A study on the effects of amino acids and arginine deiminase (*arcA*) on growth, metabolism and gene expression in *Enterococcus* faecalis V583

Et studie om effektene til aminosyrer og arginine deiminase (*arcA*) på vekst, metabolisme og genekspresjon i *Enterococcus* faecalis V583

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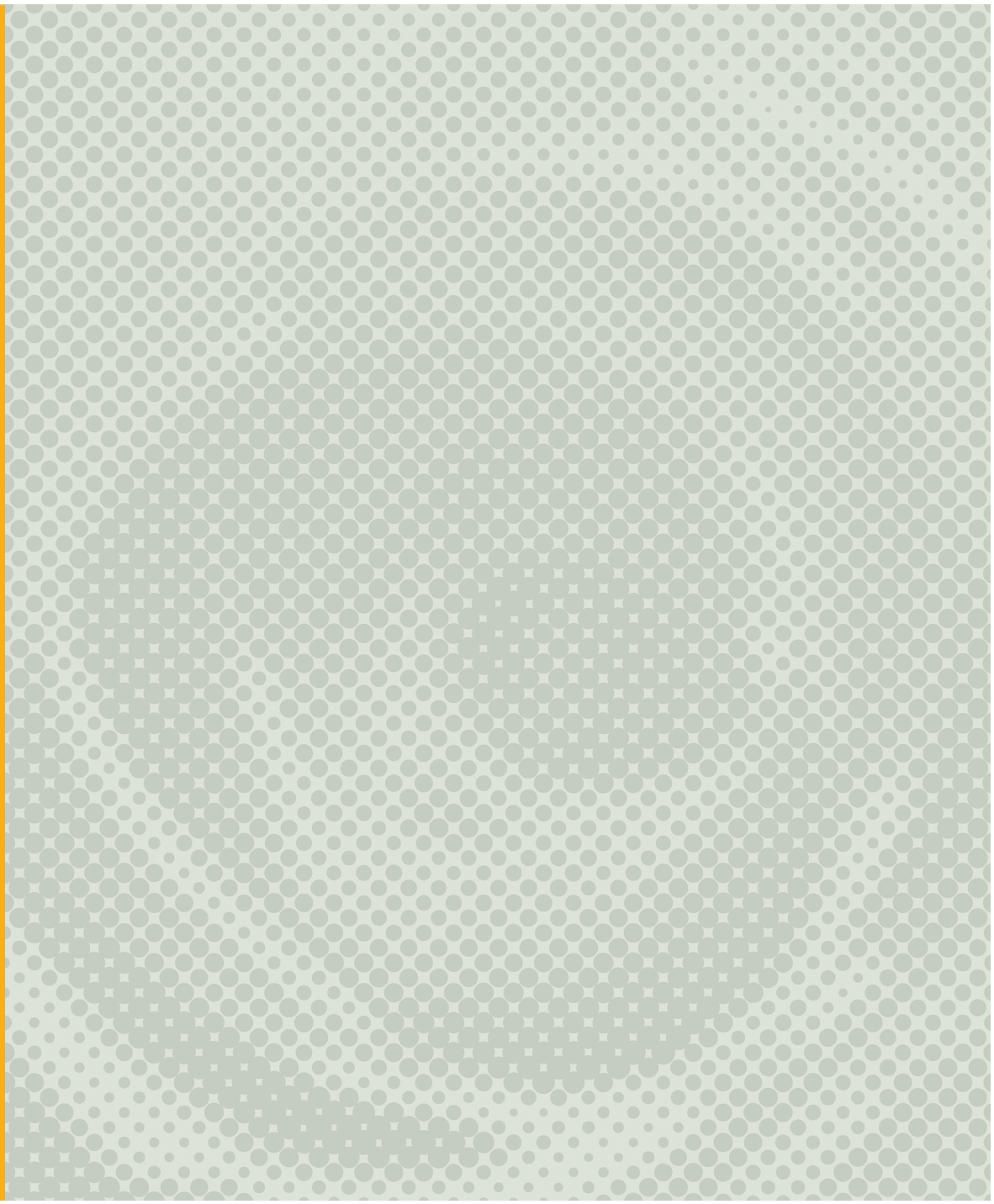

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Cyril Frantzen

Abstract

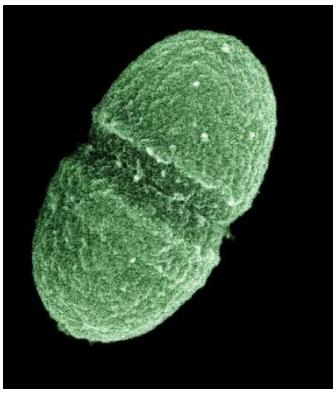
In this study the roles of amino acids and arginine deiminase (*arcA)* in *Enterococcus faecalis* V583's growth, metabolism and gene expression were investigated. Literature describes only one pathway of arginine catabolism for *Enterococcus faecalis* V583 in the ADI-pathway where arginine deiminase catalyzes the deamination of L-arginine, producing L-citrulline and ammonia. A deletion of *arcA* was constructed in *Enterococcus faecalis* V583 (Δ*arcA*), but surprisingly L-arginine was still broken down in a glucose-limited continuous culture, suggesting an alternative arginine deiminase or alternative pathway for arginine catabolism exists in the *E. faecalis* V583 genome. A significant effect of the *arcA* deletion was observed on growth and metabolism both in batch culture with no nutritional limitation, and in glucose-limited continuous culture. Growth studies in batch culture using a defined medium with amino acid leave-out compositions showed that amino acid availability played a significant role in both E. faecalis V583 wild type and mutant growth. A significant reduction in growth rate and a more pronounced death phase was observed for the Δ*arcA* mutant in comparison to wild type. Growth in glucose-limited continuous culture revealed a pH-dependency on the shift between homolactic and mixed acid fermentation. Transcriptional analysis by real-time PCR also showed pH-dependency of *pflA* transcription, revealing a down-regulation of *pflA* in pH 6.5 culture compared to pH 7.5 culture. A reduction in free phosphate concentrations in the mutant cultures, together with provisional genomic data of a polyphosphate kinase (*ppnK*) gene at EF2670 indicates *E. faecalis* V583 might be able to produce polyphosphates in stressful environments. A significant reduction in biomass for the Δ*arcA* mutant in comparison to wild type also suggests a less efficient metabolism for the Δ*arcA* mutant. Complementation of the *E. faecalis* V583Δ*arcA* mutant was performed, providing an intact *arcA* gene in trans, and construction of a double deletion mutant *E. faecalis* V583Δ*glnA*Δ*arcA* was also performed. A preliminary growth study in batch culture showed no growth difference between *E. faecalis* V583Δ*arcA* and the complemented *E. faecalis* V583Δ*arcA*, suggesting that providing an intact *arcA* gene is not sufficient to restore ADI-pathway function. A significant difference between *E. faecalis* V583Δ*glnA* and V583Δ*glnA*Δ*arcA* was observed, with V583Δ*glnA*Δ*arcA* growing significantly slower, but reaching a higher OD_{600} before initiating stationary phase.

Sammendrag

I dette arbeidet har effekten av ulike aminosyrer og arginine deiminase (*arcA*) på vekst, metabolisme og genekspresjon i *Enterococcus faecalis* V583 blitt undersøkt. Kun en vei for nedbrytning av L-arginine har så langt blitt beskrevet for *E. faecalis* V583. Her deamineres L-arginine i en reaksjon katalysert av arginine deiminase, og L-citrulline og ammoniakk blir dannet. En mutant med delesjon av *arcA* (Δ*arcA*) ble konstruert i *E. faecalis* V583. Data fra glukose-begrenset kontinuerlig kultur viste imidlertid at L- arginine fortsatt ble brutt ned i mutanten, noe som indikerer at en alternativ arginine deiminase, eller en alternativ katabolsk vei eksisterer for *E. faecalis* V583. En signifikant effekt av *arcA* delesjonen på vekst og metabolisme ble observert i kultur både med og uten glukose-begrensning. Vekstforsøk i batchkulturer i et definert medium, der enkelte aminosyrer ble utelatt, viste at tilgjengeligheten av aminosyrer har en signifikant rolle i veksten til både Δ*arcA* mutanten og villtypen av *E. faecalis* V583. Videre ble en signifikant reduksjon av veksthastigheten og en mer utpreget dødsfase observert hos Δ*arcA* mutanten sammenlignet med villtypen. Vekst i glukose-begrenset kontinuerlig kultur viste et pH-avhengig skifte mellom homolaktisk og blandet syrefermentering. Transkripsjonsanalyser, gjort med sanntids-PCR, viste også en effekt av pH på transkripsjonsnivå av *pflA*, med en nedregulering av *pflA* i pH 6.5 kulturen kontra pH 7.5 kulturen. En reduksjon i mengden fritt fosfat i mutanten, sammen med et gen med homologi til polyfosfat kinase (*ppnK*) i EF2670, foreslår at *E. faecalis* V583 kan være i stand til å danne polyfosfater under stress. En signifikant reduksjon av biomassen for Δ*arcA* mutanten, i forhold til villtypen, indikerer en mindre effektiv metabolisme hos mutanten kontra villtypen. Komplementering av Δ*arcA* mutanten der et intakt *arcA*ble tilført in trans, ,og konstruksjon av en dobbel delesjonsmutant *E. faecalis* V583Δ*glnA*Δ*arcA* ble gjort. Et innledende vekstforsøki batchkultur viste ingen forskjellig mellom *E. faecalis* V583Δ*arcA* og *E. faecalis* V583Δ*arcA* komplementert. Denne observasjonen indikerer at et intakt *arcA* gen ikke er tilstrekkelig for å gjenopprette arginine deiminase-funksjonen i cellen. En signifikant forskjell av vekst mellom *E. faecalis* V583Δ*glnA* and V583Δ*glnA*Δ*arcA* ble også observert. V583Δ*glnA*Δ*arcA* viste en signifikant reduksjon i veksthastighet, men vokser til en signifikant høyere OD_{600} før stasjonærfase innledes.

1.0 Introduction

1.1 *Enterococcus*



Domain: *Bacteria*
Phylum: *Firmicutes*
Class: *Bacilli*
Order: *Lactobacillales*
Family: *Enterococcaceae*
Genus: *Enterococcus*

Figure 1: *Enterococcus faecalis* (40).

The genus *Enterococcus* is made up from Gram-positive, facultative anaerobic bacteria and is a relatively new genus because many of today's enterococci were up until 1984, classified as part of the *Streptococcus* genus. Scientific work performed by Schleifer & Kilpper-Balz lead to the formation of the *Enterococcus* genus due to the large genetic differences of some species classified as *Streptococcus* sp. (50).

Enterococci are quite robust bacteria that can grow at pH's ranging from 4 to 9.6, as well as temperatures from 10 to 45 degrees. Enterococci tolerate temperatures up to 60 degrees for 30 minutes, and can grow in salt concentrations up to 6.5%. Enterococci are catalase negative, chemoorganotrophic, fermentative bacteria. Through homo-fermentative processing of glucose, the bacteria produce L-lactic acid and are part of the lactic acid bacteria group. (57)

Enterococci grow commensally in the gastrointestinal (GI) tract of mammals, and *E. faecalis* and *E. faecium* are the most common species found (53). Like other bacteria commensal to the gut flora, the fecal-oral route of transportation enables contamination of water, soil, vegetation, as well as foods (13). *Enterococcus* sp. are because of their role as commensal inhabitants of the GI tracts of mammals, used as indicators for fecal contamination of food stuffs or drinking water (13, 33).

The hardy nature of enterococci, combined with its ability to degrade citrate, its proteo- and lipo-lytic activity, as well as its homolactic fermentation, makes enterococci well

suited for use in production of foods such as cheeses and sausages, where it provides taste and texture (12). Enterococci are also sold as probiotic treatments (*E. faecium SF68, E. faecalis* str. Symbioflor 1) (12). On the other hand, the hardy nature of enterococci also makes them hard to get rid of in cases where they are not wanted, such as in contamination or infection (14).

The last few decades has brought forth a rise of attention for the *Enterococcus* genus due to their prevalence in nosocomial infections combined with their tendency of increased resistance to standard antimicrobial treatment (15, 29). Clinical infections caused by *Enterococcus* sp. include urinary tract infections, endocarditis, meningitis, surgical-site infection and wound infection (27). These infections are in most cases caused by *E. faecalis* or *E. faecium*, and are treated with β-lactam antibiotics (ampicillin, penicillin), or glycopeptides (vancomycin). However, the intrinsic antibiotic resistance of *Enterococcus* sp. combined with an increase in cases of acquired resistance in *Enterococcus* sp. has made nosocomial infections a lot harder to deal with (8, 15). Isolation of multi-resistant *Enterococcus* sp. from hospitalized patients is not uncommon, and in Cleveland, vancomycin-resistant enterococci (VRE) isolates outnumber clinical isolates by a factor of 10 in hospitals that actively perform VRE-screening (15). The increase of acquired resistance genes is much attributed to the high frequency of horizontal gene-transfer in *Enterococcus* sp., and the same can be said for acquired virulence genes (19). Several genes that encode virulence factors have been isolated from resistant *Enterococcus* sp. and described using molecular techniques. Amongst them are the genes *agg*, *gelE*, *ace*, *cylLS*, *esp*, *cpd*, *sprE*, *fsrB*, encoding for virulence factors such as gelatinase, cytolysin, adhesins, invasins, proteases and capacity for biofilm formation. Most virulence factors are found on mobile elements (16, 19, 54).

New antibiotics (such as quinupristin-dalfopristin, linezolid, daptomycin, tigecycline) have been developed to target the infections caused by *Enterococcus* sp. (with variable results) (15), but already VRE & multi-resistant *Enterococcus sp.* strains have been described with resistance to these new antibiotics (15). The main focus from a daily clinical standpoint is prevention of infection to begin with, through good hygiene and good hospital protocol.

The use of bacteria that are commensal to humans in foods and as a probiotic to not introduce new bacteria into the gut flora is a good idea, but coupled with the knowledge that the same bacteria is a known pathogen, makes it very important to investigate the differences between the commensal, probiotic, and pathogen bacteria. The *Enterococcus* sp. used in food

production are considered non-virulent, but even the slightest chance that bacterial strains used in food production or probiotics may carry with them unwanted traits to our commensal environments is a good enough cause for investigation (13).

1.2 *Enterococcus faecalis*

E. faecalis is as earlier described part of the mammalian gut micro flora, but is also an established pathogen, causing many nocosomial infections (10-15% of total nocosomial infections are caused by *Enterococcus* sp. (9, 17), 80-90% of them by *E. faecalis*, 5-10% by *E. faecium* (27, 48). The increasing tendency of vancomycin and multi-resistance to antibiotics has made enterococcal infections harder to treat (8). As part of the gut micro flora in mammals, it is regularly found in soil, water, and sewage. *E. faecalis* is also regularly used as an indicator for fecal contamination in drinking water and foods (45).

E. faecalis is like most enterococci, a robust bacterium and has shown the ability to withstand bile acid and other detergents (52). As part of its metabolism, *E. faecalis* produces hydrogen peroxide (H_2O_2) and superoxide (O_2-), both of which are highly reactive and can cause damage to the DNA, protein, and lipid structures of the cell. In addition *E. faecalis* produces extracellular superoxide by autoxidation of membrane-associated demethylmenaquinone (21). *E. faecalis* producing extracellular superoxide is interesting as reactive oxygen species (ROS) are correlated to chromosomal instability which is observed in colorectal cancer cells (22). Production of ROS demands a good response system for transport, and intracellular defense. Being catalase negative, *E. faecalis* still manages to grow in aerobe conditions, utilizing NADH-peroxidase to reduce hydrogen peroxide to water, and superoxide dismutase to remove super oxide remaining in the cell (22). Other regulatory responses to combat oxidative stress have been suggested, such as the synthesis of glutathione and glutathione reductase, but their exact function is not described (22). The robust nature of *E. faecalis*, its commensal nature, its role in foods, its prevalence in causing nocosomial infections and its antibiotic resistance development, certainly justifies further investigating this bacteria.

Complete genome sequences are published on many different *E. faecalis* strains, but out of these, only 5 strains are fully described (per 29.04.2013), these are *E. faecalis* V583 (reference genome) *E. faecalis* OG1RF, *E. faecalis* 62, *E. faecalis* D32, as well as *E. faecalis* str. Symbioflor 1 (sold as commercial probiotic). (Information acquired from

http://www.ncbi.nlm.nih.gov/genome/808). For this thesis, the genome reference strain *E. faecalis* V583 is used as the wild type standard.

1.3 *Enterococcus faecalis* V583

E. faecalis V583 was the first clinical isolate displaying vancomycin resistance and was also the first *E. faecalis* strain to have its genome sequenced and published (15, 45). Paulsen *et. al* described the *E. faecalis* V583 genome consisting of a 3.36Mbp circular chromosome with a GC% average of 37,4, and three plasmids, pTEF1, pTEF2, PTEF3 with an average GC% of about 34%. The *E. faecalis* V583 genome is estimated to contain 3337 open reading frames (ORF's) where about ~50% of these showed homology with genes that code for known proteins. Somewhere around $1/4^{th}$ of the *E. faecalis* V583 genome is made up of mobile or exogenous elements such as transposons, pathogen islands, resistance genes, integrated plasmid genes, bacteriophage genes, or insertion elements and the *vanB* gene mediating the vancomycin resistance is an example of one such mobile element. *E. faecalis* V583's vancomycin resistance is of the VanB-phenotype with peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala (8), this change reduces the amount of hydrogen-bond interactions available to vancomycin from 5, to 4, effectively reducing vancomycin affinity 1000-fold (47).The amount of mobile elements in *E. faecalis* V583 when compared to genomes of other bacteria, is considered as a high amount (45). The high amount of mobile elements, coupled with the suspicion that genetic material in *E. faecalis* has gone through many stages of re-arrangement, could explain why there exist so many phenotypes of *E. faecalis* (1). Paulsen *et. al* also described *E. faecalis* V583 with having many sugar phosphotransferase systems (PTS's) and ABC transporters as well as other transport mechanisms, enabling *E. faecalis* V583 to metabolize over 15 different sugars (45).

1.4 Sugar metabolism

Like other lactic acid bacteria, the fermentation process of *E. faecalis* mainly produces L-lactate from pyruvate by lactate dehydrogenase (LDH / *ldh-1, ldh-2*), regenerating NAD^+ from NADH. Smaller concentrations of other metabolites such as acetic acid, acetaldehyde, ethanol, acetoin and acetate are also produced in the process (23). Certain conditions which shift the fermentation process from homo lactic to mixed-acid, skewing the end product concentration less in the favor of L-lactate. Mixed-acid fermentation consumes pyruvate to produce formate catalyzed by pyruvate formate-lyase (PFL / *pfl*), acetate catalyzed by

phosphotransacetylase (PTA / *eutD*) followed by acetate kinase (ACK / *ack*), ethanol catalyzed by alcohol dehydrogenase (ADH / *adh*), and on rare occations also produces trace amounts of acetoin catalyzed by α-acetolactate synthase (ALS / *alsS*) (28). (23)

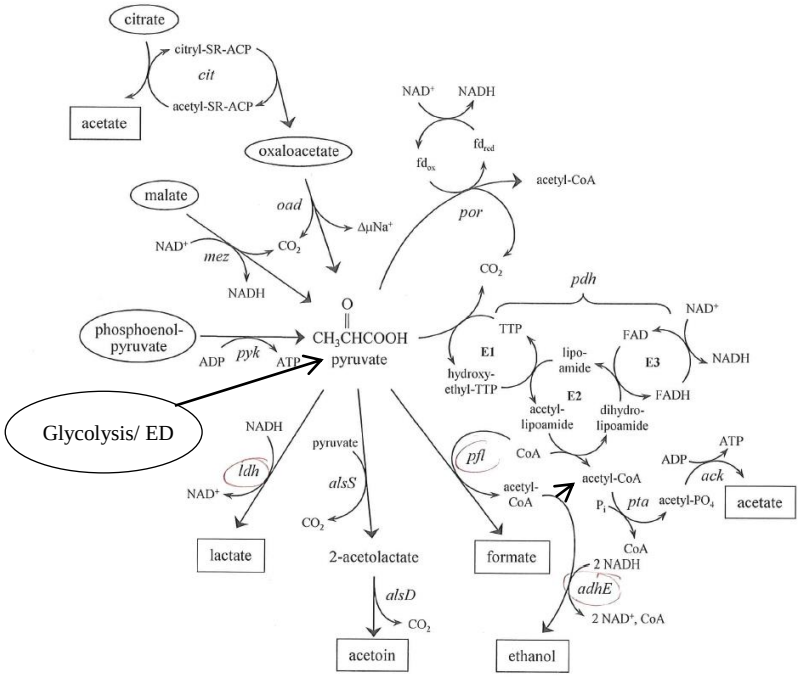


Figure 2: A schematic overview of metabolism, centered around pyruvate. Carbohydrates through the Glycolysis and ED pathways, malate by Malic enzyme (*mez)*, oxaloacetate by oxaloacetate decarboxylase (*oad)*, phosphoenol-pyruvate by pyruvate kinase (*pyk)* fuel the pyruvate center as lactate dehydrogenase (*ldh*), α-acetolactate synthase (*alsS*), alcohol dehydrogenase (*adh*), phosphotransacetylase *(eutD),* and pyruvate formate lyase (*pfl)* metabolize pyruvate. Figure modified from (23).

With pyruvate being such a central part of *E. faecalis* metabolism, many metabolic pathways and regulatory mechanisms exist to provide the cell with optimal uptake and processing of substrates for pyruvate production and ultimately, production of ATP. *E. faecalis* metabolizes over 15 different sugars through its three carbohydrate metabolic pathways, the Embden-Meyerhof-Parnas (glycolysis) pathway, the Entner-Doudoroff pathway (ED), and the pentose phosphate pathway (PPP), providing ATP, NADH, and NADPH (PPP) via substrate phosphorylation (23). In regards to the ED pathway, it is worth noting that it is uniquely exclusive to *E. faecalis* amongst the Gram-positive bacteria, which enables rapid identification of *E. faecalis* by methods that target the genes that code for enzymes involved in the ED pathway (46).

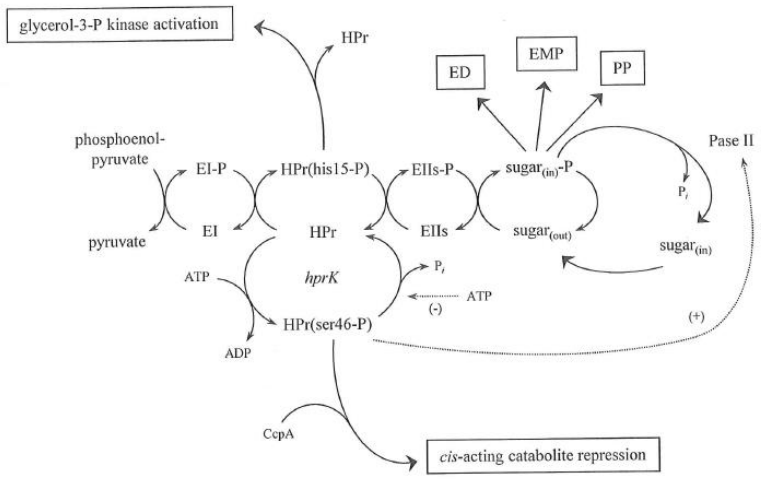


Figure 3: Schematic diagram of regulation through HPr and CcpA, as well as pathways for entering the three carbohydrate metabolic pathways, glycolysis, ED, and PPP. (23)

Three main regulatory mechanisms govern regulation of metabolism in *E. faecalis*, the phosphotransferase system (PTS), the histidine-containing phosphocarrier protein (HPr), and catabolite control protein A (CcpA).

PTS senses extracellular sugars and couples uptake of sugars with phosphorylation. PTS-mediated sugar translocation and phosphorylation, is initiated by phosphoenolpyruvate phosphorylating a small soluble cytoplasmic protein, named enzyme I (EI). EI-P transfers its phosphate to the HPr which, catalyzed by enzyme II (EII), donates its phosphate to the sugar. The function of EI and HPr is unspecific, whilst EII is sugar-specific (23). Many EII enzymes in enterococci have been characterized but one can argue that the most important PTS family is the mannose-PTS, reported to hold a substrate specificity including glucose, mannose, glucosamine, fructose, galactosamine, and *N*-acetylglucosamine (23, 32, unpublished work).

