protein, similar to dihydrolipoamide dehydrogenase/glutathione oxidoreductase and related enzymes	0.28

Group	ORF	NCBI no.	Annotation	Ratio
	1381	NCgl0798	uncharacterized membrane protein	0.21
	1477	NCgl0878	hypothetical protein	0.46
	1545	NCgl0932	hypothetical protein	0.38
	1751	NCgl1114	hypothetical membrane protein	0.40
	2690	NCgl1294	hypothetical protein	0.45
	2402	NCgl1465	hypothetical membrane protein	0.41
	2546	NCgl1576	predicted membrane protein	0.48
	2547	NCgl1577	hypothetical protein	0.49
	3392	NCgl1676	hypothetical protein	0.42
	2111	NCgl1929	hypothetical membrane protein	0.46
	2112	NCgl1930	hypothetical membrane protein	0.42
	3191	NCgl2170	hypotheical protein	0.37
	3479	NCgl2435	hypothetical protein	0.39
	2850	NCgl2504	hypothetical protein	0.36
	219	NCgl2734	hypothetical protein, similar to ABC-type transporter permease component	0.46
	2844		hypothetical protein	0.33
	1380		hypothetical protein	0.47
	1332		hypothetical protein	0.49
	2406		hypothetical protein	0.49

Tab. 7.2 Down regulated ORFs in the acid adapted *C. glutamicum*

Group	ORF	NCBI no.	Annotation	Ratio
Transport proteins	851	NCgl0375	cation transport ATPase	2.38
	855	NCgl0378	ABC-type transporter, periplasmic component ABC-type cobalamin/Fe^{3+}-siderophores transport systems, periplasmic components	3.57
	856	NCgl0379	ABC-type transporter, permease component, similar to FecCD transport family proteins and ABC-type cobalamin/Fe^{3+}-siderophores transport systems, permease components	3.22
	857	NCgl0380	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems, ATPase components	4.43
	3549	NCgl0482	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe^{3+}-siderophore transport system	5.06
	3550	NCgl0483	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe^{3+}-siderophore transport system	4.36
	3551	NCgl0484	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe^{3+}-siderophore transport system	4.32
	1168	NCgl0635	hypothetical protein, similar to siderophore-interacting proteins	3.78
	1169	NCgl0636	ABC-type transporter, ATPase component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	4.99
	1170	NCgl0637	ABC-type transporter, permease component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	5.58
	1171	NCgl0638	ABC-type transporter, permease component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	2.09
	1173	NCgl0639	ABC-type transporter, periplasmic component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	6.84
	1346	NCgl0773	siderophore-interacting protein	7.40
	1347	NCgl0774	ABC-type cobalamin/Fe^{3+}-siderophore transport system, periplasmic component	10.53
	1349	NCgl0776	ABC-type cobalamin/Fe^{3+}-siderophore transport system, periplasmic component	3.82
	1351	NCgl0778	ABC-type cobalamin/Fe^{3+}-siderophore transport system, permease component	2.79
	1352	NCgl0779	ABC-type cobalamin/Fe^{3+}-siderophore transport system, ATPase component	3.58

Group	ORF	NCBI no.	Annotation	Ratio
	1516	NCgl0909	ABC-type transporter, ATPase component, similar to ABC-type multidrug transport system	3.18
	2779	NCgl1232	Co/Zn/Cd efflux system component	6.92
	2583	NCgl1379	predicted divalent heavy-metal cations transporter	2.91
	2433	NCgl1488	cation transport ATPase	3.31
	2534	NCgl1565	ABC-type transporter, periplasmic component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	2.12
	2146	NCgl1959	ABC-type transport systems, periplasmic component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	8.43
	2969	NCgl2352	ABC-type transporter, permease component, similar to ABC-type dipeptide/oligopeptide/nickel transport systems	2.11
	2905	NCgl2463	Na+/H+-dicarboxylate symporter	2.62
	3458	NCgl2970	ABC-type transport systems, periplasmic component, similar to ABC-type cobalamin/Fe^{3+}-siderophores transport systems	2.57
Regulatory proteins	927	NCgl0430	bacterial regulatory protein, predicted arsR family transcriptional regulator	4.87
	1518	NCgl0911	two-component system sensory transduction histidine kinase	2.47
	1642	NCgl1019	transcriptional regulator	2.26
	1703	NCgl1075	DNA-directed RNA polymerase specialized sigma Subunits, similar to sigma-70 factor (ECF subfamily); and sigma24	2.03
	2000	NCgl1844	DNA-directed RNA polymerase sigma subunit SigB, similar to sigma70/sigma32 factors	2.17
	3470	NCgl2441	Mn-dependent transcriptional regulator	2.40
Metabolic proteins	422	NCgl0049	NAD-dependent aldehyde dehydrogenase	2.31
	498	NCgl0106	lactoylglutathione lyase or related lyase	2.28
	727	NCgl0294	phosphoserine phosphatase	2.75
	777	NCgl0328	nitroreductase	3.23
	1432	NCgl0842	molybdopterin biosynthesis enzyme	2.17
	1491	NCgl0888	demethylmenaquinone methyltransferase	2.15
	1504	NCgl0899	Dioxygenase, similar to 2-nitropropane dioxygenase	2.14

Group	ORF	NCBI no.	Annotation	Ratio
	1558	NCgl0943 [a]	AraC-type DNA-binding domain-containing protein	7.69
	2578	NCgl1383	hydrolase of the alpha/beta superfamily	2.20
	2428	NCgl1485	predicted nucleoside-diphosphate-sugar epimerase	2.66
	2480	NCgl1521	ammonia permease	2.90
	2006	NCgl1848	hypothetical protein, similar to archaeal enzymes of ATP-grasp superfamily	2.47
	2149	NCgl1961	thiamine monophosphate synthase	2.09
	3056	NCgl2277	aldo/keto reductase, related to diketogulonate reductase	2.03
	3700	NCgl2297	malate/lactate dehydrogenase	2.67
	64895	NCgl2297	malate/lactate dehydrogenase	2.97
	3467	NCgl2443	ribonucleotide reductase alpha subunit	5.23
	2930	NCgl2444	ribonucleotide reduction protein	4.35
	2929	NCgl2445	glutaredoxin-like protein	3.48
	2828	NCgl2521	thiamine pyrophosphate-requiring enzyme, similar to acetolactate synthase, pyruvate dehydrogenase (cytochrome), glyoxylate carboligase,phosphonopyruvate decarboxylase	3.25
	279	NCgl2785	membrane-associated phospholipid phosphatase	2.54
	309	NCgl2813	predicted flavoprotein	2.09
	1894	NCgl2908	putative mercuric reductase, similar to dihydrolipoamide dehydrogenase/glutathione oxidoreductase and related enzymes	2.94
	1922	NCgl2934	hypothetical protein, similar to phosphotransferase system mannitol/fructose-specific IIA domain (Ntr-type)	2.21
	3438	NCgl2956	hypothetical protein, similar to sugar phosphate isomerases/epimerases	2.40
	3439	NCgl2957	myo-inositol dehydrogenase, similar to predicted dehydrogenases and related proteins	2.12
	3452	NCgl2965 [a]	hypothetical membrane protein, similar to permeases of the major facilitator superfamily	5.42
Hypothetical proteins	518	NCgl0123 [a]	hypothetical protein	6.99
	534	NCgl0138	hypothetical protein	2.31
	546	NCgl0148	hypothetical protein	2.32
	588	NCgl0191	hypothetical protein	3.25
	748	NCgl0308	uncharacterized phage-associated protein	3.47
	854	NCgl0377 [a, b]	hypothetical membrane protein	7.05
	3543	NCgl0381 [a]	hypothetical membrane protein	17.53