Phosphorylation of HPr is an important regulatory mechanism for sugar uptake in *E. faecalis*, EI-P exclusively phosphorylates HPr at the specific histidyl residue his15 to initiate the phosphate transfer required for PTS-mediated uptake. However, in low GC% Gram-positive bacteria, HPr can also be phosphorylated at a specific seryl residue ser46. This ser46 phosphorylation only occurs in events of high ATP levels, as might happen with high sugar availability and metabolism (10, 23). Phosphorylation of ser46 greatly disables EI-P's ability to phosphorylate at the his15 residue, effectively inhibiting uptake by the PTS (23). The ser46 phosphorylation is reversible and is catalyzed by the bifunctional enzyme HPr

Kinase/Phosphatase HprK, which during low ATP levels has a second enzymatic site which hydrolyzes ser46-P to free HPr up for his15 phosphorylation, allowing PTS-mediated uptake to resume. (23, unpublished work)

E. faecalis, like most bacteria, has sophisticated regulatory mechanisms that prioritizes and ensures uptake of preferred carbon sources (sugars), which are rapidly metabolized. A priority system where sugars such as glucose and fructose are at the top (10). During growth in an environment providing high availability of rapidly metabolized sugars, a down-regulation in metabolism of alternate carbon sources is mediated through carbon catabolite repression (CCR). HPr phosphorylated at the ser46 residue (HPr[ser46-P]) is one such negative regulator. Another component in CCR includes the *trans*-acting factor CcpA working together with *cis*-acting nucleotide sequences termed catabolite responsive elements (*cres*) (23). CcpA is a DNA binding protein which is able to regulate expression via *cres* near promoters of genes, but in order to efficiently bind to *cres*, CcpA is dependent on interactions with HPr[ser46-P], as well as glycolytic intermediates (such as glucose-6-phosphate, or fructose-1,6-bisphosphate) (10), requiring a high ATP availability in the cell environment.

In addition to mediating CCR, HPr[ser46-P] also participates in a process called inducer expulsion (10, 23). Inducer expulsion is a phenomenon where introduction of glucose or other rapidly metabolized sugars in the bacteria environment, lead to the dephosphorylation and efflux of previously accumulated less preferred sugars (58), as well as inhibition of non-PTS's for less preferred sugars (10, unpublished work).

1.5 Alternative energy sources

High ATP levels (such as for example with high glucose availability) puts the cell in a state of high CCR, where HPr[ser46-P] and CcpA activity is high. Low ATP levels puts the cell in a state of low CCR, where HPr[ser46-P] and CcpA activity is low. The state of low CCR enables metabolism of alternative energy sources. In addition to preferred sugars such as glucose and fructose, *E. faecalis* has a wide range of alternative energy sources. Glycerol metabolism is important for lipid synthesis in the cells, in situations of high HPr[his15-P] levels (low CCR), *E. faecalis* is equipped with two pathways for glycerol catabolism . One pathway is catalyzed by glycerol kinase (GlpK) yielding glycerol-3-phosphate. The other pathway yielding dihydroxyacetone phosphate from glycerol, catalyzed first by NAD+-dependant glycerol dehydrogenase, followed by dihydroxyacetone kinase (DhaK) (7). (23)

Work by Bizzini, *et al*. showed that glycerol is mainly metabolized by the *dhaK* pathway under anaerobic conditions, whilst both pathways are available in aerobic conditions. Other metabolic pathways that lead to pyruvate formation are catabolism of malate by Malic enzyme, and catabolism of oxaloacetate by oxaloacetate decarboxylase, both being dependent on a low CCR status in the cell. Metabolism of many other substrates by *E. faecalis* is described by Mark M. Huycke in his 'Physiology of Enterococci' (2002), but not being especially relevant to this thesis, these processes are not described here. Instead the focus is on the catabolism of the amino acid arginine, as well as a slight mention of glutamate catabolism.

1.6 Arginine catabolism

The amino acid arginine is an alternative energy source used by *E. faecalis* to produce 1 mol of ATP per mole of substrate (23). Arginine is catabolized through the arginine deiminase (ADI) pathway. The genes encoding for the proteins involved in the ADI pathway are ordered in the operon *arcABCRD* (Figure 4) that encodes three enzymes, arginine deiminase (*arcA*), ornithine carbamoyltransferase (*arcB*), carbamate kinase (*arcC*), a putative Crp/Fnr-type regulator (*arcR*), and an arginine-L-ornithine antiporter (*arcD*) (4). Two regulatory genes exist in proximity to the *arcABCRD* operon, *argR1* upstream from *arcA*, and *argR2* near the 3' end of the complementary strand (4, 23).

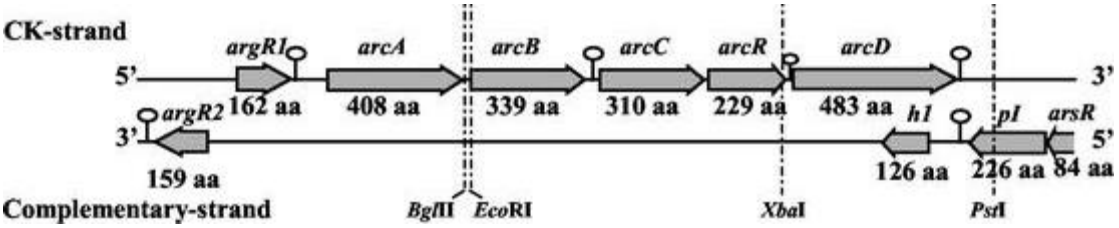


Figure 4: Schematic diagram showing the organization of the *arcABCRD* operon with amino acid residues and the *argR1* regulator upstream from *arcA*. (4)

No putative ArcR binding sequence is found in the promoter region of *arcA*, but potential binding sequences for ArgR1 and ArgR2 are found (4). Binding sequences for ArgR1 and ArgR2 are also found in the promoter regions of *argR1 and argR2*, and a potential ArcR binding sequence is found in the promoter region of *argR2*. Putative binding sequences for CcpA are also found in the promoter regions of *arcA*, as well as *argR1 and argR2*. Also, growth studies performed by Barcelona-Andrés *et al.* showed that the expression of *argR1* and *argR2* (and thereby also *arcABCRD*) is greatly influenced by the presence of arginine, and inhibited by CR mediated by CcpA (4).

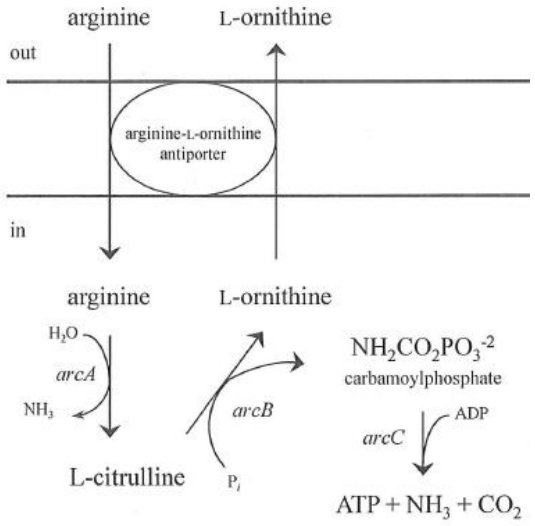


Figure 5: Schematic overview of uptake and catabolism of arginine. (23)

As shown in Figure 5, arginine enters the cell through the arginine-L-ornithine antiporter, and is deiminated via arginine deiminase to form L-citrulline and ammonia. Subsequent phosphorylation of L-citrulline is catalyzed by ornithine carbamoyltransferase to produce carbamoyl phosphate and L-ornithine. L-ornithine enables more uptake of arginine through the arginine-L-ornithine antiporter, whilst carbamoyl phosphate, containing a high energy phosphate bond, is used to generate ATP catalyzed by carbamate kinase to produce CO_2 and ammonia (23). A more detailed overview of the processes revolving around carbamoyl phosphate is found in Figure 6.

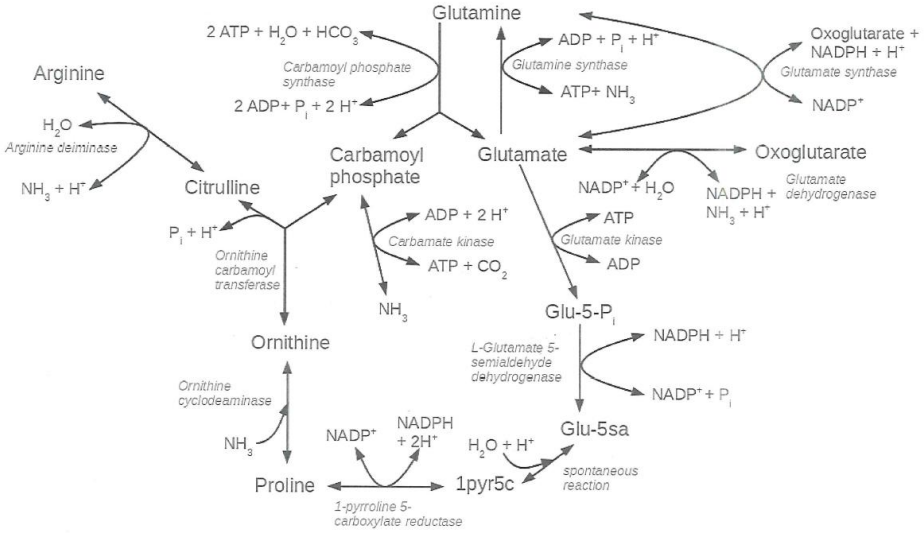


Figure 6: Schematic diagram of catabolism revolving around carbamoyl phosphate, with arginine entering through the ADI pathway, and glutamate entering through glutamine synthase followed by carbamoyl phosphate synthase (Nadine Veith, unpublished work).

1.8 Aim of the study

The work presented in this thesis was conducted as part of the SysMO-LAB2 project, as a continuation of the SysMO-LAB1 project 'Comparative systems biology of lactic acid bacteria'. The focus of the project is to develop understandable models of metabolism in four different lactic acid bacteria (LAB): *Lactococcus lactis, Lactobacillus plantarum, Streptococcus pyogenes* and *E. faecalis*. Fermentation studies conducted as part of SysMO-LAB1 showed significant differences in amino acid metabolism between the LABs, and suggested that the amino acids have an important influence on the regulation of primary metabolism. One noticeable effect observed was that of arginine, which in *L.lactis* resulted in ATP generation through the arginine deiminase pathway, with a noticable impact on the shift between homolactic and mixed acid fermentation.

Growth studies performed by Solheim, *et al.* have shown that growth of *E. faecalis* V583 is not possible without arginine, making arginine an essential amino acid for *E. faecalis* V583 (unpublished work). It also showed that growth without glutamine was possible but growth without glutamine and glutamate was not, possibly because glutamine is synthesized from glutamate catalyzed by glutamine synthetase (*glnA*) (unpublished work). Growth rate studies with cultures in a chemostat performed by Mehmeti, Faergestad *et al.* showed that 90 to 95% of arginine was consumed under glucose-limited conditions (37), as well as a shift from homolactic fermentation to mixed acid fermentation as growth rates slowed (38).

In the present work, a mutant of *E. faecalis* V583 with a deletion in the *arcA* gene was constructed. The purpose of the deletion was to investigate the role of arginine catabolism, and its effects on growth and energy metabolism in *E. faecalis* V583, as well as its effect on expression of genes related to pyruvate metabolism. The survival capabilities of the *arcA* deletion mutant in acidic environments were tested, and complementation of the *arcA* gene in *E. faecalis* V583Δ*arcA* was done to investigate whether the deletion had polar effects on the remainder of the operon.

Simultaneously a mutant of *E. faecalis* V583 with a deletion in the *glnA* gene was constructed by Margrete Solheim. A single crossover deletion in the *arcA* gene was constructed in the *E.faecalis* V583Δ*glnA* mutant, creating the double knockout mutant *E. faecalis* V583Δ*glnA*Δ*arcA*. The double mutation was performed to investigate whether or not an *arcA* deletion would affect growth yield and growth rate for the Δ*glnA* mutant.

2.0 Materials

2.1 Bacteria strains

E. faecalis V583	Lab strain LGMT 3088
E. faecalis V583Δ*arcA*	This work
E. faecalis V583Δ*arcA* with *arcA* complement	This work
E. faecalis V583Δ*glnA*Δ*arcA*	This work
E. faecalis V583Δ*glnA*	Margrete Solheim (unpublished)
E. coli DH5α	Life technologies
E. coli GeneHogs	Life technologies
E. coli EC1000	Thurlow, L. R. *et al.* (55)

2.2 Chemicals and reagents

10xBSA (10mM)	New England Biolabs
10x Taq Buffer (-$MgCl_2$)	Life technologies
4-aminobenzoic acid	Sigma
4-chloro-phenylalanin (10mM)	Sigma
5x Phusion® HF Buffer	Finnzymes
Acetic acid	Sigma
Acetonitrile	Merck
Adenine	Sigma
Agar	Merck
Agarose	Life technologies
Amino acid standard	Pierce, Boule Nordic
Ammonium chloride (NH_4Cl)	Sigma
Ammonium molybdate tetrahydrate	Sigma (Fluka)
Ampicillin	Sigma
Bacto tryptone	DIFCO laboratories
Borate buffer	Agilent Technologies
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
$CaCl_2$ x $2H_2O$	Merck
Ca-D-(+)-panthothenate	Sigma
Chloramphenicol	Sigma
Chloroform	Merck
$CoSO_4$ x $7H_2O$	Sigma
Citric acid	Sigma
$CuSO_4$ x $5H_2O$	Sigma
Cystine	Sigma
Deoxynucleotides	Life technologies
Disodium phosphate (Na_2HPO_4)	Sigma
dH_2O	Produced locally
DL-alanine	Merck

D-biotin	Sigma
DL-lactic acid	Sigma
DL-pyroglutamic acid	Sigma
DMSO	Sigma
Erythromycin	Sigma
Ethanol	Arcus
Ethidium bromide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Merck
$FeCl_2$ x $4H_2O$	Sima-Aldrich
$FeCl_3$ x $6H_2O$	Merck
Fructose	Merck
Formic acid	Sigma
Galactose	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
Guanine-HCl	Sigma
Hypochloric acid (HCl)	Merck
Inosine	Sigma
Kanamycin	Sigma
Lactose	Merck
Liquid nitrogen	AGA
L-amino acid kit	Sigma
L-arginine-HCl	Sigma
L-aspartic acid	Sigma
L-cysteine-HCl	Prolab
L-glutamic acid	Sigma
L-histidine-HCl-H_2O	Sigma
L-isoleucine	Sigma
L-leucine	Sigma
L-lysine-HCl	Sigma
L-norvalin	Merck
L-phenylalanine	Sigma
L-proline	Sigma
L-serine	Sigma
L-threonine	Sigma
L-tryptophane	Sigma
L-valine	Sigma
L-methionine	Sigma
L-aspargine	Sigma
L-glutamine	Sigma
Maltose	Merck
$MnCl_2$ x $4H_2O$	Sigma
$MgCl_2$ x $6H_2O$	Sigma

$MgCl_2$ (50mM)	Life technologies
Monopotassium phosphate (KH_2PO_4)	Sigma
NEB Buffer 3	New England Biolabs
Nicotinic acid	Sigma
Nitrogen gas	AGA
Orotic acid	Sigma
Phenol	Sigma
Propionic acid	Sigma
Pyridoxamine-HCl	Sigma
pyridoxine-HCl	Sigma
Pyruvic acid	Sigma
RNase / DNase free water	Qiagen / Life technologies
Riboflavin	Sigma
Sodium acetate anhydrous	Merck
Sodium acetate-trihydrate	Merck
Sodium bicarbonate ($NaHCO_3$)	Merck
Sodium hydroxide (NaOH)	Merck
Sodium chloride (NaCl)	Merck
Sodium acetate (NaOAc)	Merck
Spectinomycin	Sigma
Succinic acid	Sigma
Sulphuric acid	Merck
Sucrose	Merck
SYBRGreen®	Roche
T4 ligase buffer	New England Biolabs
Tetracycline	Sigma
Tetrahydrofuran	Merck
Thiamin-HCl	Sigma
Thymidine	Sigma
Titriplex III	Merck
Trichloroacetic acid	Merck
Triammonium citrate	Sigma
Tyrosine	Sigma
Uracil	Sigma
Uric acid	Sigma
Vitamin B12	Sigma
Yeast extract	Oxoid
Xanthine	Sigma
X-gal 40mg/mL	Life technologies
$ZnSO_4$ x $7H_2O$	Merck
α-ketoglutaric acid	Sigma
α-lipoic acid	Sigma
β-mercaptoethanol	Merck

2.3 Enzymes

BamHI restriction enzyme	New England Biolabs
Calf-intestinal alkaline phosphatase (CIP)	New England Biolabs
DNase I	New England Biolabs
NotI restriction enzyme	New England Biolabs
Phusion® DNA polymerase	Finnzymes
PstI restriction enzyme	New England Biolabs
RNase out	Life technologies
SnaBI restriction enzyme	New England Biolabs
T4 ligase	New England Biolabs
T4 Polynucleotide kinase	New England Biolabs
Taq® DNA polymerase	Life technologies
XhoI restriction enzyme	New England Biolabs

2.4 Equipment

1mm electroporation cuvette	Biorad
10mL culture tubes (glass)	-
2mL Cryo-tubes	-
250mL Erlendmeyer flask (glass)	-
2mm electroporation cuvette	Biorad
Acid-washed pellets ($<10^6$ microns)	Sigma
Nunc-tubes (50mL)	Thermo scientific
Nunc tubes (15 mL)	Thermo scientific
PCR capillary tubes	-
FastPrep tubes	-
Flameboy	Integra Biosciences
Gloves	VWR
Volt-meter	
Gel-electrophoresis equipment (rack, molding form, comb)	-
Gel photo system with UV spectrum	-
Scalpel knife	-
Eppendorf tubes	Eppendorf
Filter (0,025µm)	Millipore
OPA Ampoules	Agilent technologies
Petri dish	
Pincers	-
Pipette	Eppendorf
Pipette tips	VWR
Honeycomb Microplate	Bioscreen C
Biostatbplus fermentor	Sartorius Stedim Biotech
Plastic tubes	-
10L flask	VWR

5L flask	VWR
500mL flask	VWR
50mL syringes	BD Plastipak™
0,22 µm vacuum filter	Millipore
MFS-13mm CA filter, 0,2µm poresize.	-
96-Well F Microtiter plates	Sarstedt

HPLC for carbohydrates

Pump: Series 410,	Perkin Elmer
Auto-injector: Series 200,	Perkin Elmer
Column oven: LC oven 101,	Perkin Elmer
UV-detector: series 200,	Perkin Elmer
RI-detector: series 200,	Perkin Elmer
LC-terminal: TotalChrom,	Perkin Elmer
Interface: 900 series,	Perkin Elmer
Column: Aminex HPX-87H, 300x7.8 mm id,	Bio Rad
Guard column: Cation-H refill, 30x4.6mm id,	Bio Rad

HPLC for amino acids

Pump: Series 410	Perkin Elmer
Auto-injector: 1200 series	Agilent Technologies
Thermostat: 1200 series	Agilent Technologies
Column oven: Series 200	Perkin Elmer
Flourescens detector: 1200 seres	Agilent Technologies
LC-terminal: EZChrom Elite	Agilent Technologies
Column: XTera RP18, 150 x 4,7 millimeter id, particle size. 3,5µm	Waters

2.5 Growth medium

All media were autoclaved at 121°C for 15 minutes before use unless otherwise specified.

LB / LA (Luria broth / Luria agar)

10g tryptone, 5g yeast extract, 10g NaCl per liter dH_2O. To make LB-agar (LA) add 10g agar to solution per liter dH_2O.

GM17

37,25g M17-broth powder added per liter dH_2O.
After autoclaving, 10mL 40% glucose is added per liter.

2x GM17

74.5g M17-broth powder added per liter dH_2O.
After autoclaving, 10mL 40% glucose is added per liter.

Todd-Hewitt

36,4g Todd-Hewitt broth powder added per liter dH_2O. For Todd-Hewitt agar, 10g agar was added per liter dH_2O.

SOC (super optimal catabolite repression broth)

2g Bacto tryptone
0,5g yeast extract
333,3µL 3M NaCl
83,2µL 3M KCl
96mL dH2O

After autoclaving, 2mL 1M MgCl and 2mL 1M glucose is added, and solution is sterile filtrated through a 0,2 µm filter.

GYT

10mL 85% glycerol
0,125g yeast extract
0,25g tryptone
100mL dH_2O

SGM17

100mL 2xM17
100mL 1M sucrose
2mL 40% glucose

10x M9-salts

60g Na_2HPO_4
30g KH_2PO_4
5g NaCl
10g NH_4Cl
H_2O up to 1L.

MM9YE6 agar

25mL 10x M9-salts
0.625g yeast extract

3.75g agar
225mL H_2O
500mg p-chloro-phenylalanine

After autoclaving, add 3.1mL 40% glucose.

CDM-LAB

Per liter of CDM-LAB medium:
750 mL Solution A (recipe found in 2.10)
50mL AGU-cystine-xanthine mix (recipe found in 2.10)
50mL Glucose-ascorbate mix (recipe found in 2.10)
10mL 100x Vitamin stock (recipe found in 2.10)
10mL 100x Metal stock (recipe found in 2.10)
50mL amino acid solution (recipe found in 2.10)

1. AGU-cystine-xanthine mix, vitamin stock, metal stock, and glucose-ascorbate is added into autoclaved Solution A.
2. Amino acid solution is added, and final volume is adjusted to 1L
3. Solution will be approx. pH ~4, adjust to 6.5 for batch solution, or to wanted pH.
4. Filter sterilize through a 0,22µm filter

2.6 Instruments

Agilent 2100 BioAnalyzer	Agilent Technologies
Autoclave	Matachana
Biofuge (Fresco) Heraeus Centrifuge	DJB Labcare
Bioscreen C Analyzer instrument	Bioscreen C
Chip priming station for RNA 6000 Nano Chip	Agilent Technologies
Corbett Rotor Gene 6000 instrument	Corbett Life Sciences
Digital weight	Salter
Eppendorf Centrifuge 5804 R	Eppendorf
Eppendorf 5415D centrifuge	Eppendorf
FastPrep FP120	Savant
Freezer (-80°C)	Forma Scientific
Gene Pulser	Bio Rad
Gene 2 Vortex	Scientific Industries
Heraeus Multifuge X3	Thermo scientific
NanoDrop ND-1000	Nanodrop Technologies
Eppendorf Mastercycler gradient	Eppendorf
RNA 6000 Nano Chip	Agilent Technologies
SPECTROstar Nano	BMG Labtech
Ultrospec 10 Cell density meter	GE Life Sciences
SpeedVac Concentrator SPD 2010 (Savant)	Thermo Electron Corporation

2.7 Kits

Ammonia (Rapid) Assay **Megazyme**

Bottle 1: Buffer (pH 8.0) plus 2-oxoglutarate and sodium azide (0.02% w/v)
Bottle 2: NADPH
Bottle 3: Glutamate dehydrogenase suspension (2.2mL)
Bottle 4: Ammonia standard solution (5mL, 0,04 mg/mL) in 0.02% w/v sodium azide.

E.N.Z.A™ Plasmid MiniPrep Kit **VWR / Omega**

Solution I
Solution II
Solution III
Equilibration buffer
Buffer HB
DNA wash buffer
RNase A
Elution buffer
HiBind DNA mini columns
2mL collection tubes

Nucleospin® PCR Clean-up Gel Extraction kit **Macherey-Nagel**

Binding Buffer NTI
Wash Buffer NT3
Elution Buffer
NucleoSpin® Gel and PCR Clean-up Columns (yellow rings)
Collection Tubes

Phosphate Colorimetric Assay Kit **BioVision**

Phosphate reagent
Phosphate standard (10mM)

Qiagen® Plasmid Midi Kit **Qiagen**

QIAGEN-tip 20
QIAGEN-tip 100
QIAGEN-tip 500
Buffer P1
Buffer P2
Buffer P3
Buffer QBT
Buffer QC
Buffer QF
RNase A
LyseBlue

Qiagen RNeasy® Mini Kit **Qiagen**

RLT buffer
RW1 buffer
RPE buffer
RNase-free water
RNeasy mini-columns and collection tubes

RNA 6000 Kit **Agilent technologies**

RNA 6000 Nano Marker
RNA 6000 Gel matrix
RNA 6000 Nano dye concentrate
Spin filters

Superscript III reverse transcriptase kit **Life technologies**

SuperScript III (200U/µl)
5x buffer
DTT (0,1M)
PAN_6 random hexamer primers
RNase-free water

Zero Blunt® TOPO® PCR Cloning kit **Life technologies**

pCR™-Blunt II-TOPO® vector
Salt solution
dNTP mix
M13 Forward primer
M13 Reverse primer
Control Template
Water

2.8 Primers

All primers were ordered from Life technologies.

Primers for Δ*arcA* deletion mutant & complementation

Table 1: Primers used for Δ*arcA* deletion mutant & complementation

Name	Sequence	Used for:
arcA-5	5'-GGTTAACGATTTTTGAACAATTCAC-3'	Constructing the Δ*arcA* deletion construct
arcA-6	5'-CACGTACTAGTTCACTTCCTGGAAT CTCATGTGAAATAACCTCCTCAACT(*)-3'	
arcA-7	5'-ATTCCAGGAAGTGAACTAGTACGTG-3'	

arcA-8	5’-AAAATAGCACCTGTCACTAACAAGC-3’	
arcA-9	5’-GTGAATAAGCAAACACGCC-3’	Sco control
arcA-10	5’-GTAGCTGCCATGATCGC-3’	
arcA-12	5’(?)-TACGGCGGCCGC(**) ATGATGATTCCTCCTATTTTTGGGTG-3’(?)	Complementation of the Δ*arcA* deletion mutant
arcA-13	5’-atgc CTCGAG(***) AAGTAACGCATAAAAGGAAGTGAGCC-3’	
OriF	5’-CAATAATCGCATCCGATTGCA-3’	Control PCR of sco-integration with vector pLT06.
KS05SeqR	5’-CCTATTATACCATATTTTGGAC-3’	

(*) (Reverse complementary to arcA-7)
(**) NotI restriction seat
(***) XhoI restriction seat

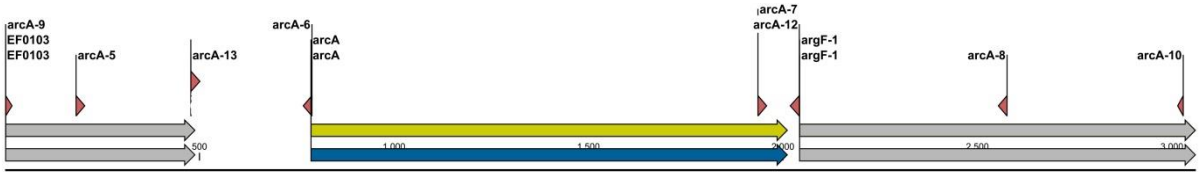


Figure 7: Schematic overview of primers in relation to *arcA* gene.

Primers for Δ*arcA*Δ*glnA* double deletion mutant.

Table 2: Primers used for Δ*arcA*Δ*glnA* double deletion mutant.

Name	**Sequence**
arcA-sco1	5’-CATCGTCCAGGTAAGGAATTAG-3’
arcA-sco2	5’-TTCATCGCCACCTTCAATTC-3’

Primers for Real-Time PCR

Table 3: Primers used for Real-Time PCR

Name	**Sequence**	**Target**
ldhI-F	5’-CGCAGGGAATAAAGATCACCA-3’	*ldh1*
ldhI-R	5’-GCAATCGTCATAAGTAGCAGCA-3’	*ldh1*
adhE-F	5’-TCTGAGCAAGCGGTCCATTGTGG-3’	*adhE*

adhE-R	5’-AGTCGAATTAGAAGGTGCAGGTCCAG-3’	*adhE*
pflA-F	5’-GGAAGCATTACGTTTTCGCTCTTATTGGG-3’	*pflA*
pflA-R	5’-CCACACGTATCTAAGGTTGTATGAATGCC-3’	*pflA*
EF0082-F	5’-GCTTGCACGACTTTTCATGGGGAAAC-3’	EF0082
EF0082-R	5’-GGGCCATTTATTGGGATGTTATTG-3’	EF0082
arcC-F	5’-CGGCTACTGGTTGTCCAATGCGC-3’	*arcC*
arcC-R	5’-CTTCAGCTTCTGTTAAAAATGGACCGATCG-3’	*arcC*
23S-F	5’-CCTATCGGCCTCGGCTTAG-3’	23S
23S-R	5’-AGCGAAAGACAGGTGAGAATCC-3’	23S

2.9 Software

BioEdit	Ibis biosciences
Bioscreen EZ experiment software	BioScreen C
CLC Workbench	CLC Bio
Google Chrome Version 26.0.1410.64 m	Google
NanoDrop 3.0.0	Nanodrop technologies
Microsoft Word 2010	Microsoft
Microsoft Excel 2010	Microsoft
Rotor Gene 6000 Series software 1.7	Corbett

2.10 Solutions mixed by student

1kb ladder (50ng/µl)

50µg ladder mix was dissolved in 167µl loading buffer 6x and 783µl H_2O.

1xTE buffer

5mL 1M Tris HCl (pH 8,0) and 1mL 0,5M EDTA (pH 8,0) dissolved in 494mL Milli-Q dH2O and autoclaved. 1xTE diluted to 0,1xTE before use.

50x TAE buffer

242g Tris base, 57,1 mL ice-vinegar, 18,7g EDTA dissolved in 900 mL dH_2O, volume adjusted to 1L. 50x TAE diluted to 1x before use.

Loading buffer 6x (20mL)

8g sucrose
200µl 0.5M EDTA
En spatelspiss bromfenolblått

H_2O to 20mL

Solution A for CDM-LAB
Per 1 liter of CDM-LAB:
1g K_2HPO_4
5g KH_2PO_4
0,6g $(NH_4)_3$-citrate
1g Na-acetate (anhydrous)
2,5g $NaHCO_3$
0,25g Tyrosine

Dissolve in 0,75L dH_2O. Autoclave at 121°C for 20 minutes.

AGU-cystine-xanthine mix for CDM-LAB
Per liter of CDM-LAB:
50mg cystine
38,5mg adenine
27,5mg guanine-HCl
22mg uracil
10mg xanthine

1. Dissolve cystine, adenine in 20mL 1M HCl one component at a time, start with cystine.
2. Dissolve guanine-HCl, uracil, xanthine in 20mL dH_2O by ding drops of 10M NaOH. Xanthine last.

Glucose-ascorbate mix for CDM-LAB
Per liter of CDM-LAB:
11g D-(+)-glucose monohydrate
0,5g L-ascorbic acid

Dissolve in 0,05L dH_2O.

100x Vitamin stock for CDM-LAB
Per liter 100x Vitamin stock:
500 mg pyridoxamine-HCl
250 mg D-biotin
100 mg Ca-D-(+)-panthothenate
100 mg vitamin B12
250 mg α-lipoic acid
200 mg pyridoxine-HCl
100 mg nicotinic acid
100 mg Riboflavin

100 mg thiamin-HCl
1 mg 4-aminobenzoic acid
500 mg orotic acid
500 mg thymidine
500 mg inosine

Bring the pH up to 10 to dissolve all the vitamins.

Thereafter, bring pH back to 6,8. Filter sterilize through a 0,22µm filter and freeze down (-20°C) in aliquots of 50mL.

100x Metal stock for CDM-LAB
Per liter 100x Metal stock:
0,5g $FeCl_2 \times 4H_2O$
0,3g $FeCl_3 \times 6H_2O$
5g $CaCl_2 \times 2H_2O$
1,6g $MnCl_2 \times 4H_2O$
20g $MgCl_2 \times 6H_2O$
0,5g $ZnSO_4 \times 7H_2O$
0,25g $CoSO_4 \times 7H_2O$
0,25g $CuSO_4 \times 5H_2O$
0,25 Ammonium molybdate tetrahydrate $((NH_4)_6Mo_7O_{24} \times 4H_2O)$

1. Dissolve the $FeCl_2 \times 4H_2O$ in 10mL 17% HCl
2. Dissolve the $FeCl_3 \times 6H_2O$ in 200mL dH_2O.
3. Dissolve all the other components in 700mL dH_2O.
4. After all three solutions are dissolved, mix them together and adjust the final volume to 1L.
5. Filter sterilize through a 0,22µm filter and freeze (-20°C) down in aliquots of 50mL.

Amino acid stock for CDM-LAB
Per liter of amino acid stock:
4,8g DL-alanine
10g L-arginine-HCl
8,4g L-aspartic acid
2,6g L-cysteine-HCl
10g L-glutamic acid
3g L-histidine-HCl-H_2O
4,2g L-isoleucine
9,5g L-leucine

8,8g L-lysine-HCl
5,5g L-phenylalanine
13,5g L-proline
6,8g L-serine
4,5g L-threonine
1g L-tryptophane
6,5g L-valine
3,5g glycine
2,5g L-methionine
2g L-aspargine
4g L-glutamine

Dissolve in 1L dH2O by adjusting pH to 6,8. Filter sterilize through a 0,22µm filter and freeze (-20°C) down in aliquots of 50mL.

2.11 Vectors

pCR™-Blunt II-TOPO® vector

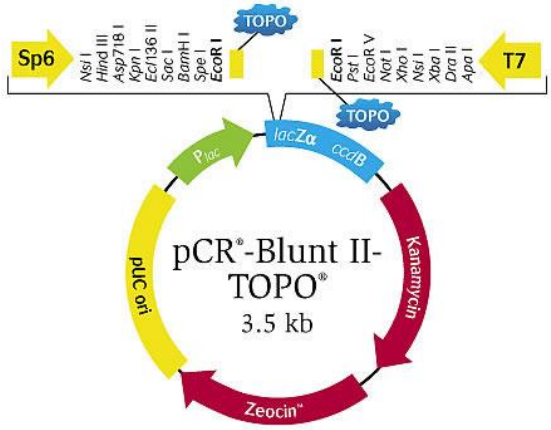


Figure 8: Schematic of pCR™-Blunt II-TOPO® vector, its gene placements and restriction seats placed around insertion area. Most noticeable is the kanamycin resistance gene, and the insertion site being placed between the promoter P_{lac} and the gene *LacZα*. (Figure acquired from Invitrogen homepages, http://products.invitrogen.com/ivgn/product/K283020)

pLT06

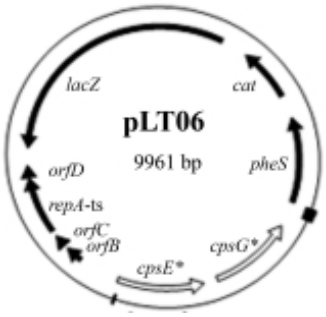


Figure 9: Schematic of pLT06 vector and its gene placements. Most noticeable is the *LacZ* gene, the *cat* gene providing chloramphenicol resistance, the thermo-sensitive *RepA-ts*, and the P-*PheS* cassette inhibiting vector replication in the presence of 4-chloro-phenylalanine, provided by Thurlow. (55)

pÅS222

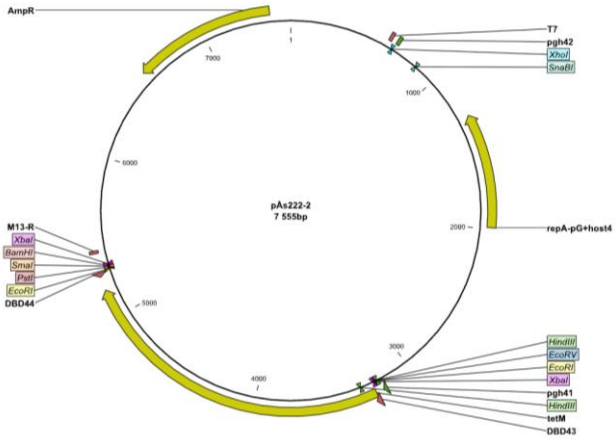


Figure 10: Schematic of the pÅS222 vector, with its gene placements and restriction seats. pÅS222 is made thermo sensitive through its *repA-pG+host4* gene, and includes genes providing resistance for tetracycline and ampicillin, provided by Jonsson, M. (28)

pREG

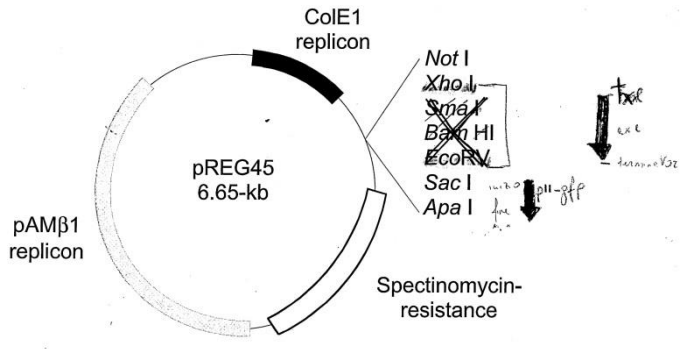


Figure 11: Schematic of the pREG vector, with its gene placements and some restriction seats. pREG a spectinomycin resistance marker allowing for selection on agar, and a axe-txe system, allowing for selection without antibiotics in broths, provided by Grady, R. (18).

3.0 Method

3.1 Cultivation of bacteria

3.1.1 Overnight culture (ON-culture)

Bacteria inoculated into medium and grown over night prior to usage are referred to as overnight cultures (ON-cultures). The culture will then be in its stationary phase with a cell number of approximately 10^9.

3.1.2 Cultivation of *Escherichia coli*

Strains of *E. coli* were incubated overnight (ON) in LB or on LA at 37°C. Liquid cultures were incubated with shaking at 250rpm. Strains carrying genes providing resistance to an antibiotic were incubated with the antibiotic added to the medium. Concentrations of antibiotic differ depending on which type of antibiotic-resistance the bacteria carried.

Table 4: Antibiotic concentrations used when working with *E. coli*.

Antibiotics used when working with *E. coli*	
Antibiotic name	Concentration (µg/mL)
Tetracycline	12.5
Chloramphenicol	15

Ampicillin	100
Erythromycin	300
Kanamycin	50
Spectinomycin	150

3.1.3 Cultivation of *Enterococcus faecalis*

Strains of *E. faecalis* were incubated overnight (ON) in TH-broth, in CDM-medium, or on TH-agar at 37°C. Strains carrying genes providing resistance to an antibiotic were incubated with the antibiotic added to the medium. Concentrations of antibiotic differ depending on which type of antibiotic-resistance the bacteria carried.

Table 5: Antibiotic concentrations used when working with *E. faecalis*.

Antibiotics used when working with *E. faecalis*	
Antibiotic name	Concentration (µg/mL)
Tetracycline	25
Chloramphenicol	34
Erythromycin	10-20
Spectinomycin	500

3.2 Storage

3.2.1 Storage at 4°C

Agar plates and liquid medium was stored at 4°C. Agar plates and liquid medium containing bacterial growth were stored at 4°C for up to two weeks. If used, agar plates and liquid medium was heated up to room temperature prior to inoculation.

3.2.2 Storage at -20°C

All genetic material as well as reagents with -20°C storage requirements were stored at -20°C until further use.

3.2.3 Storage at -80°C

Long-term storage of competent cells and bacterial isolates were stored at -80°C. All *E. coli* and *E. faecalis* bacterial strains (wild-type, intermediates, or mutants), were stored as freeze stocks. Freeze stocks were made in Cryo-tubes (1mL). The Cryo-tube was 265 µl 80%

glycerol, and 735 µl ON bacterial culture, for a final concentration of ~20% glycerol, and stored in a freezer at -80°C for future use. *E. coli* strains were stored in LB-medium. *E. faecalis* strains were stored in TH-medium. Competent *E. coli* cells were stored in GYT-medium in aliquots of 50/100µl. Competent *E. faecalis* cells were stored in SGM17-medium in aliquots of 50/100µl.

3.3 Schematic overview of study progression

In figure 12 an overview of study progression is described, originally the structure of the study only involved production of the Δ*arcA* deletion mutant. Complementation of *arcA* and the Δ*arcA*Δ*glnA* double mutant was added to the study at a later stage. The overview of study progression is set up in chronological order, reflecting the flow of lab work through the thesis. The written thesis is also built up in the same chronological order.

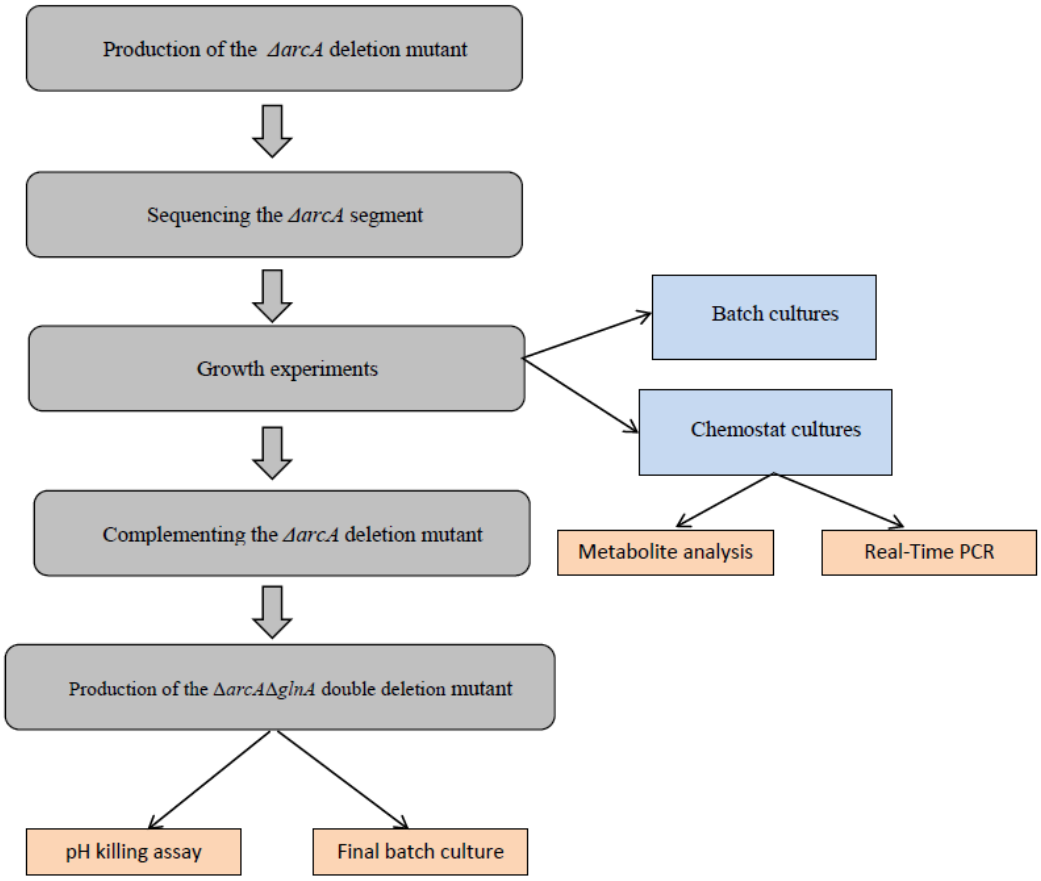


Figure 12: Schematic overview of study progression.

3.4 Construction of *E. faecalis* V583Δ*arcA*

Molecular cloning was used to produce a deletion mutant with a deletion in the *arcA* gene. Genomic DNA from V583 wild type was used as template for two-step PCR procedure where the flanking regions of the arcA gene were amplified and fused together, producing the *arcA* omitted Δ*arcA* construct. The Δ*arcA* insert was cloned into a commercial vector PCR®-, and subsequently transformed into electro-competent *E. coli*, producing the pTOPOΔ*arcA* construct. The pTOPOΔ*arcA* construct was isolated from successful transformants and the Δ*arcA* construct was cut out of pTOPOΔ*arcA* using restriction enzymes BamHI and PstI. The thermo-sensitive pLT06 vector was also cut using restriction enzymes BamHI and PstI, and subsequently the Δ*arcA* construct was ligated into pLT06 before transformation into electro-competent *E. coli* EC1000. The pLT06Δ*arcA* vector construct was then isolated from successful transformants and transformed into electro-competent *E. faecalis* V583. The thermo-sensitive qualities of the pLT06 were utilized by cultivation under set temperatures 30°C and 42°C to set up an integration of the vector construct into the bacterial chromosome causing a single crossover in the *arcA* gene. Double crossover was subsequently achieved by cultivation at 30°C combined with growth medium containing p-chloro-phenylalanine, resulting in a markerless deletion of the *arcA* gene.

A more detailed description of the individual steps in the construction of the mutant is listed below:

3.4.1 Preparation of electro-competent *E. coli*

In order for cells to be transformable (able to absorb DNA), they have to be in a state of competence. *E. coli* cells were made electro-competent to function as a production factory for vectors containing DNA fragments to higher concentrations, before the vector containing our construct was transformed into *E. faecalis* V583.

Materials:
E. coli DH5α, *E. coli* EC1000 and *E. coli* GeneHogs
10% glycerol
LB medium
GYT medium
Nunc-tubes (50mL)
10mL culture tubes (glass)
250mL Erlendmeyer flask (glass)

Ultrospec 10 Cell density meter
Eppendorf Centrifuge 5804 R

Procedure:

1. 5mL LB-medium was inoculated and incubated ON at 37°C in a shaker at 250rpm.
2. 1mL of the ON-culture was inoculated into 100mL LB-medium, and incubated at 37°C with shaking at 250rpm until a cell density of 0,6 OD_{600} was reached (Measured on Ultrospec 10 density meter).
3. 100mL culture was chilled on ice for 30 minutes.
4. After chilling, culture was centrifuged for 15 minutes at 4000 rpm at 4°C.
5. Pellet was washed twice using 50mL ice cold GYT-medium, and re-suspended in 200µl ice-cold GYT medium.
6. Suspension was distributed into aliquots of 100/50 µl and stored in a freezer at -80°C for later use.

3.4.2 Preparation of electro-competent *E. faecalis* V583.

In order to ready *E. faecalis* V583 cells for transformation, *E. faecalis* V583 cells were made electro-competent through a method described by Holo & Nes (20).

Materials:
E. faecalis V583
E. faecalis V583Δ*arcA*
E. faecalis V583Δ*glnA*
1M sucrose
40% glucose
20% glycine
GM17 medium
GM17 (2x) medium
SGM17 medium
Nunc-tubes (50mL)
10mL culture tubes (glass)
Ultrospec 10 Cell density meter
Eppendorf Centrifuge 5804 R

Procedure:

1. *E. faecalis* V583 was inoculated into 5mL GM17 and grown ON at 37°C.
2. A gradient of glycine in SGM17 was made by adding to each tube (in the follow order to ensure mixing):

- 5mL 1M sucrose
- 125µl 40% glucose
- glycine and 2x M17 according to the table below

Table 6: Volume and concentrations of glycine-gradient tubes.

[glycine]	**4%**	**4.5%**	**5%**	**5.5%**	**6%**
20% glycine (mL)	2	2.25	2.5	2.75	3
2x M17 (mL)	3	2.75	2.5	2.25	2

3. 100µl from the 5mL GM17 ON-culture was added to each tube and grown ON at 37°C.
4. OD_{600} was measured, and competent cells of culture with an OD_{600} between 0,2 - 0,3 were made. (If two cultures are in the range, they can be mixed before proceeding).
5. Culture was pelleted by centrifugation at 4°C.
6. Pellet was washed twice with ice-cold 0,5M sucrose.
7. Washed pellet was re-suspended 2-400µl 0.5M sucrose+10% glycerol.
8. Aliquots of 50/100µl was frozen down for later use (can also leave on ice for 30 minutes before usage the same day).

3.4.3 Isolating genomic DNA from *E. faecalis* V583.

Genomic DNA (gDNA) from *E. faecalis* V583 was isolated using the E.N.Z.A™ Plasmid MiniPrep Kit (MiniPrep) in combination with FastPrep. The MiniPrep kit is optimized for smaller DNA fragments such as vectors, but can be used to effectively isolate genomic DNA when used in combination with FastPrep cell lysis. This combined procedure mechanically lyses cells by violent shaking with acid-washed glass beads, and proceeds to separate the gDNA from the rest of the cell material through the MiniPrep kit. After elution, gDNA concentration was measured using NanoDrop ND-1000.

Materials:
5mL ON culture of *E. faecalis* V583
FastPrep tubes
FastPrep FP120 (Savant)
Acid-washed pellets ($<10^6$ microns)
10mL culture tubes (glass)
Eppendorf tubes
E.N.Z.A™ Plasmid MiniPrep Kit

NanoDrop ND-1000
Eppendorf Centrifuge 5804 R

Procedure:

1. 5mL ON-culture inoculated with E. faecalis V583 was pelleted by centrifugation at 6000 rpm for 5 minutes.
2. Supernatant was decanted and pellet re-suspended in 300µl Solution I/RNaseA (From MiniPrep kit).
3. Suspension was transferred to a FastPrep tube containing 0,5g acid-washed glass pellets ($<10^6$ microns).
4. FastPrep tubes were shaken for 20 seconds at 6,0 m/s in the FastPrep FP120 to mechanically lysate cells.
5. FastPrep tubes were centrifuged for 3 minutes at 13000 rpm, and the supernatant transferred to an Eppendorf tube.
6. gDNA separated from the rest of the cell material by MiniPrep using the protocol for the MiniPrep kit, starting at step 4. (See appendix, attachment 2).

After the elution step in the MiniPrep kit protocol, concentration of gDNA was measured using NanoDrop ND-1000 and stored at -20°C until further use.

3.4.4 Producing *arcA* flanking fragments arcA5-6 and arcA7-8 through PCR

Polymerase chain reaction (PCR) is a method used to amplify a specific sequence of DNA. The method consists of the three temperature regulated phases; denaturation, annealing, elongation. In the denaturation phase, double-stranded DNA is separated and made single-stranded by incubation at 95-98°C. In the annealing phase, short synthetic strands of DNA called primers, attach to a site complementary to their primer sequence. Primers serve as a starting location for sequence elongation, and frame the area of interest for amplification. The temperature of this phase is decided by the primer sequence, but usually annealing is performed at a temperature between 58-62°C. In the elongation phase, DNA polymerase synthesize new DNA based on the primer annealing sites, the elongation phase is usually performed at 72°C. Amplification through PCR is exponential as the three phases are repeated usually around 29-35 times. The cycling of the three phases, is usually preceded by a longer denaturation stage to ensure denaturation of template DNA, and followed by a longer elongation stage to ensure complete elongation of all synthesized product.