Group	ORF	NCBI no.	Annotation	Ratio
	3544	NCgl0382	hypothetical membrane protein	6.23
	1059	NCgl0549	hypothetical membrane protein	2.04
	1080	NCgl0565	putative membrane protein	2.46
	1106	NCgl0584	hypothetical membrane protein, similar to putative stress-responsive transcriptional regulator	2.42
	1152	NCgl0623 [a]	hypothetical protein	5.45
	1168	NCgl0635 [b]	hypothetical protein, similar to siderophore-interacting proteins	3.78
	1239	NCgl0691	hypothetical protein, similar to dihydrofolate reductase	2.17
	1293	NCgl0734 [a]	hypothetical protein, similar to transcription factor WhiB	2.82
	1495	NCgl0891	hypothetical protein	2.26
	1540	NCgl0927	hypothetical protein	2.62
	1541	NCgl0928	hypothetical membrane protein, similar to esterases	2.46
	1631	NCgl1011	hypothetical protein	3.28
	1704	NCgl1076	hypothetical protein	2.06
	1718	NCgl1090	hypothetical protein	2.07
	1851	NCgl1197	hypothetical protein	2.14
	2706	NCgl1287	hypothetical protein	2.26
	2705	NCgl1288	hypothetical protein	3.23
	2703	NCgl1289 [a]	hypothetical protein	4.88
	2645	NCgl1327	hypothetical protein	2.24
	2579	NCgl1382	hypothetical protein	2.16
	2431	NCgl1487	hypothetical protein	2.07
	2489	NCgl1528	hypothetical protein	2.45
	3347	NCgl1646 [a]	hypothetical protein	4.29
	3348	NCgl1647	hypothetical protein	2.32
	2006	NCgl1848	hypothetical protein, similar to archaeal enzymes of ATP-grasp superfamily	2.47
	2974	NCgl2356	hypothetical protein	2.21
	2920	NCgl2450 [a]	hypothetical protein, involved in propionate catabolism	35.16
	2919	NCgl2451 [a]	hypothetical protein	13.75
	21	NCgl2584 [a]	hypothetical protein, involved in biosynthesis of extracellular polysaccharides	5.21
	242	NCgl2753	hypotheical protein, similar to vancomycin resistance protein	2.04
	1884	NCgl2900	hypothetical protein	2.28
	1898	NCgl2912	hypothetical membrane protein	2.42
	1922	NCgl2934	hypothetical protein, similar to phosphotransferase system mannitol/fructose-specific IIA domain (Ntr-type)	2.21
	1938	NCgl2944	hypothetical protein	2.63
	1941	NCgl2946 [a]	hypothetical protein	3.89
	535		hypothetical protein	2.08

Group	ORF	NCBI no.	Annotation	Ratio
	963		hypothetical protein	2.08
	1940		hypothetical protein	2.09
	810		hypothetical protein, similar to transposases	2.12
	749		hypothetical protein	2.15
	1939		hypothetical protein	2.36
	325		hypothetical protein	2.40
	3190		hypothetical protein	2.45
	853		hypothetical protein	2.71
	2147		hypothetical protein	3.57
	517	[a]	hypothetical protein	9.96
	2754		hypothetical protein	25.11

Tab. 7.3 Up regulated ORFs in the acid adapted *C. glutamicum*

Curriculum vitae

Personal data:

Name:	Kinga Jakob
Date of Birth:	Nov. 5 1972
Place of Birth:	Cluj-Napoca, Romania
Nationality:	American
Marital status:	Married

Education:

9/1979-3/1987	Brassai Samuel Liceum in Cluj-Napoca, Romania
4/1987-6/1988	Junior High School 189, Flushing, New York, USA
9/1988-6/1991	Flushing High School, Regents diploma, Flushing, New York, USA
9/1991-5/1995	Bachelor of Science, in Biology, at St. John's University, New York, USA
9/1995-5/1997	Master of Science, in Genetics, at St. John's University, New York, USA
12/2000-present	PhD student at the Department of Microbiologie, Zentral Institut für Ernährungs und Lebensmittelforschung Weihenstephan

Experience:

9/1999-11/2000	Teaching assistant – Rutgers University
8/1998-8/1999	Food quality control test assistant – Rutgers University
1/1997-8/1998	Tutor at Biology Learning Center – Nassau Community College