In this thesis, the DNA polymerases Phusion® and Taq® are used. These differ quite significantly in that Taq® polymerase works at a rate of 1000 base pairs per 1 minute, whilst Phusion® polymerase works at a rate of 1000 base pairs per 20 seconds. In addition, Phusion® Polymerase has proof-reading, which minimizes its chance of incorporating wrong nucleotides into the sequence (Taq® polymerase on average incorporates 1 wrong nucleotide per 1000 bases, Phusion® polymerase is listed as having an error rate 50-fold lower than that (44). A typical PCR reaction mix consists of; a buffer specific for the DNA polymerase, $MgCl_2$, forward and reverse primers, nucleotides (dNTPs), a DNA polymerase, template DNA containing sequence of interest, and water. In this thesis, the general PCR reaction mix and reaction conditions using Phusion® and Taq® polymerase are as follows:

Phusion® polymerase PCR reaction mix:

- 5x Phusion® HF Buffer 10 µl
- dNTPs (10mM) 1 µl
- Forward primer (10mM) 1 µl
- Reverse primer (10mm) 1 µl
- Template DNA ~50 ng
- Phusion® DNA polymerase 0.5 µl
- dH_2O Added up to a total volume of 50 µl.

Phusion® polymerase PCR reaction conditions:

- Initial denaturation 98 °C for 1 minute
- Denaturation 98 °C for 30 seconds (x35)
- Primer annealing * °C for 30 seconds (x35)
- Polymerization 72 °C for 30 seconds (x35)
- Final polymerization 72 °C for 4 minutes
- Cooling 4 °C ∞

* Usual temperature interval is 58-62°C,but other temperatures can be used for annealing phase depending on the primers used. Where other annealing temperatures are used, this is specified in the method.

Taq® polymerase PCR reaction mix:

- 10x Taq Buffer (-$MgCl_2$) 5 µl
- $MgCl_2$ (50mM) 1 µl
- dNTPs (10mM) 1 µl
- Forward primer (10mM) 1 µl
- Reverse primer (10mm) 1 µl

- Template DNA ~50 ng
- Taq® DNA polymerase 0.5 µl
- dH_2O Added up to a total volume of 50 µl.

Taq® polymerase PCR reaction conditions:

1. Initial denaturation 95 °C for 1 minute
2. Denaturation 95 °C for 30 seconds (x29)
3. Primer annealing * °C for 30 seconds (x29)
4. Polymerization 72 °C for 1 minute 30 seconds (x29)
5. Final polymerization 72 °C for 4 minutes
6. Cooling 4 °C ∞

* Usual temperature interval is 58-62°C, but other temperatures can be used for annealing phase depending on the primers used. Where other annealing temperatures are used, this is specified in the method.

To produce the *arcA* deletion construct (Δ*arcA*), the four primers arcA-5, arcA-6, arcA-7, and arcA-8 were used. Primers arcA-5 and arcA-6 covers the elements upstream from the *arcA* gene, and primers arcA-7 and arcA-8 covers the elements downstream from the *arcA* gene. Two PCRs were run using gDNA isolated from *E. faecalis* V583 wild-type as template. The product of the PCRs being two constructs, arcA5-6, and arcA7-8 where arcA6 contains a sequence reverse complementary to a sequence in arcA7.

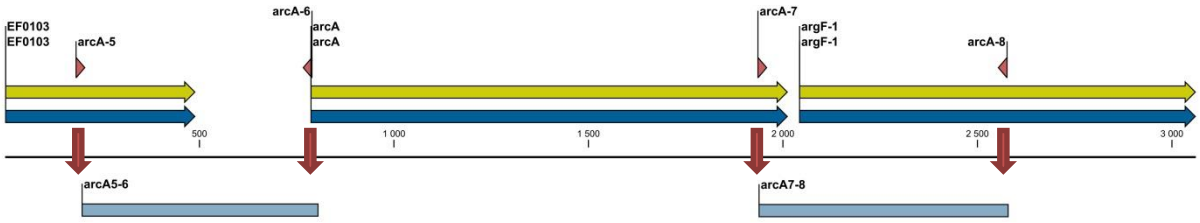


Figure 13: Diagram of the *arcA* gene with primers arcA-5F, arcA-6R, arcA-7F, arcA-8R. Figure also shows the arcA5-6 and arcA7-8 constructs which are produced in this first round of PCRs.

Materials:
5x Phusion® HF Buffer
dNTPs (10mM)
Primer arcA-5 (10mM)
Primer arcA-6 (10mM)
Primer arcA-7 (10mM)
Primer arcA-8 (10mM)
Template DNA

Phusion® DNA polymerase
dH_2O

Procedure:

Two separate PCRs were set up using a PCR reaction mix for Phusion® DNA polymerase as described above. The 1st PCR with arcA-5 (forward primer) and arcA-6 (reverse primer), and the 2nd PCR with arcA-7 (forward primer) and arcA-8 (reverse primer). PCR reaction conditions for Phusion® DNA polymerase reaction was set as specified above.

3.4.5 Gel electrophoresis, extraction and purification of arcA5-6 and arcA7-8.

To validate the presence and length of the two constructs arcA5-6 (609bp) and arcA7-8 (643bp) after PCR, the PCR product was run through a 1% agarose gel by electrophoresis. DNA is negatively charged at neutral pH, and migrates towards the positive terminal when in an electric field. The 1% agarose gel contains pores that allow separation of DNA fragments based on size and configuration. Small fragments migrate faster than large fragments through the gel, and supercoiled DNA will migrate faster than linear DNA and open circular DNA. Ethidium bromide added to the gel forms a complex with the DNA during migration. This complex formation fluoresces under a UV-light spectrum, and allows visualization of the DNA. This fluorescent visualization allows observation of DNA size and presence through fluorescent bands, and enables extraction of DNA with specific band sizes.

The respective bands arcA5-6 (609bp) and arcA7-8 (643bp) were cut out from the gel and cleaned up using the Nucleospin® PCR Clean-up Gel Extraction kit. After elution, product concentration was measured using NanoDrop ND-1000.

Materials:

1% Agarose
1x TAE buffer
Ethidium bromide (10mg/mL) (EtBr)
6x loading buffer
1kb. ladder
Volt-meter
Gel-electrophoresis equipment (rack, molding form, comb)
Gel photo system with UV spectrum.
Scalpel knife
Nucleospin® PCR Clean-up Gel Extraction kit
NanoDrop ND-1000

Procedure:

1. 50µl ~1% agarose gel with 2µl EtBr added was made.
2. First well was added 10µl 1kb. Ladder.
3. PCR-mixture with1/6th of its volume in loading buffer was vortexed, and added to available wells.
4. Gel-electrophoresis was run at 80V for 30-40 minutes.
5. Gel was then placed under UV-spectrum, bands observed and photographed by the Gel Photo System.
6. Gel fragments containing the bands were cut out from gel with a scalpel knife (new knife for each band), and transferred to pre-weighed eppendorf tubes.
7. Weight of gel fragments was calculated using pre-weight of the eppendorf tubes, and 200 µl NTI-buffer per 100µg gel was added to each tube.
8. NTI/gel mixture was heated up to 50°C on a heat-block, and vortexed every 5 minutes until the gel was completely dissolved.
9. NTI/dissolved gel mixture was added to a Nucleospin® PCR Clean-up Column, and was washed, then eluted as advised in the Nucleospin® PCR Clean-up Extraction Kit manual. (See appendix, attachment 3).

After elution, concentration of both arcA5-6 and arcA7-8 product was measured using NanoDrop ND-1000, and stored at -20°C until further use.

3.4.6 Fusing arcA5-6 and arcA7-8 together to Δ*arcA* through fusion-PCR.

The complementary sequence overlap in arcA-6 and arcA-7 allows for sequence overlap extension fusing using PCR. The two constructs arcA5-6 and arcA7-8 were fused together through sequence overlap extension PCR using primers arcA-5 and arcA-8. The result of this PCR was the full arcA5678, where the *arcA* gene is omitted (hereafter referred to as the Δ*arcA* construct). The construct had a size of 1,2kb.

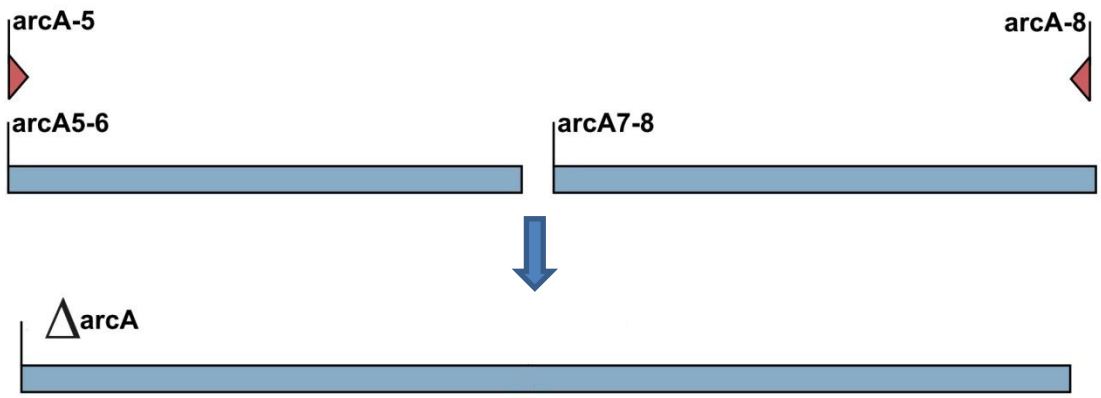


Figure 14: Diagram of the primer positions on arcA5-6 and arcA7-8 in the sequence overlap extension PCR reaction. The reverse complementary part of arcA-6 and arcA-7 enabling the PCR to fuse together the arcA5678 (Δ*arcA*) construct.

Materials:
5x Phusion® HF Buffer
dNTPs (10mM)
Primer arcA-5 (10mM)
Primer arcA-8 (10mm)
The arcA5-6 construct
The arcA7-8 construct
Phusion® DNA polymerase
dH_2O

Procedure:

A PCR was set up using a PCR reaction mix using arcA-5 (forward primer) and arcA-8 (reverse primer) for Phusion® DNA polymerase as described in 3.4.4. PCR reaction conditions for Phusion® DNA polymerase reaction was set as described in 3.4.4.

3.4.7 Gel electrophoresis, extraction and purification of Δ*arcA*.

Materials:
Materials as is listed in 3.4.5.

Procedure:

The Δ*arcA* construct was run through a gel by electrophoresis before extraction and purification from the gel in the same way as explained in 3.4.5. After elution, concentration of Δ*arcA* insert was measured on NanoDrop, and stored at -20°C until further use.

3.4.8 Ligating Δ*arcA* into PCR®-Blunt II-TOPO® vector, and transformation into *E. coli*

To be able to transform the Δ*arcA*-fragment into electro-competent *E. coli* cells, the Δ*arc*A-fragment was first ligated into the pCR®-Blunt II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning kit (producing the pTOPOΔ*arcA* vector construct). The pTOPOΔ*arcA* vector construct was then transformed into electro-competent *E. coli* GeneHogs by electroporation. The pCR®-Blunt II-TOPO® vector carries a resistance marker for kanamycin, and carries the *LacZα* gene encoding for the α-subunit of β-galactosidase. The blunt insertion site of the pCR®-Blunt II-TOPO® is placed between the *LacZ* promoter (P_{lac}) and *LacZα* gene. This means that when cloning the Δ*arcA* construct in the vector, the *LacZα* gene expression is inactivated.

Using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (hereafter referred to as X-gal), a lactose analog made up by galactose and indole bonded by a β-glycosidic bond. Expression of the *LacZα* gene produces β-galactosidase, which in turn cleaves the β-glycosidic bond between galactose and indole. Cleaved indole molecules spontaneously dimerize, providing an insoluble strong blue colour on the agar plates (2).

The feature of the *LacZα* inactivation on insertion into the pCR®-Blunt II-TOPO® vector was utilized in collaboration with kanamycin antibiotic selection, to select for transformants containing pTOPOΔ*arcA*, by isolating white (*LacZα* negative) colonies.

Materials:
Δ*arcA* insert from 3.4.7.
Electro-competent *E. coli* GeneHogs made in 3.4.1.
Zero Blunt® TOPO® PCR Cloning kit
SOC medium
Gene Pulser from Bio Rad
1mm electroporation cuvette
Eppendorf tubes
LA with 50µg/mL kanamycin
X-gal 40mg/mL
LB with 50µg/mL kanamycin

Procedure:
1. Cloning reaction was performed as instructed under "Performing the TOPO® Cloning reaction", in the manual for the Zero Blunt® TOPO® PCR Cloning kit. (See appendix, attachment 1)
2. Transformation was performed as described under "One Shot® Electroporation", in the manual for the Zero Blunt® TOPO® PCR Cloning kit. (See appendix, attachment

1). Using a modification of the method described by Wai Lin Tung, King-C. Chow (56), electroporation was performed in a pre-chilled 1mm electroporation cuvette using 1.7kV, 25 µF, 200 ohms. As selective media LA with 50µg/ml kanamycin and 40µl X µg/mL x-gal was used.

3. 3-4 White (*LacZα*-negative) Colonies from ON-culture on LA with 50µg/ml kanamycin were isolated and inoculated in 5mL LB added 50µg/ml kanamycin. Inoculated LB was incubated ON at 37°C.

3.4.9 Isolating the pTOPOΔ*arcA* vector construct from transformants

After incubation ON, freeze stock of *E. coli* + pTOPOΔ*arcA* was made, and the pTOPOΔ*arcA* vector construct isolated using the E.N.Z.A™ Plasmid MiniPrep Kit.

Materials:

5mL ON culture of *E. coli* + pTOPOΔ*arcA* in LB-medium with 50µg/mL kanamycin
80% glycerol
10mL culture tubes (glass)
Eppendorf tubes
E.N.Z.A™ Plasmid MiniPrep Kit
NanoDrop ND-1000

Procedure:

1. Freeze stocks of *E. coli* + pTOPOΔ*arcA* were made as explained in 3.2.3.
2. pTOPOΔ*arcA* vector construct was isolated using the E.N.Z.A™ Plasmid MiniPrep Kit and performed as instructed in the E.N.Z.A™ Plasmid MiniPrep manual. (See appendix, attachment 2).
3. Vector construct concentration was measured on NanoDrop ND-1000, and stored at -20°C, until further use.

3.4.10 Validating Δ*arcA* in the pTOPOΔ*arcA* vector construct

Control PCR using Taq® DNA polymerase with primers arcA-5 and arcA-8, followed by gel electrophoresis was performed to validate presence and size of Δ*arcA*, using the pTOPOΔ*arcA* vector construct as template for the PCR reaction.

Materials:
10x Taq Buffer (-$MgCl_2$)
$MgCl_2$ (50mM)
dNTPs (10mM)
Primer arcA-5 (10mM)
Primer arcA-8 (10mm)
Isolated pTOPOΔ*arcA* from 3.4.9

Taq® DNA polymerase
dH_2O

Procedure:

A PCR was set up using a PCR reaction mix using arcA-5 (forward primer) and arcA-8 (reverse primer) for Taq® DNA polymerase as described in 3.4.4. PCR reaction conditions for Taq® DNA polymerase reaction was set as described in 3.4.4, except for the elongation phase being adjusted to the expected sequence length. The PCR mixture was run through a gel by electrophoresis, as explained in 3.4.5 steps 1 to step 5.

3.4.11 Restriction cutting Δ*arcA* insert out of pTOPOΔ*arcA* & restriction cutting the pLT06 vector

After cloning the Δ*arcA* construct into the pCR®-Blunt II-TOPO® vector, which only contains a Gram-negative replicon, the Δ*arcA* construct was cut out of the pTOPOΔ*arcA* vector using restriction enzymes BamHI and PstI. Subsequent ligation into the vector pLT06 was performed. The vector pLT06 contains both a Gram-negative, and a Gram-positive replicon and is thermo-sensitive in Gram-positives, made Ts by its *repA*-ts gene which only initiates replication in Gram-positives. In order to replicate in Gram-negatives, the vector is dependent on a copy of the *repA* gene being provided chromosomally *in trans* by its Gram-negative cell host (35).

Materials:

Materials as listed in 3.4.5 with the exception of the cutting reaction mix.

Cutting reaction mix

- NEB Buffer 3 — 2 µl
- 10xBSA (10mM) — 2 µl
- BamHI restriction enzyme — 1 µl
- PstI restriction enzyme — 1 µl
- DNA (Vector) — ~1.5 µg
- dH_2O — Added up to a total volume of 20 µl.
- CIP — Only added when auto re-ligation is a problem, 1µl is added 1 hour into cutting reaction.

Procedure for pTOPOΔ*arcA*:

1. Approximately 1.5 µg pTOPOΔ*arcA* was added into cutting reaction mix.
2. Solution was incubated at 37°C for 2 hours.

3. Solution was run through a gel by electrophoresis and the ~1.2kb band was extracted and purified, as explained in 3.4.5.
4. Concentration of eluted Δ*arcA* construct was measured on NanoDrop ND-1000, and stored at -20°C until further use.

Procedure for pLT06:

1. Approximately 1.5 µg pLT06 was added into cutting reaction mix.
2. Solution was incubated at 37°C for 1 hour.
3. 1 µl CIP was added to the cutting reaction mix 1 hour into incubation, and solution was incubated another hour at 37°C.
4. Solution was run through a gel by electrophoresis together with uncut pLT06 as a control. The ~7.9 kb band was extracted and purified, as explained in 3.4.5.
5. Concentration of eluted cut pLT06 vector was measured on NanoDrop ND-1000 and stored at -20°C until further use.

3.4.12 Ligating Δ*arcA* into pLT06 vector

The Δ*arcA* construct was ligated into the pLT06 vector using T4 DNA ligase. With the Δ*arcA* insert size being 1.2kb, and the pLT06 vector being 7.9kb this results in a construct total size of 9.1kb (hereafter referred to as pLT06Δ*arcA* vector construct).

Materials:

Ligation reaction mix :

- T4 ligase buffer 2µl
- T4 ligase enzyme 1µl
- Vector pLT06 28,8ng
- Δ*arcA* construct ~45ng
- dH_2O Added up to a total volume of 20 µl.

Procedure:

To calculate a good insert / vector ratio, a general formula is used.

$$\frac{10 * X\ bp\ insert * Y\ ng\ vector}{Z\ bp\ vector} = amount\ of\ insert\ needed\ (ng)$$

$$\frac{amount\ of\ insert\ needed\ (ng)}{amount\ of\ insert\ in\ solution\ (\frac{ng}{\mu l})} = \mu l\ of\ insert\ solution\ needed$$

Example using the data from the Δ*arcA* + pLT06 ligation mix:

$$\frac{10\ x\ 1.2kb\ x\ 28{,}8ng\ pLT06}{7.9kb} = 43{,}7ng\ \Delta arcA\ insert\ needed$$

Concentration of Δ*arcA* insert solution = 12,4ng/µl.

$$\frac{43{,}2ng}{12{,}4ng/\mu l} = \sim 3{,}5\mu l$$

Reaction mix incubated for 16 hours at 16°C, followed by 20 minutes at 72°C for enzyme inactivation. Ligation mix was stored at -20°C until further use.

3.4.13 Dialyzing the pLT06Δ*arcA* ligation mix

To avoid arcing caused by salts or other compounds that may increase conductivity in the solution, it is advised to dialyze the solution before electroporation. Arcing can lead to cell death and a failed transformation (34).

Materials:

pLT06Δ*arcA* ligation mix
0,1xTE buffer
Millipore filter (0,025µm)
Petri dish
Pincers
Eppendorf tube

Procedure:

1. Petri dish was poured half-full of 0,1xTE buffer.
2. Filter was carefully placed upon 0,1xTE buffer fluid using a pincer.
3. Ligation mix was applied on the filter and dialyzed for at least an hour before electroporation.

3.4.14 Transforming the pLT06Δ*arcA* vector construct into *E. coli* EC1000

After dialyzing the pLT06Δ*arcA* ligation mix, the pLT06Δ*arcA* vector construct was transformed into electro-competent *E. coli* EC1000 by electroporation. *E. coli* EC1000 was used because it provides a copy of the *repA* gene chromosomally *in trans*, as required for replicaton of the pLT06 vector in Gram-negatives (35), x-gal selection (as described in 3.4.8)

for *LacZα* positive transformants (blue colonies) was utilized in collaboration with chloramphenicol antibiotics selection.

Materials:

Dialyzed pLT06Δ*arcA* ligation mix from 3.4.13
Electro-competent *E. coli* EC1000
SOC medium
Gene Pulser from Bio Rad
1mm electroporation cuvette
Eppendorf tubes
LA with 15µg/mL chloramphenicol
X-gal 40mg/mL
80% glycerol
2mL Cryo-tubes

Procedure:

1. Dialyzed ligation mix was mixed with 40µL electro-competent *E. coli* EC1000 and incubated for 5 minutes.
2. Solution was transferred to a pre-chilled 1mm electroporation cuvette.
3. Cuvette was placed into Gene Pulser and electroporated under conditions 1.7kV, 25µF, 200 ohms.
4. 250µL SOC medium was immediately added to solution after electroporation and carefully mixed.
5. Transformation solution was transferred to an Eppendorf tube and incubated at 37°C with 250rpm shaking for 1 hour.
6. Transformation solution was applied on two LA+15µg/mL chloramphenicol agar dishes with 40µL 40mg/mL x-gal added. (50µL from the transformation solution on one agar dish, and the rest on the other agar dish)
7. Agar dishes were incubated ON at 30°C.
8. Blue (*LacZα*-positive) colonies on the agar dishes were harvested and inoculated in 5mL LB+15µg/mL chloramphenicol ON at 30°C.
9. Freeze stocks of *E. coli* with the pLT06Δ*arcA* vector (hereby *E. coli* + pLT06Δ*arcA*) were made as described in 3.2.3.

3.4.15 Isolating pLT06Δ*arcA* from E. coli + pLT06Δ*arcA*

After incubation ON, the pLT06Δ*arcA* vector construct was isolated using the Qiagen® Plasmid Midi Kit. The concentration of vector isolated was measured on NanoDrop ND-1000.

Materials:

100mL ON culture of *E. coli* + pLT06Δ*arcA* in LB-medium with 15µg/mL chloramphenicol
250mL culture flasks (glass)
Eppendorf tubes
Qiagen® Plasmid Midi Kit
NanoDrop ND-1000

Procedure:

1. 100mL ON culture of *E. coli* + pLT06Δ*arcA* in LB-medium with 15µg/mL chloramphenicol was set up and incubated ON at 30°C.
1. The pLT06Δ*arcA* vector construct was isolated using the Qiagen® Plasmid Midi Kit and performed as instructed in the Qiagen® Plasmid Midi Kit Quick-Start Protocol. (See appendix, attachment 4).
2. Vector concentration was measured on NanoDrop ND-1000, and stored at -20°C until further use.

3.4.16 Dialyzing the pLT06Δ*arcA* MiniPrep elution

The pLT06Δ*arcA* MiniPrep elution was dialyzed with the same materials and procedure as described in 3.4.13.

3.4.17 Transforming the PLT06Δ*arcA* into electro-competent *E. faecalis* V583.

After dialyzing the pLT06Δ*arcA* MiniPrep elution, the pLT06Δ*arcA* vector construct was transformed into electro-competent *E. faecalis* V583 by electroporation. X-gal selection (as described in 3.4.8) for *LacZα* positive transformants (blue colonies) was utilized in collaboration with chloramphenicol antibiotics selection.

Materials:

Dialyzed pLT06Δ*arcA* MiniPrep elution from 3.4.16
Electro-competent *E. faecalis* V583
SGM17 medium
Gene Pulser
2mm electroporation cuvette
Eppendorf tubes
TH-agar with 34µg/mL chloramphenicol
X-gal 40mg/mL

80% glycerol
2mL Cryo-tubes
30°C Incubator

Procedure:

1. Dialyzed pLT06Δ*arcA* MiniPrep elution was mixed with 40µL electro-competent *E. faecalis* V583 and incubated for 5 minutes
2. Solution was transferred to a pre-chilled 2mm electroporation cuvette
3. Cuvette was placed into Gene Pulser and electroporated under conditions 2kV, 25µF, 200 ohms.
4. 1mL SGM17 medium was immediately added to solution after electroporation and carefully mixed.
5. Transformation solution was transferred to an eppendorf-tube and incubated at 37°C for at least 3 hours.
6. Transformation solution was applied on two TH+34µg/mL chloramphenicol agar dishes with 40µL 40mg/mL x-gal added. (350µL from the transformation solution on one agar dish, and the rest on the other agar dish)
7. Agar dishes were incubated ON at 30°C.
8. Blue (*LacZα*-positive) colonies on the agar dishes were harvested and inoculated in 5mL LB+15µg/mL chloramphenicol ON at 30°C.
9. Freeze stock of *E. faecalis* with the pLT06Δ*arcA* vector construct (hereafter referred to as *E. faecalis* + pLT06Δ*arcA*) was made as described in 3.2.3.

3.4.18 Engineering the single-crossover (sco) in *E. faecalis* V583 + pLT06Δ*arcA*

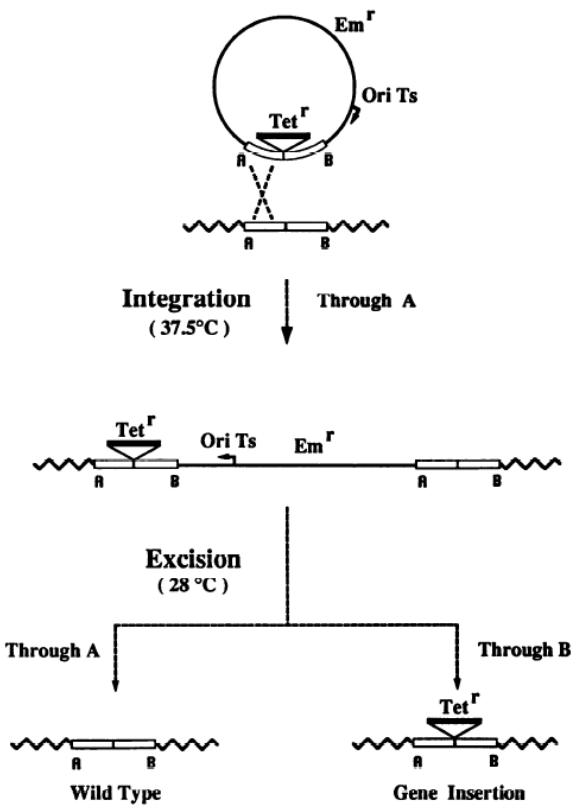


Figure 15: Picture originally shows principle of repA-pG+Host4. The same principle is applied to use of vector pLT06 in creating the Δ*arcA* deletion mutant. (6)

The vector pLT06 is a thermo-sensitive vector made thermo-sensitive by its *repA-ts* fragment, which at 37°C-42°C (or even higher) is integrated into the bacterial chromosome (sco), as the temperature is reduced to 28°C the integration becomes unstable and the vector excised from the chromosome (dco). Depending on which end of the integrated vector the excision is done, gene mutant or wild type is generated. Inserting a gene for antibiotics resistance inside the vector with the Δ*arcA* insert provides opportunity for antibiotics selection during single crossover, as bacterial cell cannot survive at 37°C in an environment with antibiotics, without the resistance gene from the vector. We do not use the antibiotics gene for selection during double crossover, as we wish our mutant to be as close to the wild type as possible, and do not wish to end up with a resistant mutant in the case of future studies using the mutant.

Materials:

10mL culture tubes
TH broth with 34µL/mL chloramphenicol
TH agar with 34µL/mL chloramphenicol
40mg/mL x-gal
30°C Incubator
42°C Incubator
Cryo-tubes

Procedure:

1. One of the ON-cultures from 3.4.17 was diluted 1000x into fresh TH broth, and grown at 30°C for 2 ½ hours (this to generate cell number and reset growth phase)
2. Culture was transferred to a 42°C incubator and grown for 2 ½ hours.
3. -1 to -8 dilutions were made from this culture and 5 µL from each dilution was applied on to TH agar with 34µL/mL chloramphenicol and 40µL x-gal.
4. Incubated ON at 42 °C.
5. 2 to 4 blue (*LacZα*-positive) colonies were harvested and inoculated into TH broth with 34µL/mL chloramphenicol, and incubated ON at 42 °C.
6. Freeze stock *E. faecalis* with the pLT06Δ*arcA* vector integrated (hereafter referred to as *E. faecalis*+ pLT06Δ*arcA* sco) was made as described in 3.2.3.

3.4.19 Validating the sco

Before proceeding to the double crossover (dco) step, we wished to first validate the presence of the sco. This was done by isolating gDNA from *E. faecalis*+ pLT06Δ*arcA* sco. When gDNA was eluated, then control PCR using the two plasmid specific primers OriF and KSo5SeqR, along with Δ*arcA* primers arcA-9 and arcA-10 was performed. These primers had a low annealing temperature at 50°.

Materials:

E. faecalis+ pLT06Δ*arcA* sco from 3.4.18
Materials for gDNA isolation as described in 3.4.3
Materials for gel electrophoresis as described in 3.4.5
Materials for PCR reaction using Taq® DNA polymerase as described in 3.4.10 with the exception of primers;
Primer oriF (10mM)
Primer arcA-10 (10mm)
Primer arcA-9 (10mM)
Primer KS05SeqR (10mm)

Procedure:

1. Genomic DNA was isolated as explained in 3.4.3.
2. Two PCR reactions were set up using reaction mix for Taq® DNA polymerase as described in 3.4.4. One PCR reaction using primers OriF (forward primer) with arcA-10 (reverse primer), and the other PCR reaction using primers arcA-9 (forward primer) with KS05SeqR (reverse primer). PCR reaction conditions for both reactions were set as described in 3.4.4 for Taq® DNA polymerase, except for the annealing temperature

which was adjusted to 50°C, and the elongation phase time was adjusted to match the expected sequence length.

3. PCR product was run through a gel by electrophoresis, as explained in 3.4.5 steps 1 to step 5.

3.4.20 Engineering the double crossover (dco) in *E. faecalis*+ pLT06Δ*arcA* sco.

After validation of sco, dco was engineered on MM9YE6-agar with 10mM p-chloro-phenylalanin (PCP) added. No antibiotics added. MM9YE6-agar with PCP added selects for bacteria which undergo dco and don't harbor the pLT06 vector (integrated or not) due to the presence of PCP and the P-*PheS* cassette on pLT06 (55). It is expected that the process of dco will yield a higher amount of wild type revertants than it will mutants, and it may take a few passages for the dco trigger (55). For that reason the procedure of ON-culturing, diluting, and streaking on MM9YE6-agar with PCP is recommended to be repeated for 2-3 days.

Materials:

E. faecalis+ pLT06Δ*arcA* sco from 3.4.18
10mL culture tubes
TH-broth
MM9YE6-agar with 10mM p-chloro-phenylalanin

Procedure:

1. ON-cultures from 3.4.18 were diluted 1000x into TH-broths with no antibiotics added, and incubated ON at 30°C.
2. ON-cultures were diluted -2, -3, -4, -5 and 10 µL from each dilution streaked on MM9YE6-agar with PCP added, incubated ON at 30°C.
3. 8-12 Colonies on MM9YE6-agar were harvested and inoculated into 5mL TH-broth, then incubated ON at 30°C.

As this is expected to take a few passages, the -3 dilution from step 2 is incubated ON at 30°C to repeat step 1 through 3 the next day.

3.4.21 Validating the double crossover

The dco was validated by control PCR. The goal was to investigate whether or not the dco had yielded the Δ*arcA* mutant, hereafter referred to as *E. faecalis* V583Δ*arcA* (band of ~1.2kb) or reverted back to the wild type *E. faecalis* V583 (band of ~2.5 / no band). This was

done by first isolating gDNA from the *E. faecalis*+ pLT06Δ*arcA* dco cultures. When gDNA is eluated, control PCR using Δ*arcA* primers arcA-5 and arcA-8 is performed.

Materials:

E. faecalis+ pLT06Δ*arcA* dco cultures in 5mL TH-broth from 3.4.20
Materials for gDNA isolation as described in 3.4.3
Materials for gel electrophoresis as described in 3.4.5
2mL Cryo-tubes
Materials for PCR reaction using Taq® DNA polymerase as described in 3.4.10 with the exception of primers;
Primer arcA-5 (10mM)
Primer arcA-8 (10mm)

Procedure:

1. Genomic DNA was isolated as explained in 3.4.3.
2. PCR reaction was set up using primers arcA-5 (forward primer) and arcA-8 (reverse primer) in a reaction mix for Taq® DNA polymerase as is described in 3.4.4. PCR reaction conditions were set as is described in 3.4.4 for Taq® DNA polymerase, expect elongation phase time was adjusted to match the expected sequence length of mutant *arcA*.
3. PCR product was run through a gel by electrophoresis, as explained in 3.4.5 steps 1 to step 5.
4. After PCR-confirmation of dco, freeze stock of *E. faecalis* V583Δ*arcA* was made as described in 3.2.3.

3.5 Sequencing the *arcA* region in *E. faecalis* V583Δ*arcA*

Sequencing of the *arcA* gene region was performed by GATC Biotech AG, returned sequence was aligned with wild type *arcA* using the CLC Workbench software.

Materials:

5mL culture of *E. faec*alis V583Δ*arcA* in TH-broth
Primer arcA-5
Primer arcA-8
Materials for gDNA isolation as described in 3.4.3
Materials for PCR as described in 3.4.6
BioEdit software
CLC Workbench software

Procedure:

1. Genomic DNA was isolated from 5mL culture of *E. faec*alis V583Δ*arcA* in TH-broth as described in 3.4.3.
2. PCR run on *E. faec*alis V583Δ*arcA* gDNA was performed with conditions as described in 3.4.6
3. Sequencing mix was prepared as described in LightRun brochure (See appendix, attachment 5), and sequencing mix was shipped to GATC Biotech AG. Seq.IDs: 94EB49 & 94EB50.
4. Upon return, sequence was imported and treated in BioEdit, and aligned to wild type *arcA* sequence using CLC Workbench software.

3.6 Growth experiments

In an attempt to unveil phenotypical changes in the *E. faecalis* V583Δ*arcA* mutant, growth experiments were performed. Wild type *E. faecalis* V583 and mutant *E. faecalis* V583Δ*arcA* were grown in batch culture, and in glucose-limited cultures in a chemostat.

3.6.1 Batch cultures in Bioscreen C Analyzer

Growth studies using batch cultures were performed to observe differences in growth patterns between wild type *E. faecalis* V583 and mutant *E. faecalis* V583Δ*arcA*. The batch cultures were performed with the defined medium CDM-LAB adjusted to pH 7.5, as well as five amino acid omission modifications of CDM-LAB, also adjusted to pH 7.5. Cultures were incubated in Bioscreen C analyzer instrument connected to a computer with EZ Experiment software installed for 48 hours, measuring OD_{600} every 15 minutes with pre-emptive shaking for 10 seconds. Study was performed with 3 biological replicates in 3 technical replicates per sample (a total of 9 parallels per sample). For the batch culture in full CDM-medium, the deletion mutant *E. faecalis* V583Δ*glnA,* engineered by Margrete Solheim (unpublished) was also included in the experiment.

Materials:

CDM-LAB medium, full medium (pH7.5)
CDM-LAB medium, glycine omitted (pH7.5)
CDM-LAB medium, glutamate omitted (pH7.5)
CDM-LAB medium, glutamine omitted (pH7.5)
CDM-LAB medium, with $1/8^{th}$ glutamine concentration (pH7.5)
CDM-LAB medium, serine omitted (pH7.5)
10mL culture tubes
3x5mL cultures *E. faecalis* V583 wild type in CDM-LAB

3x5mL cultures *E. faecalis* V583Δ*arcA* in CDM-LAB
3x5mL cultures *E. faecalis* V583Δ*glnA* in CDM-LAB
Honeycomb Microplate
Bioscreen C Analyzer instrument
Computer with EZ Experiment software installed

Procedure:

1. 5mL CDM-LAB was inoculated with V583-wildtype (3x5mL), V583ΔarcA (3x5mL), and V583ΔglnA (3x5mL) and incubated at 37 °C ON.
2. ON-cultures were diluted 1000x in 999mL fresh CDM-medium, and from this inoculate, 300µl was applied to a Honeycomb Microplate, in three wells per sample. Negative Control (sterile medium) was also applied to three wells.
3. The Honeycomb Microplate was incubated in the BioScreen C analyzer instrument for 48 hours, with OD_{600} measurements every 15 minutes (with 10 seconds pre-emptive shaking).

3.6.2 Glucose-limited cultures in a chemostat

Glucose-limited continuous cultures in a chemostat were set up to investigate metabolic and changes in *E. faecalis* V583Δ*arcA compared to E. faecalis* V583 wild type, as well as changes in gene expression for genes related to pyruvate metabolism. Dry weight of cultures was also measured. *E. faecalis* V583 wild type and *E. faecalis* V583Δ*arcA* were grown anaerobically at 37°C in the defined medium CDM-LAB, *E. faecalis* V583 wild type at pH 7.5 and *E. faecalis* V583Δ*arcA* at both pH 6.5 and pH 7.5 (*E. faecalis* V583 wild type pH 6.5 already performed by Margrete Solheim). The continuous cultures were grown in a Biostatbplus fermentor with a working volume of 750 mL medium at a dilution rate (D) of 0.15 h^{-1} (112,5mL flow-through per hour). The medium pH was kept stable by addition of 5M NaOH and the anaerobic environment was kept stable by the addition of nitrogen gas at 60mL/min. Mixing of culture was performed with a stirring speed of 100 rpm. Samples were extracted when cells were considered to be in a steady-state. Steady-state is defined as when there is no detectable glucose in the culture supernatant, and OD_{600}, cell dry weight and product concentrations in the environment are constant. With a D of 0.15 h-1, steady-state is reached approximately 2½ days after inoculation (Ibrahim Mehmeti, personal communication). After sample extraction, at least six generations had passed (steady-state

reached) before new samples were extracted. Samples were extracted in triplets three times per chemostat run, resulting in a total of 9 samples.

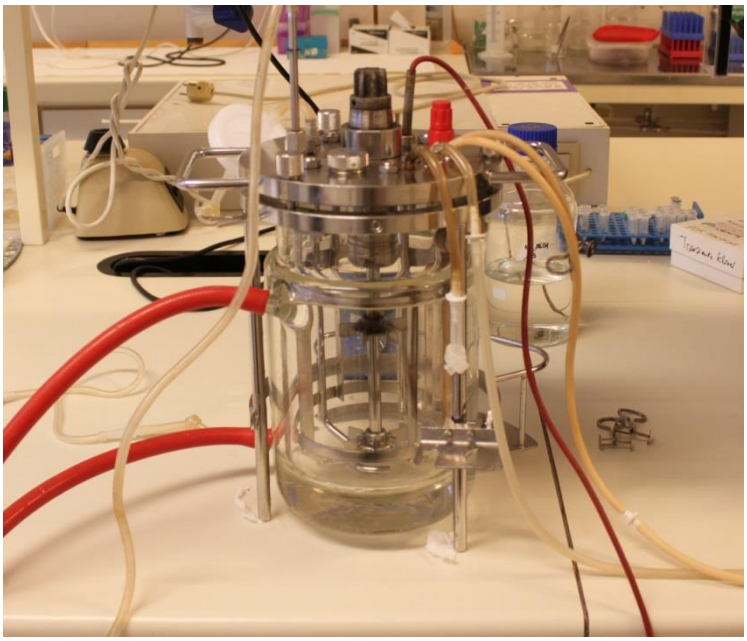


Figure 16: Fermentor container with tubes and pH-meter attached, picture taken by Margrete Solheim.

Materials:

CDM-LAB (adjusted to pH6.5)
CDM-LAB (adjusted to pH7.5)
5mL cultures *E. faecalis* V583 wild type in CDM-LAB
5mL cultures *E. faecalis* V583Δ*arcA* in CDM-LAB
5M NaOH
Nitrogen gas
Biostatbplus fermentor (Sartorius Stedim Biotech)
Plastic tubes
Flaske 10L (medium flask)
Flaske 5L (waste flask)
Flaske 500mL (NaOH flask)
Nunc tubes (50 mL)
Nunc tubes (15 mL)
BD Plastipak™ 50mL syringes
Eppendorf Centrifuge 5804 R
Liquid Nitrogen
0,22 µm Millipore vacuum filter

Procedure:

1. 10 and 1 liters of CDM-LAB medium was made, adjusted to appropriate pH, and filtered through a 0,22 µm Millipore vacuum filter.
2. 750mL of the 1 liter was poured into the fermentor and incubated ON with 100 rpm mixing to allow the system to detect a stable pH level in the medium, and to ensure no contamination of medium.
3. 25mL medium was extracted prior to inoculation to use as blank sample for later analysis.
4. Medium in fermentor was inoculated with 5mL of selected culture.
5. Upon reaching steady-state, 4x50mL samples were extracted into 50mL Nunc tubes.
6. Samples were centrifuged at 4°C at 7,000 x for 6 minutes.
7. The supernatant was transferred to another 50mL Nunc tube and stored at -20°C for metabolic analysis.
8. Cell pellets were flash-frozen in Liquid Nitrogen and stored at -80°C for transcriptional analysis.

Procedure was performed three times, once with *E. faecalis* V583 wild type at pH 7.5, once with *E. faecalis* V583Δ*arcA* at pH 6.5, and once with *E. faecalis* V583Δ*arcA* at pH 7.5. Step 4 to 7 was performed three times total (triplicate experiment) per chemostat run, with an interval of at six generations.

3.6.3 Chemostat cultured sample analysis

3.6.3.1 Metabolite analysis

To quantify metabolites in the supernatant in samples extracted from chemostat culture, High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) was utilized. Analysis was performed by Kari Olsen and the materials and procedure is as described by Kari Olsen.

Materials:

15mL Nunc tubes
MFS-13mm CA filter, 0,2µm poresize.
Eppendorf 5415D centifuge

Instrumentation:
Pump: Series 410, Perkin Elmer
Auto-injector: Series 200, Perkin Elmer
Column oven: LC oven 101, Perkin Elmer

UV-detector: series 200, Perkin Elmer
RI-detector: series 200, Perkin Elmer
LC-terminal: TotalChrom, Perkin Elmer
Interface: 900 series, Perkin Elmer
Column: Aminex HPX-87H, 300x7.8 mm id, Bio Rad.
Guard column: Cation-H refill, 30x4.6mm id, Bio Rad.

Factors:

Chromatography factors: Mobilphase 5mM H_2SO_4
Flow: 0.4ml/min
Columntemp: 30 degree
Detector: UV-detector 210nm wavelength
RI-detector

Chemicals and reagents:

Citric acid
Orotic acid
Pyruvic acid
Succinic acid
DL-lactic acid
Uric acid
DL-pyroglutamic acid
α-ketoglutaric acid
Propionic acid
Acetic acid
Formic acid
Glucose
Lactose
Maltose
Fructose
Galactose
Sulphuric acid
Acetonitrile

Procedure for HPLC:

1. Dilute sample 1:10
2. Weight out 1.00g sample in 15mL Nunc tube with screw cap.
3. Add 2.5 ml deionized water.
4. Add 0.200mL 0.5M sulphuric acid.
5. Add 8.0mL acetonitrile.

6. Mix for 30 min.
7. Centrifuge for 15 min at 3500 rpm.
8. Extract supernatant and run through a 0.2 micrometer filter into a HPLC-vial, close vial with a septum and plastic cap.
9. 25ul of this preparation is injected into the HPLC.

Procedure for GC:

10 grams of supernatant was weighted up and diluted 1:10, blank sample was analyzed with no dilution.

3.6.3.2 Amino acid analysis

To quantify amino acids in the supernatant in samples extracted from chemostat culture, High-Performance Liquid Chromatography (HPLC) was utilized. Analysis was performed by Kari Olsen and the materials and procedure is also as described by Kari Olsen.

Materials:

50mL Nunc Tube
MFS-13mm CA filter, 0,2µm poresize.
Eppendorf 5415D centrifuge
Heraeus Multifuge X3
Gene 2 Vortex

Instrumentation:

Pump: Series 410 (Perkin Elmer, Connecticut, USA)
Auto-injector: 1200 series (Agilent Technologies, Germany)
Thermostat: 1200 series (Agilent Technologies)
Column oven: Series 200 (Perkin Elmer)
Flourescens detector: 1200 seres (Agilent Technologies)
LC-terminal: EZChrom Elite (Agilent Technologies)
Column: XTera RP18, 150 x 4,7 millimeter id, particle size. 3,5µm (Waters, Massachusetts, USA)

Factors:

Mobile phase A: 30 mmol/l NaOAc pH 7,20 + 0,25% tetrahydrofuran + 0,1 mol/l titriplex III
Mobile phase B: 100 mmol/l NaOAc pH 7,20 + 80% acetonitrile + 0,1 mol/l titriplex III

A linear gradient is run from 3,3% to 20,7% B in 12 minutes, 20.7% to 30% B in 12 minutes, 30% to 100% B in 4 minutes, column is kept at 100% B for 7 minutes before returning back to 3,3% B in 7 minutes.

Flow: 0.7 ml/min
Column temperature: 42°C
Detector wavelength: OPA-derivates (primary amino acids) are detected by excitations at 340nm and emissions at 455nm.

Chemicals and reagents:

Amino acid standard
OPA
Boratbuffer
Sodium acetate-trihydrate
titriplex III
tetrahydrofuran
L-norvalin
L-amino acid kit
4% trichloroacetic acid
0,1M HCl

Procedure:

1. Weigh in 5 grams of sample in a 50mL Nunc tube.
2. Add 5mL 0,1M HCl with 0,4µmol/ml L-norvalin added
3. Put in ultrasound bath for 30 minutes.
4. Centrifuge at 3400 rpm for 40 minutes at 4°C.
5. Extract 1mL of supernatant and add 1mL 4% trichloroacetic acid.
6. Vortex and put on ice for 30 minutes (or in cooler at 4°C overnight).
7. Centrifuge at 13000 rpm for 5 minutes.
8. Filter supernatant though MFS-13mm CA filter, and store sample in -20°C freezer until analysis.
9. 50µl sample is then added 350 µl boratbuffer before analysis, and derivatized. Samples are held at 5°C in auto-injector.

Result is multiplied with a factor of 2 to get mM.

3.6.3.3 Dry weight measurement

In addition to samples extracted for metabolic and genomic analysis, samples were extracted for dry weight measurement. Dry weight samples were extracted in triplets, and only extracted once per full glucose-limited chemostat run.

Materials:

As described in 3.6.2. In addition:
Eppendorf tube
SpeedVac Concentrator SPD 2010 (Savant)
0.9% NaCl

Procedure:

1. 3x10mL was extracted from glucose-limited cultures in a chemostat, in 3.6.2.
2. Samples were centrifuged 4°C at 7,000 x for 6 minutes.
3. Supernatant was decanted, cell pellet re-suspended in 0.9% NaCl and transferred to pre-weighed eppendorf tube.
4. Eppendorf tubes were centrifuged at 8,000 x for 4 minutes.
5. Supernatant was decanted.
6. Cell pellet was dried in the SpeedVac Concentrator SPD 2010 (Savant) at 50°C for 15 minutes and weighed. Dry weight was established by subtracting weight of empty eppendorf tube.

3.6.3.4 Ammonium quantification

To quantify ammonium concentration in samples, Megazyme Ammonia (Rapid) Assay kit was used. Incubation and photometric measurements at 340nm were done on a SPECTROstar Nano instrument.

Materials:

Sample supernatants from 3.6.2
Megazyme Ammonia (Rapid) Assay
SPECTROstar Nano
96-Well F Microtiter plates (Sarstedt)

Procedure:

Procedure and calculation of results was performed as described in Megazyme Ammonia (Rapid) Assay Procedure manual. (See appendix, attachment 6).

3.6.3.5 Phosphate quantification

To quanitify phosphate concentration in samples extracted from the chemostat cultures, BioVision Phosphate Colorimetric Assay Kit was used. Incubation and photometric measurements at 650nm were performed on a SPECTROstar Nano instrument.

Materials:

Sample supernatants from 3.6.2
BioVision Phosphate Colorimetric Assay Kit
SPECTROstar Nano
96-Well F Microtiter plates (Sarstedt)

Procedure:

Procedure and calculation of results was performed as described in BioVision Phosphate Colorimetric Assay Kit manual. (See appendix, attachment 7).

3.6.3.6 Transcriptional analysis by Real-Time PCR

We wanted to investigate variations in gene expression between the wild type and Δ*arcA* mutant, to do this we utilized Real-Time Quantitative PCR (qPCR). The process of qPCR involves RNA isolation from cells, and subsequent cDNA synthesis from this RNA. The cDNA was used as template in a PCR, with primer pairs for each gene we want to quantify expression of. In addition, the fluorescent marker (SYBRGreen®) is added to the reaction. This fluorescent marker fluoresces when binding to double threaded DNA, and the amount of DNA (PCR-product) is proportional to the fluorescent signal. By comparing the intensity of fluorescent signals from target genes with fluorescence intensity of a housekeeping gene (gene that is presumed expressed at a stable level in all cells), we can estimate the expression level of target genes in relation to this housekeeping gene through the Pflaffl method (5).

3.6.3.6.1 RNA isolation

Isolation of RNA from samples extracted during glucose-limited chemostat culture was performed as the first step of running qPCR. This was done by using a combination of FastPrep and the Qiagen RNeasy Mini Kit.

Materials:

β-mercaptoethanol
Chloroform
96% EtOH
Biofuge (Fresco) Heraeus Centrifuge
FastPrep FP 120 (Savant)
FastPrep tubes
Qiagen RNeasy® Mini Kit

Procedure:

Important! Wash work space with water, 70% EtOH and RNase-zap / RNase-away or other RNase removal product before beginning procedure.

1. Weight up 0,6 grams acid-washed glass pellets (<106 microns, Sigma) in FastPrep tubes.
2. 280ul chloroform was added to FastPrep tubes (cover pellets).
3. Frozen cell pellets were thawed.
4. Cells were re-suspended in 350ul RLT w/ 10% β-mercaptoethanol, and transferred to the FastPrep-tubes.
5. Cells were lysed in the FastPrep FP 120 at 6m/s for 20 seconds, and placed on ice to avoid vacuum when opening.
6. FastPrep tubes were spun down in a centrifuge.
7. Supernatant was transferred from the FastPrep tubes to Eppendorf tubes, Eppendorf tubes were spun down, and supernatant transferred to new eppendorf tubes. This is to get rid of the chloroform-phase.
8. 250µl 96% EtOH was added, and Eppendorf tubes were vortexed to mix solution.
9. Sample was applied to RNeasy Mini Columns.
10. Columns were centrifuged for 15 seconds at 13,000 rpm. Flow-through was discarded.
11. 350µl RW1 (from RNeasy Mini Kit) was added to the column. Columns were centrifuged for 15 seconds at 13,000 rpm. Flow-through was discarded.
12. 10µl DNase I stock was added to 70µl RDD-buffer per sample and mixed carefully.
13. 80µl DNase I/RDD mix was added to each column. Columns were incubated at room temperature for 15 minutes.
14. 350µl RW1 was added to each column. Each column was centrifuged for 15 seconds at 13,000 rpm. Flow-through discarded.

15. Column was transferred to a new collection-tube.
16. 500µl RPE-buffer was added to each column. Each column was centrifuged for 15 seconds at 13,000 rpm. Flow-through discarded.
17. Point 16 was repeated, but centrifugation was done for 2 minutes. Flow-through discarded.
18. Columns were centrifuged for another minute to dry filter. Flow-through discarded.
19. Each column was transferred to an Eppendorf tube, 30µl RNase-free water was added. RNA was eluated by centrifugation for 1 minute at 13,000 rpm.
20. Point 19 was repeated by adding flow-through to column, and centrifuging for 1 minute at 13,000 rpm again. (Double eluation).

3.6.3.6.2 DNase-treatment of RNA

To get rid of all DNA traces, in additional off-column DNase-treatment of RNA was performed.

Materials:

Isolated RNA from 3.6.3.6.1
RNase out (40 U/µl)
DNase I (2 U/µl)
RDD buffer from the Qiagen RNeasy Mini Kit
96% EtOH
70% EtOH
Phenol
Chloroform
3M NaAc
Biofuge (Fresco) Heraeus Centrifuge
Vacuum centrifuge with temperature regulation

Procedure:

1. All components in the DNase treatment reaction were mixed accordingly:

Per 20µg RNA:

- 2µl RNase out
- 10µl DNase I
- 70µl RDD buffer

2. The reaction was incubated at 37°C for 30 minutes.
3. DNase-treated RNA was extracted by phenol:chloroform extraction as follows:
    a. 50µl phenol, 50µl chloroform and 50µl DEPC-water added to RNA, mixed by vortexing.
    b. Solution centrifuged for 1 minute at 10,000x rpm to separate RNA from DNase I (RNA in watephase).
    c. Waterphase transferred to a new eppendorf tube containing 960µl 96% EtOH + 40µl 3M NaAc.
    d. Solution incubated ON at -20°C.
    e. RNA precipitated by centrifugation at 10,000x rpm for 30 minutes at 4°C.
    f. Supernatant removed, and pellet washed with 70% EtOH.
    g. Pellet dried in vacuum centrifuge at 45°C for approx. 15 minutes.
    h. Pellet re-suspended in 20 µl RNase-free water.

3.6.3.6.3 Control of RNA concentration and quality

Before proceeding to cDNA synthesis, concentration and quality of RNA was controlled by using NanoDrop to measure the RNA concentration, and BioAnalyzer (Agilent) for RNA quality.

Materials:

NanoDrop ND-1000
Agilent 2100 BioAnalyzer
RNA 6000 Kit
RNA 6000 Nano Chip
Chip priming station

Procedure:

- RNA concentration measurement on NanoDrop was performed by applying 2µl from each sample to the NanoDrop ND-1000 spectrophotometer with RNA settings enabled.
- RNA Quality test on BioAnalyzer was performed as described in RNA 6000 kit. (See appendix, attachment 8).

3.6.3.6.4 cDNA synthesis

In order to perform the qPCR-reactions, cDNA synthesis was performed on the isolated RNA.

Materials:

Isolated RNA from 3.6.3.6.1
RNase out
PAN_6 random hexamer primers
DEPC water
Superscript III reverse transcriptase kit
PCR-machine

Procedure:

1. RNA primer mix was made as follows:
   - 1µg total RNA
   - 1µg RNase out
   - 1µg PAN_6 random hexamer primers
   - DEPC water for a total volume of 12,5 1µl
2. RNA mix was incubated at 65°C for 5 minutes, then for 5 minutes at 4°C.
3. Reverse transcriptase mix (RT-mix) was made as follows (per 1 sample):
   - 1µl RNase-free dNTP (10mM)
   - 1µl 0.1M DTT
   - 0.7µl Superscript III (200 U/µl)
   - 0.8µl DEPC water
   - 4µl First strand Buffer (5x)
4. 7.5µl RT-mix was added to each RNA primer mix for a total volume of 20µl.
5. Reaction solution was incubated for 5 minutes at 25°C, followed by 2 hours at 50°C.
6. Enzyme inactivation was done by 15 minutes incubation at 70°C.
7. 1µl RNase A (20µg/µl) was added, and solution was incubated for 20 minutes at 37°C.
8. Solution was stored at -20°C until Real-Time PCR was performed.

3.6.3.6.5 Real-time PCR

Real-time PCR was run to quantify the expression level of the 5 genes *ldhI, adhE, pflA,* EF0082 and *arcC,* in relation to the housekeeping gene encoding for 23S rRNA. Real-time PCR was performed in a Corbett Rotor Gene 6000 using SYBRGreen® as the fluorescence agent. Result analysis was performed on Rotor Gene 6000 Series Software 1.7. A standard run using 10^{-1}, 10^{-2}, 10^{-3} dilutions of cDNA, was performed in order to estimate primer efficiency and decide upon which dilution to use for the Real-Time PCR on the 6 genes.

Materials:

cDNA from 3.6.3.6.4
SYBRGreen®
PCR capillary tubes
RNase / DNase free water
Primers as described in table 3 (Materials → Primers).
Corbett Rotor Gene 6000 instrument
Rotor Gene 6000 Series software 1.7

Procedure for standard qPCR runs:

1. Wild type cDNA from 3.6.3.6.4 was mixed and diluted -1 (1/10), -2 (1/100), and -3 (1/1000).
2. Diluted samples were mixed into SYBRGreen® master mixes for each primer pair, and run through qPCR with two parallels per dilution per sample plus two non-template controls. qPCR conditions were set as follows:

- 95°C for 10 minutes. Initial denaturation.
- 95°C for 15 seconds. Denaturation, run 40x times.
- 60°C for 30 seconds. Annealing, run 40x times.
- 72°C for 30 seconds. Elongation, run 40x times.

3. Efficiency on all primer pairs was calculated using Rotor Gene 6000 series software 1.7, and dilution with highest efficiency on average was selected for sample qPCR runs.

Procedure for sample analysis qPCR runs:

1. 10^{-2} dilutions of cDNA from 3.6.3.6.4 were made for all samples.
2. All sampels were mixed into SYBRGreen® master mixes for each primer pair and run through qPCR with three parallels per sample, plus two non-template controls. qPCR conditions were set as is decribed in standard qPCR run.
3. Results are normalized to primer efficiency and gene expression of genes calculated in relations to expression level of 23S rRNA.

3.7 Complementation of *E. faecalis* V583Δ*arcA*

In order to investigate whether or not the *arcA* deletion had a polar effect on upstream / downstream elements near *arcA*, complementation of *arcA* in our deletion mutant was performed. Complementation was performed by producing *arcA* construct with adjacent XhoI and NotI restriction seats by PCR. Construct was ligated into the vector pREG containing Gram-negative and Gram-positive replicons, a spectinomycin resistance marker, as well as a toxin-antitoxin system (axe-txe) providing stability of the plasmid in the absence of antibiotics (18), and transformed into *E. coli*, isolated from *E. coli* + pREG*arcA*, before subsequent transformation into *E. faecalis* V583Δ*arcA*.

A more detailed description of the individual steps in the complementation of the mutant is listed below:

3.7.1 Producing the *arcA* complementation insert with XhoI and NotI restriction seats.

The *arcA* complementation construct with adjacent XhoI and NotI restriction seats was made using primers arcA-12 and arcA-13.

Materials:
E. faecalis V583 wild type genomic DNA
Phusion® HF Buffer
dNTPs (10mM)
Primer arcA-12 (10mM)
Primer arcA-13 (10mM)
Phusion® DNA polymerase
dH_2O

Procedure:

A PCR was set up using a PCR reaction mix using arcA-13 (forward primer) and arcA-12 (reverse primer) for Phusion® DNA polymerase as described in 3.4.4. PCR reaction conditions for Phusion® DNA polymerase reaction was set as described in 3.4.4.

3.7.2 Digesting *arcA* complementation insert and pREG-vector with XhoI and NotI restriction enzymes

In order to ligate the *arcA* complementation construct and the pREG-vector, both had to be digested by restriction enzymes XhoI and NotI.

Materials:
Materials as listed in 3.4.5, with the exception of the two cutting reactions listed below.

Cutting reaction mix for the *arcA* complementation construct

- NEB Buffer 3 — 2 µl
- 10xBSA (10mM) — 2 µl
- XhoI restriction enzyme — 1 µl
- NotI restriction enzyme — 1 µl
- DNA (arcA construct) — ~1.5 µg
- dH_2O — Added up to a total volume of 20 µl.

Cutting reaction mix for the pREG-vector

- NEB Buffer 3 — 2 µl
- 10xBSA (10mM) — 2 µl
- XhoI restriction enzyme — 1 µl
- NotI restriction enzyme — 1 µl
- DNA (Vector) — ~1,5 µg
- dH_2O — Added up to a total volume of 20 µl.
- CIP — 1µl is added 1 hour into cutting reaction.

Procedure for *arcA* complementation construct:

5. Approximately 1.5 µg *arcA* was added into the cutting reaction mix.
6. Solution was incubated at 37°C for 2 hours.
7. Solution was run through a gel by electrophoresis and the ~1.2kb band was extracted and purified, as explained in 3.4.5.
8. Concentration of eluted *arcA* insert was measured on NanoDrop ND-1000, and stored at -20°C until further use.

Procedure for pREG-vector:

1. Approximately 1.5 µg pREG was added into the cutting reaction mix.
2. Solution was incubated at 37°C for 1 hour.
3. 1µl CIP was added to the solution, and solution was incubated for an additional hour.
4. Solution was run through a gel by electrophoresis and the ~1.2kb band was extracted and purified, as explained in 3.4.5.

5. Concentration of eluted pREG-vector was measured on NanoDrop ND-1000, and stored at -20°C until further use.

3.7.3 Ligating *arcA* complementation construct and the pREG-vector

The *arcA* complementation construct was ligated into the pREG-vector using T4 DNA ligase.

Materials:

Ligation reaction mix :

- T4 ligase buffer 2µl
- T4 ligase enzyme 1µl
- Vector pREG ~100ng
- *arcA* complementation construct ~600ng
- dH_2O Added up to a total volume of 20 µl.

Procedure:

To calculate a good insert / vector ratio, the general formula described in 3.4.12 was used. Reaction mix incubated for 16 hours at 16°C, followed by 20 minutes at 72°C for enzyme inactivation. Ligation mix was stored at -20°C until further use.

3.7.4 Dialyzing the pREG*arcA* ligation mix

The pREG*arcA* ligation mix was dialyzed with the same materials and procedure as described in 3.4.13.

3.7.5 Transforming pREG*arcA* into E. coli

After dialyzing the pREG*arcA* ligation mix, pREG*arcA* was transformed into electro-competent *E. coli* GeneHogs by electroporation.

Materials:

Dialyzed pREG*arcA* ligation mix from 3.7.4
Electro-competent *E. coli* GeneHogs
SOC medium
Gene Pulser from Bio Rad
1mm electroporation cuvette
Eppendorf tubes
LA with 150µg/mL spectinomycin
80% glycerol
2mL Cryo-tubes

Procedure:

1. Dialyzed ligation mix was mixed with 40µL electro-competent *E. coli* GeneHogs and incubated for 5 minutes.
2. Solution was transferred to a pre-chilled 1mm electroporation cuvette.
3. Cuvette was placed into Gene Pulser and electroporated under conditions 1.7kV, 25µF, 200 ohms.
4. 250µL SOC medium was immediately added to solution after electroporation and carefully mixed.
5. Transformation solution was transferred to an eppendorf-tube and incubated at 37°C with 250rpm shaking for 1 hour.
6. Transformation solution was applied on two LA+150µg/mL spectinomycin agar. (50µL from the transformation solution on one agar dish, and the rest on the other agar dish)
7. Agar dishes were incubated ON at 37°C.
8. Colonies on the agar dishes were harvested and inoculated in 5mL LB+150µg/mL spectinomycin ON at 37°C.
9. Freeze stocks of *E. coli* with the p pREG*arcA* vector construct (hereby *E. coli* + pREG*arcA*) were made as described in 3.2.3.

3.7.6 Isolating pREG*arcA* from *E. coli*

After incubation ON the pREG*arcA* vector construct was isolated from *E. coli* + pREG*arcA* using the E.N.Z.A™ Plasmid MiniPrep Kit.

Materials:

5mL ON culture of *E. coli* + pREG*arcA* in LB-medium with 150µg/mL spectinomycin
10mL culture tubes (glass)
Eppendorf tubes
E.N.Z.A™ Plasmid MiniPrep Kit
NanoDrop ND-1000

Procedure:

1. pREG*arcA* vector construct was isolated using the E.N.Z.A™ Plasmid MiniPrep Kit and performed as instructed in the E.N.Z.A™ Plasmid MiniPrep manual (See appendix, attachment 2).
2. Vector concentration was measured on NanoDrop ND-1000, and stored at -20°C, until further use.

3.7.7 Dialyzing pREG*arcA* MiniPrep solution

The pREG*arcA* MiniPrep elution was dialyzed with the same materials and procedure as described in 3.4.13.

3.7.8 Transforming pREG*arcA* into *E. faecalis* V583Δ*arcA*

Post dialyzing the pREG*arcA* MiniPrep elution, the pREG*arcA* construct was transformed into electro-competent *E. faecalis* V583Δ*arcA* by electroporation. 500µg/mL spectinomycin was used in growth media to select for transformants.

Materials:

Materials as described in 3.4.17 except for:
Dialyzed pREG*arcA* MiniPrep elution from 3.7.7
Electro-competent *E. faecalis* V583Δ*arcA*
TH-agar with 500µg/mL spectinomycin

Procedure:

Transformation procedure performed as described in 3.4.17, except for spectinomycin, and no x-gal was used.

3.7.9 Validating the complementation

After transforming the pREG*arcA* vector construct into *E. faecalis* V583Δ*arcA*, validation that the arcA construct had not been excised out of the vector was performed with a control PCR. This was done by isolating pREG*arcA* using MiniPrep, and using it as template for a control PCR using primers arcA-12 and arcA-13.

Materials:

E. faecalis V583Δ*arcA* + pREG*arcA* cultures in 5mL TH-broth 500µL/mL spectinomycin from 3.7.8
Materials for gel electrophoresis as described in 3.4.5
E.N.Z.A™ Plasmid MiniPrep Kit
2mL Cryo-tubes
Materials for PCR reaction using Taq® DNA polymerase as described in 3.4.10 with the exception of primers;
Primer arcA-12 (10mM)
Primer arcA-13 (10mm)

Procedure:

1. pREG*arcA* vector construct was isolated using the E.N.Z.A™ Plasmid MiniPrep Kit and performed as instructed in the E.N.Z.A™ Plasmid MiniPrep manual. (See appendix, attachment 2).
2. PCR reaction was set up using primers arcA-13 (forward primer) and arcA-12 (reverse primer) in a reaction mix for Taq® DNA polymerase as is described in 3.4.4. PCR reaction conditions were set as is described in 3.4.4 for Taq® DNA polymerase, except the elongation phase time was adjusted to match the expected sequence length.
3. PCR product was run through a gel by electrophoresis, as explained in 3.4.5 steps 1 to step 5.
4. After PCR-confirmation of *arcA* insert in the vector, freeze stock of *E. faecalis* V583Δ*arcA*+pREG*arcA* was made as described in 3.2.3.

3.8 Construction of *E. faecalis* V583Δ*glnA*Δ*arcA*

A single crossover disruption of *arcA* was engineered in *E. faecalis* V583Δ*glnA* with the use of the thermo-sensitive pÅS222 vector. An *arcA* homologous sequence construct with a size of 700bp was produced by PCR. The construct was blunt ligated into the SnaBI restriction site of pÅS222, and pÅS222*arcA* was transformed into *E. coli*. pÅS222*arcA* was isolated from *E.coli* + pÅS222*arcA* and transformed into *E. faecalis* V583Δ*glnA*. The single crossover disruption of the *arcA* gene was then engineered with use of the thermo-sensitive vector pÅS222.

A more detailed description of the individual steps in the construction of the double mutant is listed below:

3.8.1 Constructing the arcA homologous sequence

An *arcA* homologous sequence was made using primers arcA-sco1 and arcA-sco2.

Materials:
E. faecalis V583 wild type genomic DNA
Phusion® HF Buffer
dNTPs (10mM)
Primer arcA-sco1 (10mM)
Primer arcA-sco2 (10mM)
Phusion® DNA polymerase
dH_2O

Procedure:

A PCR was set up using a PCR reaction mix using arcA-sco1 (forward primer) and arcA-sco2 (reverse primer) for Phusion® DNA polymerase as described in 3.4.4. PCR reaction conditions for Phusion® DNA polymerase reaction was set as described in 3.4.4.

3.8.2 Restriction cutting pÅS222 using SnaBI

In order to ligate the *arcA* homologous construct into the pÅS222 vector, pÅS222 had to be digested with the restriction enzyme SnaBI.

Materials:

Materials as listed in 3.4.5, with the exception of the cutting reaction

Cutting reaction mix for the pÅS222-vector

- NEB Buffer 3 — 2 µl
- 10xBSA (10mM) — 2 µl
- SnaBI restriction enzyme — 1 µl
- DNA (Vector) — ~1.5 µg
- dH_2O — Added up to a total volume of 20 µl.
- CIP — 1µl is added 1 hour into cutting reaction.

Procedure:

6. Approximately 1.5 µg pÅS222 was added into the cutting reaction mix.
7. Solution was incubated at 37°C for 1 hour.
8. 1µl CIP was added to the solution, and solution was incubated for an additional hour.
9. Solution was run through a gel by electrophoresis and the ~1.2kb band was extracted and purified, as explained in 3.4.5.
10. Concentration of eluted pÅS222-vector was measured on NanoDrop ND-1000, and stored at -20°C until further use.

3.8.3 Phosphorylating the *arcA* homologous construct

Prior to ligation into pÅS222, the arcA homologous construct made by PCR (blunt ended) had to be phosphorylated using T4 polynucleotide kinase.

Materials:

Ligation reaction mix :

- T4 polynucleotide kinase buffer — 2µl
- T4 polynucleotide kinase — 1µl

- *arcA* homologous construct ~1µg
- dH_2O Added up to a total volume of 20 µl.

Procedure:

Reaction mix incubated for 1 hour at 37°C, followed by 20 minutes at 72°C for enzyme inactivation.

3.8.4 Ligating arcA (sco) with pÅS222

The *arcA* homologous construct was ligated into the pÅS222-vector using T4 DNA ligase.

Materials:

Ligation reaction mix :

- T4 ligase buffer 2µl
- T4 ligase enzyme 1µl
- Vector pÅS222 ~200ng
- *arcA* homologous construct ~1µg
- dH_2O Added up to a total volume of 20 µl.

Procedure:

To calculate a good insert / vector ratio, the general formula described in 3.4.12 was used. Reaction mix incubated for 16 hours at 16°C, followed by 20 minutes at 72°C for enzyme inactivation. Ligation mix was stored at -20°C until further use.

3.8.5 Dialyzing the pÅS222*arcA* (sco) mix

The pÅS222*arcA* (sco) ligation mix was dialyzed with the same materials and procedure as described in 3.4.13.

3.8.6 Transforming pÅS222*arcA* (sco) into E. coli

After dialyzing the pÅS222*arcA* (sco) ligation mix, pÅS222*arcA* (sco) was transformed into electro-competent *E. coli* GeneHogs by electroporation.

Materials:

Materials as is described in 3.4.14 except for:
Dialyzed pÅS222*arcA* (sco) ligation mix from 3.8.5
LA with 12,5µg/mL tetracycline

Procedure:

Transformation procedure performed as described in 3.4.14, except tetracycline antibiotic and no x-gal was used.

3.8.7 Isolating pÅS222*arcA* (sco) from *E. coli*+pÅS222*arcA* (sco)

After incubation ON the pÅS222*arcA* (sco) vector construct was isolated from *E. coli* + pÅS222*arcA* (sco) using the E.N.Z.A™ Plasmid MiniPrep Kit.

Materials:

5mL ON culture of *E. coli* + pÅS222*arcA* in LB-medium with 12,5µg/mL tetracycline
10mL culture tubes (glass)
Eppendorf tubes
E.N.Z.A™ Plasmid MiniPrep Kit
NanoDrop ND-1000

Procedure:

1. pÅS222*arcA* (sco) vector construct was isolated using the E.N.Z.A™ Plasmid MiniPrep Kit and performed as instructed in the E.N.Z.A™ Plasmid MiniPrep manual (See appendix, attachment 2).
2. Vector concentration was measured on NanoDrop ND-1000, and stored at -20°C, until further use.

3.8.8 Dialyzing pÅS222*arcA* (sco) MiniPrep solution

The pÅS222*arcA* (sco) MiniPrep solution was dialyzed as described in 3.4.13.

3.8.9 Transforming pÅS222arcA (sco) into E. faecalis V583Δ*glnA*

Post dialyzing the pÅS222*arcA* (sco) MiniPrep elution, pÅS222*arcA* (sco) was transformed into electro-competent *E. faecalis* V583Δ*glnA* by electroporation. Tetracycline antibiotic was used in growth media to select for transformants.

Materials:

Materials as described in 3.4.17 except for:
Dialyzed pÅS222*arcA* (sco) MiniPrep elution from 3.8.8
Electro-competent *E. faecalis* V583Δ*glnA*

TH-agar with 25µg/mL tetracycline

Procedure:

Transformation procedure performed as described in 3.4.17, except tetracycline antibiotic and no x-gal is used.

3.8.10 Making freeze stock of complemented *E. faecalis* V583Δ*glnA* + *pÅS222arcA*

After pÅS222*arcA* (sco) was transformed into *E. faecalis* V583Δ*glnA,* freeze stocks were made as described in 3.2.3.

3.8.11 Engineering the single crossover in E. faecalis V583Δ*glnA* + pÅS222arcA (sco)

The vector pÅS222 is a thermo-sensitive vector made thermo-sensitive by its *repA-pG+Host4* fragment, which cannot replicate at 37°C and has to be integrated into the bacterial chromosome in order to provide the cell with antibiotic resistance. As the vector with the *arcA* homologous sequence is integrated at the site of the chromosomal *arcA* copy, transcription of the *arcA* gene also transcribes the vector, rendering the arginine deiminase protein production dysfunctional. This procedure effectively nullifies the cells ability to produce arginine deiminase.

Materials:

10mL culture tubes
TH broth with 25µL/mL tetracycline
40mg/mL x-gal
37°C Incubator

Procedure:

1. Transformant colonies from 3.8.9 was harvested, inoculated, and grown ON at 30°C in TH-broth with 25µL/mL tetracycline.
2. ON-cultures from step 1 was diluted 100x into fresh TH broth with 25µL/mL tetracycline, and grown at 37°C ON to facilitate single crossover integration.

3.8.12 Validating the single crossover

After growth at 37°C, we expect single crossover integration to have taken place in most living cells in the culture. Still wanting to validate this, control PCR was performed. This was done by first isolating gDNA by FastPrep coupled with MiniPrep (this will also destroy remaining non-integrated vector) from *E. faecalis* V583Δ*glnA* +pÅS222*arcA(sco)*, then using this gDNA as template for a control PCR using primers arcA-sco1 and arcA-sco2.

Materials:

E. faecalis V583Δ*glnA* + pÅS222*arcA(sco)* cultures in 5mL TH-broth 25µL/mL tetracycline from 3.8.10
Materials for gDNA isolation as described in 3.4.3
Materials for gel electrophoresis as described in 3.4.5
2mL Cryo-tubes
Materials for PCR reaction using Taq® DNA polymerase as described in 3.4.10 with the exception of primers;
Primer arcA-sco1 (10mM)
Primer arcA-sco2 (10mm)

Procedure:

5. Genomic DNA was isolated as explained in 3.4.3.
6. PCR reaction was set up using primers arcA-sco1 (forward primer) and arcA-sco2 (reverse primer) in a reaction mix for Taq® DNA polymerase as is described in 3.4.4. PCR reaction conditions were set as is described in 3.4.4 for Taq® DNA polymerase, except the elongation phase time was adjusted to match the expected sequence length.
7. PCR product was run through a gel by electrophoresis, as explained in 3.4.5 steps 1 to step 5.
8. After PCR-confirmation of sco, freeze stock of *E. faecalis* V583Δ*glnA*+pÅS222*arcA*(sco) was made as described in 3.2.3.

3.10 Acidic environment survival test

Construction of an *arcA* negative S. pyogenes strain in the SysMo-LAB project showed that the mutant suffered a decreased survival capability in acidic environments. With the *E. faecalis* V583Δ*arcA* mutant constructed and complemented. Investigations on whether *E. faecalis* V583Δ*arcA* suffered the same consequences were performed.

Materials:

E. faecalis V583Δ*arcA* ON-culture

E. faecalis V583Δ*arcA* with *arcA* complemented ON-culture
E. faecalis V583 wild type ON-culture
1mM $MgCl_2$
20mM $Na_2HPO_4^-$
25mM L-arginine adjusted to pH 4.0
TH-broth
TH-agar

Procedure:

1. ON-cultures were diluted 1000x in fresh TH-broth and incubated for 3 hours to ensure the culture was in the exponential growth phase.
2. 1mL from the culture was centrifuged and washed with 0.9% NaCl.
3. Pellets were re-suspended in 1mL 1mM $MgCl_2$, 20mM $Na_2HPO_4^-$, and 25mM L-arginine adjusted to pH 4.0.
4. 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spotted on TH-agar after 0, 2, 4, and 6 hours.
5. TH-agar was incubated ON.

3.11 Growth comparison of all constructed mutants

As a follow up to construction of the *E. faecalis* V583Δ*glnA*Δ*arcA* double mutant and the complementation of the *E. faecalis* V583Δ*arcA* mutant, a growth comparison in batch culture was performed on all constructed *E. faecalis* V583 mutants and the V583 wild type. Growth study was performed as described in chapter 3.6.1, with the exception of only performing the experiment in full CDM-LAB, and the added tetracycline in the *E. faecalis* V583Δ*glnA*Δ*arcA*'s growth medium for mutant stability.

4.0 Results

4.1 Construction of *E. faecalis* V583Δ*arcA*

Construction of *E. faecalis* V583Δ*arcA* was done by amplifying the sequences flanking the *arcA* gene, producing the two constructs arcA5-6 (609 bp) and arcA7-8 (643 bp) (Figure 17). Part of the sequence in arcA-6 was reverse complementary to part of the sequence in arcA-7, allowing for fusing of arcA5-6 and arcA7-8 into the arcA5678 (Δ*arcA* (1252 bp)) construct through sequence overlap extension PCR (Figure 18).

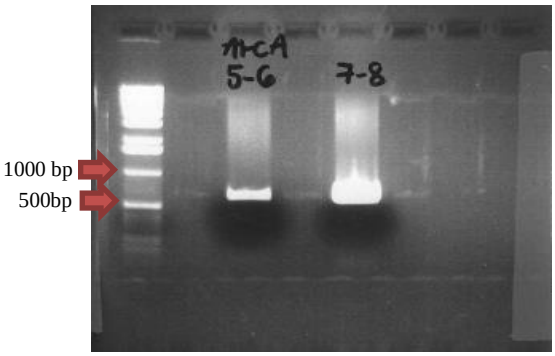


Figure 17: Photo of arcA5-6 (well 2, 609 bp) and arcA7-8 (well 4, 643 bp) products run through an agarose gel by electrophoresis.

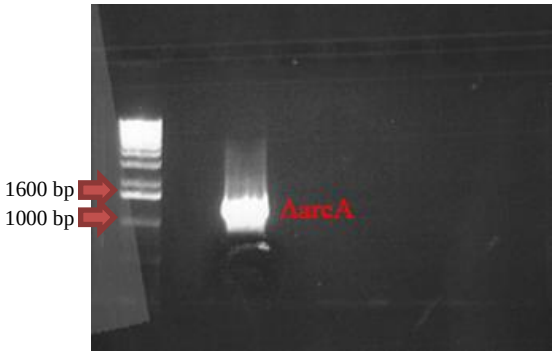


Figure 18: Photo of the Δ*arcA* construct (well 2, 1252 bp) run through an agarose gel by electrophoresis.

The Δ*arcA* construct was ligated into the commercial pCR®-Blunt II-TOPO® vector before transformation into *E. coli*. The pTOPOΔ*arcA* construct was then isolated and the Δ*arcA* construct digested out, using restriction enzymes BamHI and PstI. The thermo-sensitive vector pLT06 was digested with BamHI and PstI, run through a gel by electrophoresis, extracted and purified from the gel before the Δ*arcA* construct was ligated to it. The pLT06Δ*arcA* construct was transformed into *E. coli*, isolated from transformants and subsequently transformed into *E. faecalis* V583. Single crossover integration into the chromosome was staged by incubation at 42°C and validated by PCR, using vector-specific primers with primers flanking the Δ*arcA* construct (Figure 19). The cultures exhibiting single

crossover integration, were diluted 1000x into medium with no antibiotics and incubated ON at 30°C. ON-cultures were further diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and streaked on MM9YE6 agar with 10mM 4-chloro-phenylalanine and 40µl 40µg/mL x-gal. The agar plates were incubated ON at 30°C, and LacZα-negative (white) colonies were harvested for double crossover control PCR using primers arcA-5 and arcA-8. One in 20 colonies on MM9YE6 contained the Δ*arcA* mutation (shown in well 9 of figure 20), whilst 19 reverted to wild type.

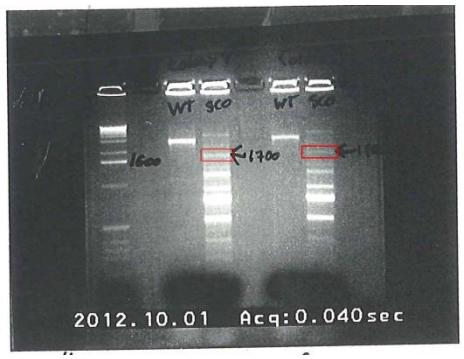


Figure 19: Photo of control PCR using vector-specific primers with primers flanking the Δ*arcA* construct. The length validating integration is seen as the 1700 bp band.

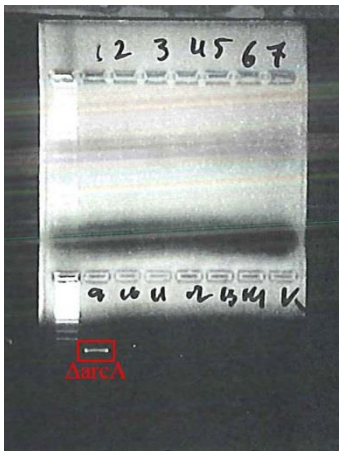


Figure 20: Photo of control PCR following double crossover procedure. All except well 9 reverted to wild type, well 9 shows mutant *arcA*. The elongation in the PCR-reaction was set to match the sequence length of mutant *arcA* (1252 bp), which is why the other wells are not displaying wild type bands.

4.2 Sequencing the mutated region of *arcA*

Sequencing of the *arcA* gene in *E. faecalis* V583Δ*arcA* was performed to match sequence of Δ*arcA* with wild type *arcA*. The sequence was amplified using primers arcA-5 and arcA-8, flanking the entirety of the *arcA* gene, and sent to GATC Biotech AG for sequencing. The sequence was then aligned with *E. faecalis* V583 wild type *arcA* using the CLC Workbench software. A simplified version is shown in figure 21. For a full description of the sequence alignment, see the appendix, attachment 9.

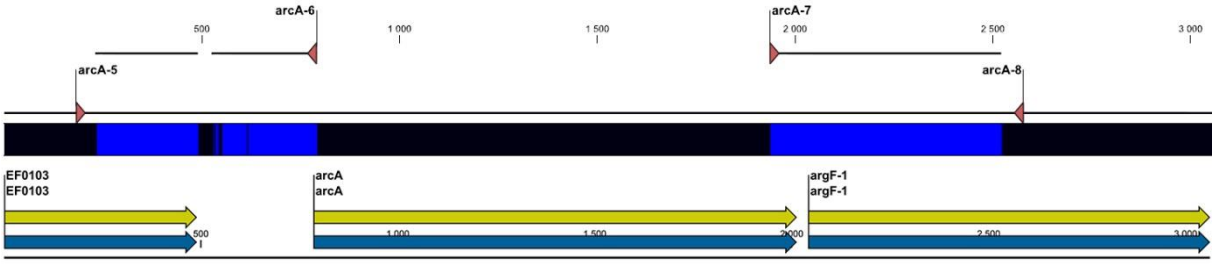


Figure 21: Sequence alignment of Δ*arcA* region versus wild type *arcA*. Below, the genomic data from *E. faecalis* V583 is shown with gene annotations, above line displays the alignment. Wild type genomic data is shown as the black background, whilst the *ΔarcA* deletion mutant sequence amplified using primers arcA-5 and arcA-8 is shown in blue. For full description of sequence alignment, see appendix, attachment 9.

In aligning the sequence for *arcA* in Δ*arcA* deletion mutant to wild type *arcA*, it is established that the *arcA* gene has successfully been deleted out of the *E. faecalis* V583 chromosome.

4.3 Batch culture growth experiments

To study the *arcA* deletion's effect on *E. faecalis* V583 growth in batch cultures. Growth studies were performed in the defined medium CDM-LAB with 6 different amino acid compositions. Amino acid compositions were chosen based on earlier studies in the SysMo-LAB project where indications that *E. faecalis* V583 consumes glutamine, glutamate, and serine for energy metabolism, and that omitting glycine has shown to retard growth of *E. faecalis* V583 even though it seems to successfully be producing glycine from serine (Helge Holo, Margrete Solheim – personal communication).

The 6 different compositions of CDM-LAB were as follows:

1. CDM-LAB medium with all components.
2. CDM-LAB with glutamine omitted.
3. CDM-LAB with 1/8th of the full glutamine concentration
4. CDM-LAB with glutamate omitted.
5. CDM-LAB with serine omitted.
6. CDM-LAB with glycine omitted.

Three biological replicates of each *E. faecalis* strain (V583, V583Δ*arcA*, V583Δ*glnA* (only in full medium) were cultivated ON and diluted 1000x in the six configurations of CDM-LAB. From the 1000x dilutions, three technical replicates of each strain in each configuration of CDM-LAB were applied on Honeycomb microplates. An average of technical and biological replicates was calculated and plotted into growth curves with legend as follows;

- *E. faecalis* V583 wild type
- *E. faecalis* V583Δ*arcA*
- *E. Faecalis* V583Δ*glnA* (only in full medium)

All P-values are calculated using student's t-test.

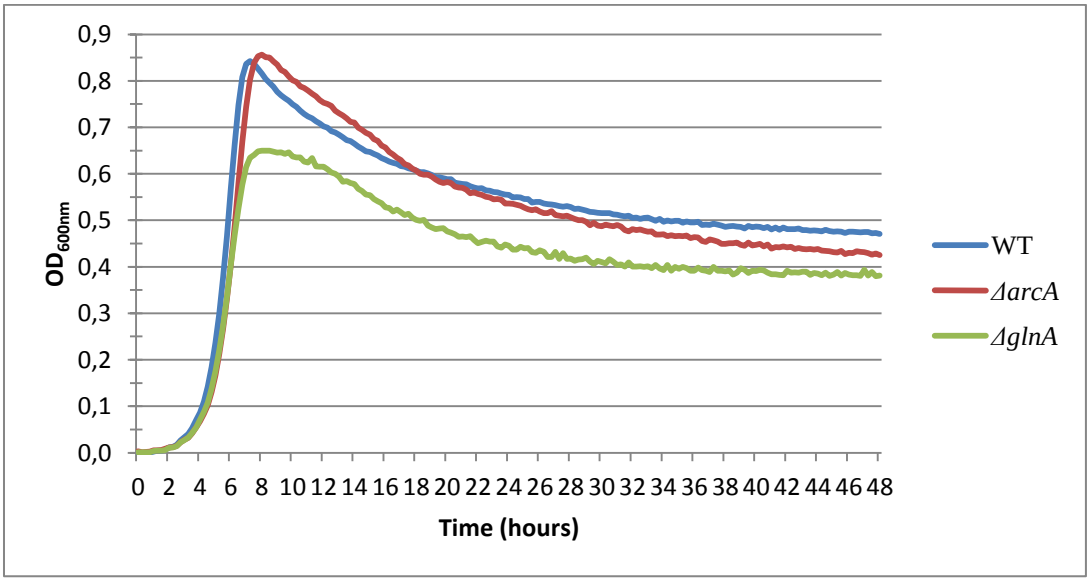


Figure 22: Growth of V583 wild type, V583Δ*arcA*, V583Δ*glnA* in full CDM-LAB medium.

Growth of V583 wild type, Δ*arcA*, and Δ*glnA* (Figure 22) showed a similar pattern at the beginning of the exponential growth phase. Growth of Δ*glnA* was halted at a lower OD_{600} than wild type, peaking growth at an OD_{600} of ~0.65. Figure 22 visually describes the Δ*arcA* mutant growing to a slightly higher OD_{600} than wild type before halting, reaching a OD_{600} peak of 0,856 where wild type reaches a OD_{600} peak at 0.8400. The difference in OD_{600} peak was observed in all three replicates of the experiment, and also in other CDM-LAB configurations. However, the difference was not statistically significant in any of the cases ($p=0.19$). A relatively short stationary phase is observed for all strains. The following reduction in OD_{600} following the stationairy phase was significantly less for Δ*glnA* mutant (dropping ~0.25 OD_{600}) in comparison to wild type and Δ*arcA*, which both dropped ~0.4 OD_{600}. The difference in OD_{600} reduction between Δ*glnA* and wild type was of statistical significance ($p<0.01$).

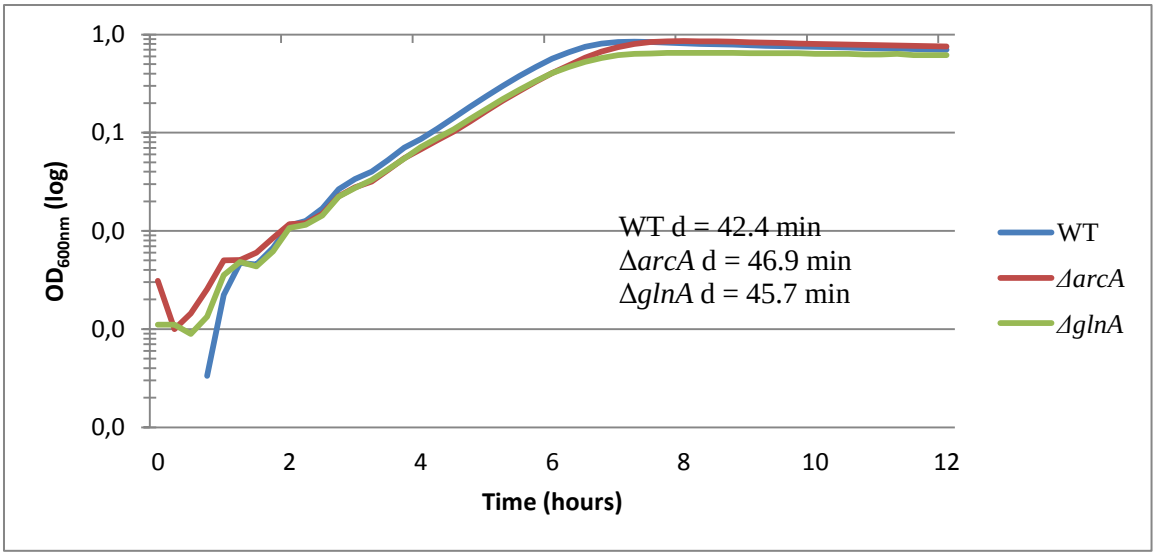


Figure 23: Logarithmic transformation of Y-axis with V583 wild type, V583Δ*arcA*, V583Δ*glnA* growth in full CDM-medium with calculated doubling times. Note that the graph is truncated compared to Figure 22, this is because only the exponential growth phase (linear when log transformed) is of interest when calculating the doubling time. Doubling time was calculated using the equations: [log (OD_{t2}/OD_{t1})] / log(2) = g, doubling time (d) = (t2-t1) mins / g.

A logarithmic transformation of growth data in full CDM-LAB medium showed a reduced growth rate of both deletion mutants in comparison to wild type , with Δ*arcA* displaying the longest doubling time (Figure 23). The differences in doubling times between both mutants in comparison to wild type was statistically significant ($p<0.01$), while the differences in doubling time between Δ*arcA* mutant and Δ*glnA* mutant was not ($p=0.26$).

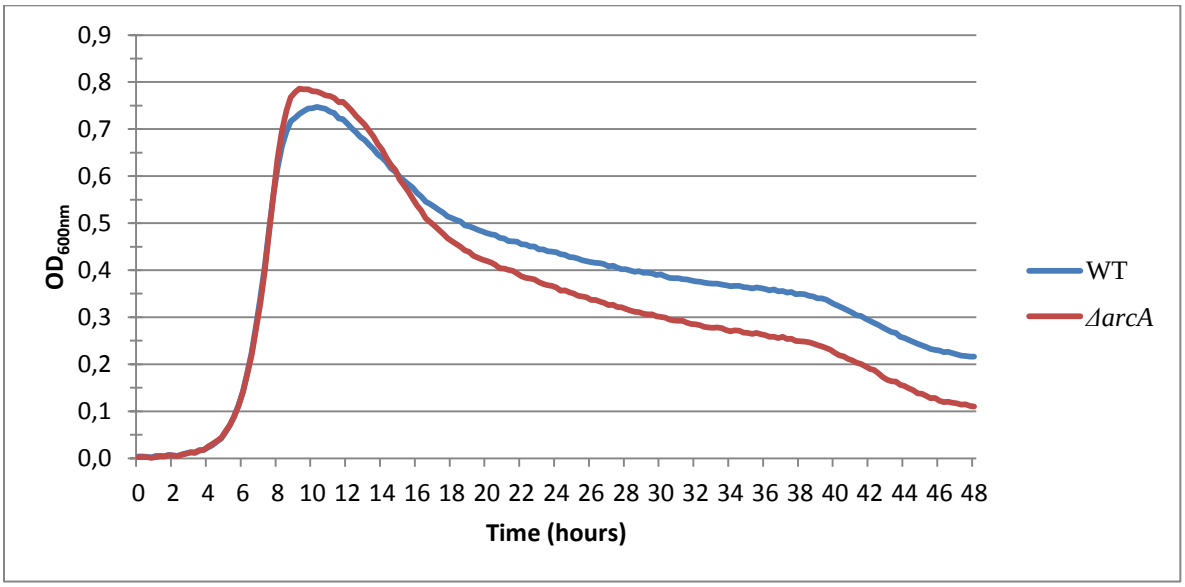


Figure 24: Growth of V583 wild type and V583Δ*arcA* in CDM-LAB medium with glutamine omitted.

Growth in CDM-LAB medium with glutamine omitted displays same trend as in full CDM-LAB medium, but the OD_{600} maximum peak achieved for both wild type and Δ*arcA* mutant, was slightly reduced in comparison to the full CDM-LAB medium (Figure 24). Wild type and mutant growth in exponential growth phase was very similar, but the mutant grew to a slightly higher OD_{600} in comparison to the wild type. This was similar to observations in full CDM-LAB medium. The difference in OD_{600} peak between wild type and mutant was not statistically significant ($p=0.18$). The mutant had a larger and more rapid reduction in OD_{600} following OD_{600} peak in comparison to the wild type. The mutant had a reduction of ~0.6 OD_{600} in the decline phase, whilst the wild type OD_{600} reduction was ~0.45. The difference in OD_{600} reduction is statistically significant ($p<0.01$).

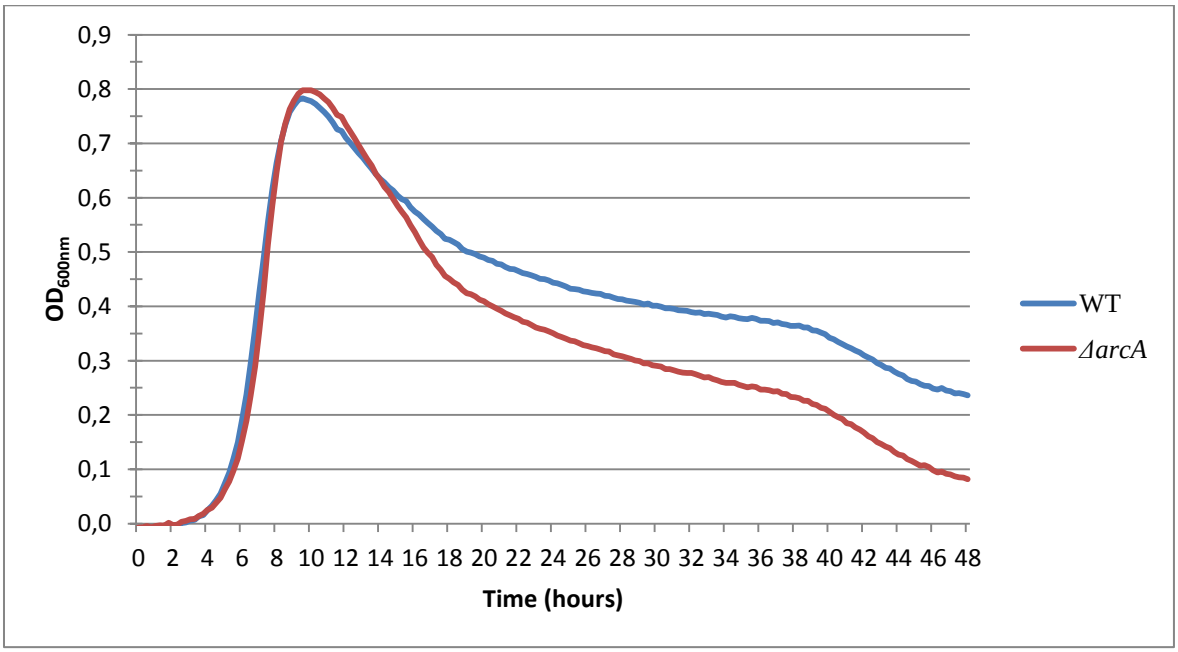


Figure 25: Growth of V583 wild type and V583Δ*arcA* in CDM-LAB medium with low glutamine concentrations ($1/8^{th}$ of the glutamine concentration in full CDM-LAB).

Growth in CDM-LAB medium with low glutamine concentrations (Figure 25) displayed growth very similar to growth in CDM-LAB with glutamine omitted. Wild type and mutant growth in exponential growth phase was very similar, but the mutant grew to a slightly higher OD_{600} in comparison to the wild type ($p=0.16$, not significant). Also, similar to the growth in

CDM-LAB with no glutamine, the mutant had a larger and more rapid reduction in OD_{600} following OD_{600} peak in comparison to the wild type. The mutant had a reduction of ~0.6 OD_{600} in the decline phase, whilst the wild type OD_{600} reduction was ~0.45. The difference in OD_{600} reduction is statistically significant ($p<0.01$).

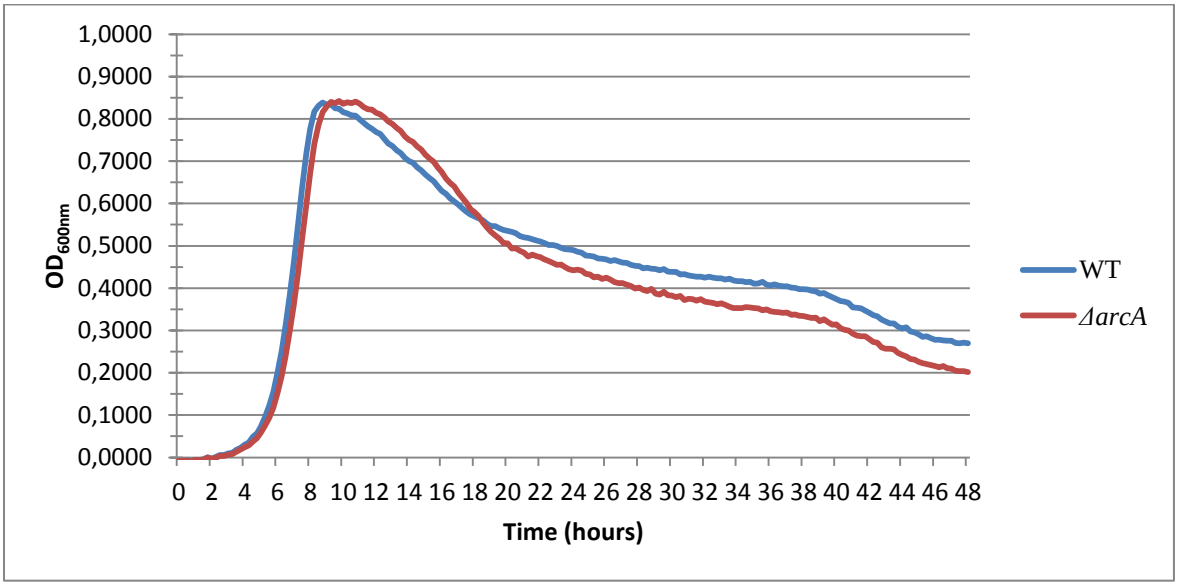


Figure 26: Growth of V583 wild type and V583Δ*arcA* in CDM-LAB medium with glutamate omitted.

Growth in CDM-LAB medium with glutamate omitted (Figure 26) displayed a similar pattern observed in CDM-LAB medium with glutamine omitted (Figure 24) and in CDM-LAB with low glutamine concentrations (Figure 25). However, the differences were less pronounced in CDM-LAB with glutamate omitted. The mutant did not grow to a visibly higher OD_{600} than the wild type ($p=0.71$), and the differences in OD_{600} reduction following the OD_{600} peak was more similar than in full CDM-LAB medium and in the CDM with no or low glutamine. The wild type had an OD_{600} reduction of ~0.45 following the OD_{600} peak, whilst the mutant had an OD_{600} reduction of ~0.55 ($p=0.02$).

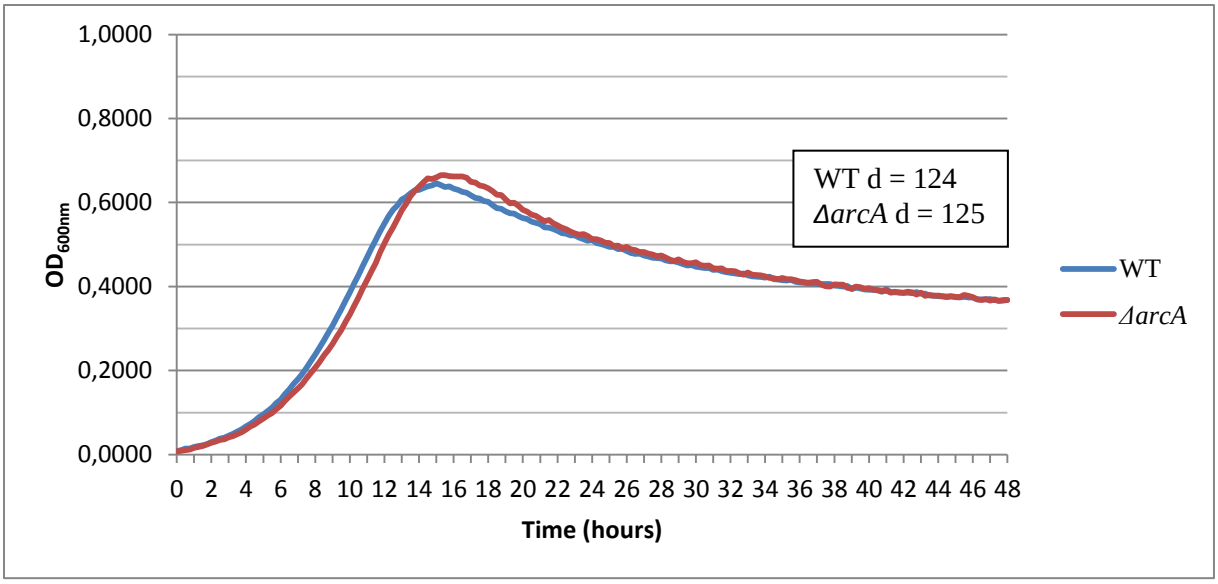


Figure 27: Growth of V583 wild type and V583Δ*arcA* in CDM-LAB medium with serine omitted.

The growth patterns observed in serine omitted CDM-LAB medium (Figure 27) was significantly different than in the previous compositions. The OD_{600} peak in serine omitted CDM-LAB is reached 15-16 hours into the growth experiment with a doubling time of ~124-125, whereas the OD_{600} peak is reached approximately 10 hours into the experiment in the previous compositions (d = 42-46). The mutant reached a slightly higher OD_{600} peak ($p=0.67$), whilst the wild type grew to its OD_{600} peak slightly faster than the mutant. A very similar OD_{600} pattern after peaking was observed, both dropping ~0.3 OD_{600}, but with mutant dropping slightly more because of a higher OD_{600} peak ($p=0.06$).

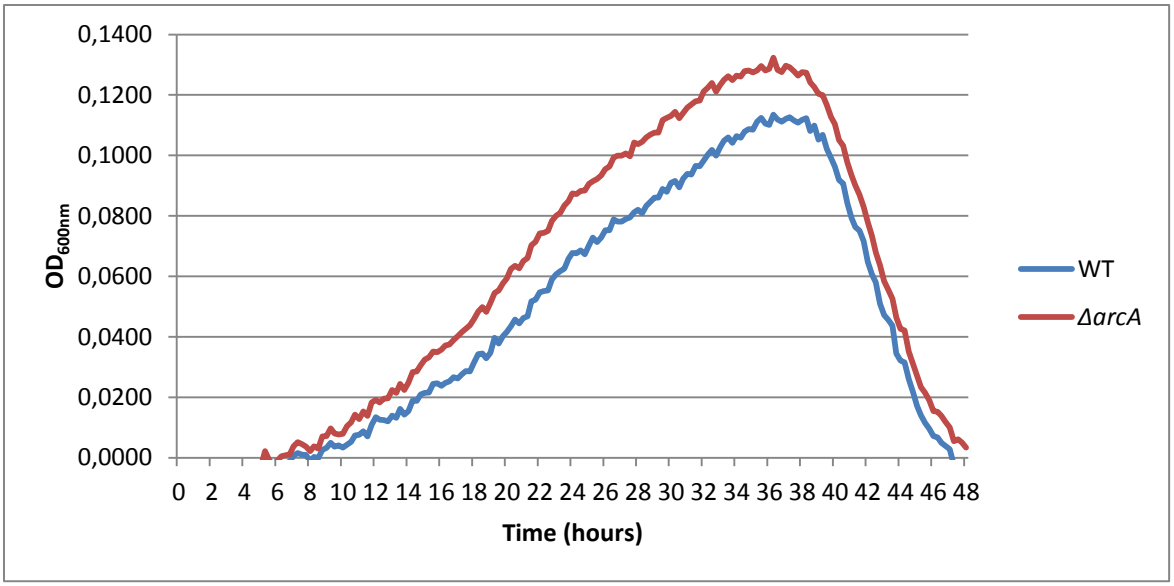


Figure 28: Growth of V583 wild type and V583Δ*arcA* in CDM-LAB medium with glycine omitted. Note that the Y-axis has been scaled down (0,0-0,14) as growth is severely hampered in this CDM-LAB configuration, when compared to the others.

Growth in CDM-LAB medium with glycine omitted displayed a very different pattern than the other configurations. Growth was severely impeded, reaching a OD_{600} peak of only ~0.12. Even so, the trend shown in the other CDM-LAB configurations, where the mutant grew to a higher OD_{600} than the wild type ($p<0.01$) was also observed in this CDM-LAB configuration with glycine omitted.

4.4 Glucose-limited continuous cultures in a chemostat

To investigate the *arcA* deletion's effect on *E. faecalis* V583 metabolism, *E. faecalis* V583 wild type and V583Δ*arcA* were grown in a chemostat at a dilution rate (D) of 0.15 h^{-1}.The wild type was grown at pH 7.5, while V583Δ*arcA* was grown at pH 6.5 and pH 7.5. Similar experiment with wild type at pH 6.5 was previously performed by Margrete Solheim.

All P-values are calculated using student's t-test.

4.4.1 Metabolite, dry weight, ammonium and phosphate analysis

During continuous cultures in chemostat, samples were extracted for metabolic and transcriptional analysis. Supernatant of samples extracted were used in measurement analysis of organic acids, carbohydrates, and amino acids using HPLC and GC, and quantification of ammonium and phosphate using commercial kits. Cell pellets from samples extracted were

used for transcriptional analysis by Real-Time PCR. In addition, samples were also extracted for dry weight measurements.

The effect of the mutation in *arcA* was that less formate is formed in the mutant compared to wild type. Effect of lowering the pH was that less formate and more lactate was formed at pH 6.5 than at pH 7.5. As shown in Table 7 and Figure 29, formate was the major metabolic end product for both wild type (pH 7.5) and mutant (pH 7.5 and pH 6.5). The pH 7.5 cultures (both wild type and mutant) produced large amounts of acetate and ethanol in addition to formate, while the pH 6.5 mutant culture produced more lactate. Trace amounts of acetaldehyde were detected in all cultures, and trace amounts of acetoin was detected in pH 6.5 mutant culture. A skew away from homolactic into a more mixed-acid fermentation is observed in all continuous cultures.

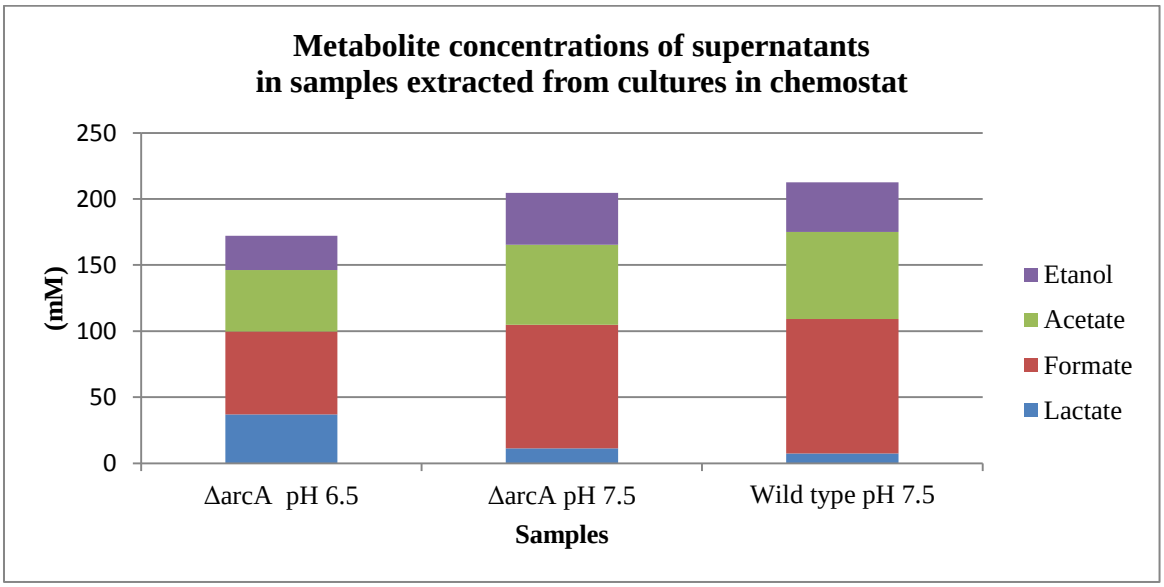


Figure 29: Concentrations (mM) of metabolites in supernatants of samples extracted from the chemostat cultures (*E. faecalis* V583 wild type in pH 7.5, *E. faecalis* V583*ΔarcA* in pH 7.5 and pH 6.5).

Table 7: Metabolites of *E. faecalis* V583 wild type (pH 7.5) and Δ*arcA* (pH 6.5 and pH 7.5) mutant harvested during steady-state in the chemostat cultures.

Metabolite	Medium (mM)	Δ*arcA* pH 6.5 Mean concn (mM)±SD	Δ*arcA* pH 7.5 Mean concn (mM)±SD	Wild type pH 7.5 Mean concn (mM)±SD
Glucose	61.0	0.0	0,0	0.0
Citrate	2.5	0.0	0.0	0.0
Pyruvate	0.0	0.6±0.04	0.5±0.12	0.4±0.05
Succinate	0.0	0.0	0.0	0.0
Lactate	0.0	37.0±5.6	11.4±3.5	7.4±1.2

Formate	0.0	62.7±2.7	93.4±1.5	101.7±4.7
Acetate	12.2	46.6±2	60.5±1.2	66.0±3.2
Acetate formed	0.0	34.6±2	48.5±1.2	54.0±3.2
Acetaldehyde	0.0	0.9±0.0	1.0±0.0	1.1±0.0
Ethanol	0.0	25.8±3.5	39.4±1.1	37.5±5.6
Acetoin	0.0	3.0±0.0	0.0	0.0

Carbon balance

The carbon balance was calculated for each of the samples. Carbon balance was calculated using the total amount of carbon in supernatants divided by total amount of carbon in medium. A carbon balance of ~82-83% was achieved for all samples (Table 8).

Table 8: Carbon balance calculations using metabolite data in table 7.

Chemostat culture	**Carbon balance (Carbon retrieval percentage)**
ΔarcA pH 6,5	82%
ΔarcA pH 7,5	82%
Wild type pH 7.5	83%

Dry weight

From the continuous cultures, samples were extracted for dry weight measurements (Table 9).

Table 9: Dry weight measurements done in samples extracted from the continuous cultures.

Dry weight measurements	
***E. faecalis* V583 wild type pH 7.5**	2.2 g/L
***E. faecalis* V583Δ*arcA* pH 6.5**	1.6 g/L
***E. faecalis* V583Δ*arcA* pH 7.5**	1.9 g/L

Dry weight measurements revealed a significant reduction in biomass for the *E. faecalis* V583 Δ*arcA* mutant compared to the wild type at pH 7.5 ($p<0.01$), and an even larger reduction of biomass for mutant culture at pH 6.5 compared to mutant and wild type at pH 7.5 ($p<0.01$).

Ammonium

The ammonium concentrations of the supernatants were measured using the Megazyme Ammonia (Rapid) Assay kit (Table 10).

Table 10: Ammonium concentration measured in sample supernatants from chemostat culture extractions.

Ammonium concentrations	
***E. faecalis* V583 wild type pH 7.5**	4.04 mM
***E. faecalis* V583Δ*arcA* pH 6.5**	4.04 mM
***E. faecalis* V583Δ*arcA* pH 7.5**	3.93 mM

No significant differences in ammonium production were observed between wild type and mutant ($p=0.65$).

Phosphate

The amount of phosphate in the supernatants was quantified using the BioVision Phosphate Colorimetric Assay Kit (Table 11).

Table 11: Phosphate concentration measured in sample supernatants from chemostat culture extractions.

Phosphate concentration	
***E. faecalis* V583 wild type pH7.5**	50±3,4mM
***E. faecalis* V583Δ*arcA* pH6.5**	40±2,1mM
***E. faecalis* V583Δ*arcA* pH7.5**	34±1,7mM

A significant reduction in phosphate concentration was observed in both Δ*arcA* mutant cultures compared to the wild type culture ($p<0.05$). A significantly lower concentration of phosphate was measured in the supernatant of the mutant culture cultivated at pH 7.5 in comparison to the mutant culture cultivated at pH 6.5 ($p<0.05$).

4.4.2 Amino acid analysis

Supernatant in samples extracted during chemostat runs and amino acid concentrations were measured using HPLC.

The results from the amino acid measurements (Figure 30, Table 12) showed that all of the L-serine had been consumed in all three cultures, and very surprisingly, so had all of the L-arginine. Consumption of L-aspartic acid, L-glutamic acid, glycine, L-valine, L-leucine, and L-alanine in Δ*arcA* mutant culture in the pH 6.5 environment was significantly reduced, compared to both Δ*arcA* mutant and wild type cultures in a pH 7.5 environment. The mutant cultivated at pH 7.5 had consumption of amino acids quite similar to wild type cultivated at

pH 7.5, with the exception of L-valine and L-alanine, which was significantly less consumed in mutant than wild type. The increased levels of L-alanine in both mutant cultures exceed even the concentrations of L-alanine in fresh CDM-LAB medium. In the supernatants from all three chemostat cultures, the level of L-citrulline and L-ornithine was higher than in fresh CDM-LAB medium. L-citrulline was detected in trace amounts (0.05 – 0.11 mM), and L-ornithine in larger amounts (1.28-1.56 mM). Only trace amounts of L-arginine was detected in supernatants from all chemostat cultures.

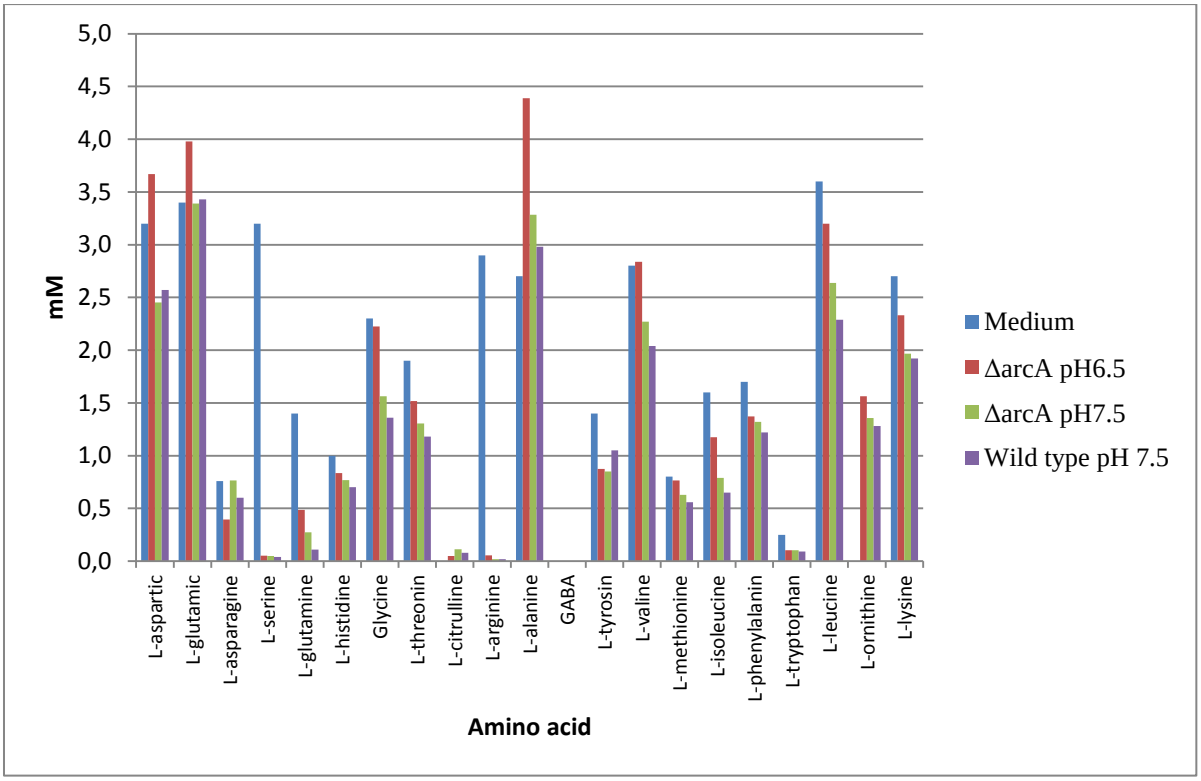


Figure 30: Amino acid concentrations in supernatants extracted from the chemostat cultures wild type at pH 7.5, mutant at pH 7.5 and mutant at pH 6.5. Samples are also compared to medium composition.

Table 12: Amino acid concentrations in sample supernatants and medium composition.

Amino acid	Medium (mM)	*ΔarcA* pH 6.5 Mean concn (mM)±SD	*ΔarcA* pH 7.5 Mean concn (mM)±SD	WT pH 7.5 Mean concn (mM)±SD
L-aspartic	**3.20**	3.67±0.76	2.45±0.27	2.57±0.11
L-glutamic	**3,40**	3.98±0.88	3.39±0.36	3.43±0.07
L-asparagine	**0.76**	0.39±0.08	0.77±0.07	0.60±0.02
L-serine	**3.20**	0.05±0.01	0.05±0.07	0.04±0.01
L-glutamine	**1.40**	0.49±0.11	0.27±0.06	0.11±0.02
L-histidine	**1.00**	0.84±0.17	0.77±0.10	0.70±0.02
Glycine	**2.30**	2.22±0.44	1.56±0.19	1.36±0.04
L-threonin	**1.90**	1.52±0.31	1.30±0.10	1.18±0.02
L-citrulline	**0.00**	0.05±0.01	0.11±0.02	0.08±0.01
L-arginine**	**2.90**	0.06±0.00	0.02±0.00	0.02±0.00
L-alanine	**2.70**	4.39±0.79	3.28±0.32	2.98±0.09
GABA*	**0.00**	0.00±0.00	0.00±0.00	0.00±0.00
L-tyrosin	**1.40**	0.87±0.13	0.85±0.05	1.05±0.12
L-valine	**2.80**	2.84±0.60	2.27±0.25	2.04±0.05
L-methionine	**0.80**	0.77±0.17	0.63±0.07	0.56±0.01
L-isoleucine	**1.60**	1.17±0.26	0.79±0.09	0.65±0.02
L-phenylalanin	**1.70**	1.37±0.30	1.32±0.13	1.22±0.02
L-tryptophan	**0.25**	0.10±0.04	0.10±0.01	0.09±0.02
L-leucine	**3.60**	3.20±0.73	2.64±0.28	2.29±0.06
L-ornithine	**0.00**	1.56±0.31	1.36±0.08	1.28±0.01
L-lysine	**2.70**	2.33±0.49	1.97±0.20	1.92±0.04
L-Proline	**5.90**	Not measured	Not measured	Not measured

* γ-Aminobutyric acid (GABA)
** Arginine measurements are unreliable because they are hard to separate in HPLC analysis in such low amounts, numbers might as well be 0 (Kari Olsen – personal communications)

4.4.3 RNA quality and concentration

RNA was isolated from the cell pellets in samples extracted from the chemostat for use in transcriptional analysis by Real-Time PCR. RNA was isolated using the RNeasy kit from Qiagen. Quantity was checked on NanoDrop, measuring all RNA samples to a concentration between ~1500ng/µL to ~2200ng/µL. Quality was checked on BioAnalyzer using the RNA 6000 Kit.

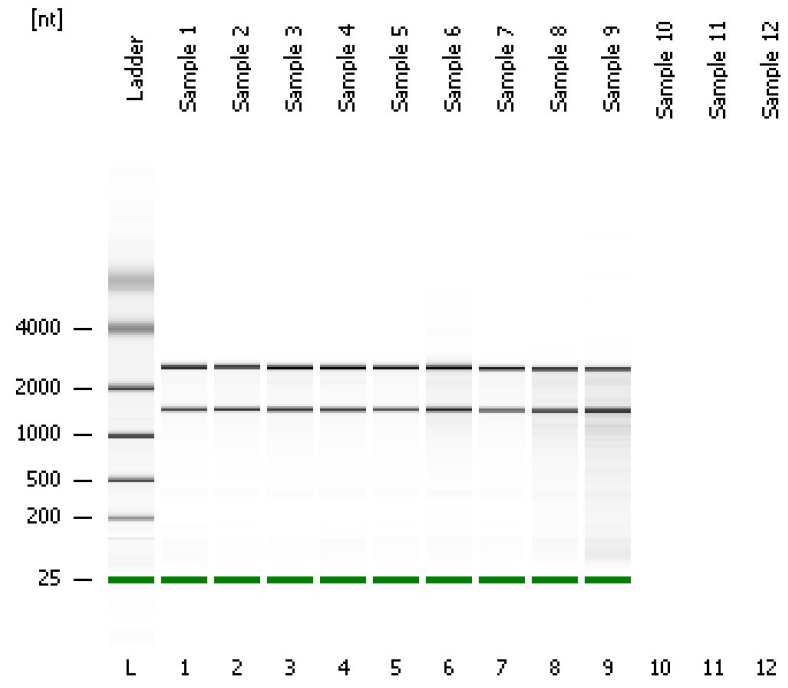


Figure 32: Picture from RNA 6000 Kit Gel Electrophoresis performed in BioAnalyzer. Samples 1 through 9 are samples extracted from the chemostat cultures. Two clear bands are seen, representing 16S and 23S rRNA.

The presence of two distinct bands for each sample in the gel (Figure 32) indicated that the RNA was intact and of a sufficient quality for transcriptional analysis.

4.4.4 Transcriptional analysis using Real-Time PCR

Real-Time PCR was performed using isolated RNA from chemostat as template. Genes targeted were *ldh-1, adhE, pflA, arcC* and EF0082, with 23S as reference. Relative gene expression ratio was calculated using the Pflaffl method (5).

Results from Real-Time PCR (Figure 33) showed a down regulation of *pflA* and EF0082, and an up regulation of *ldh-1*, *adhE*, and *arcC* in mutant from chemostat cultivation in a pH 7.5 environment compared to wild type chemostat cultivation at pH 7.5.

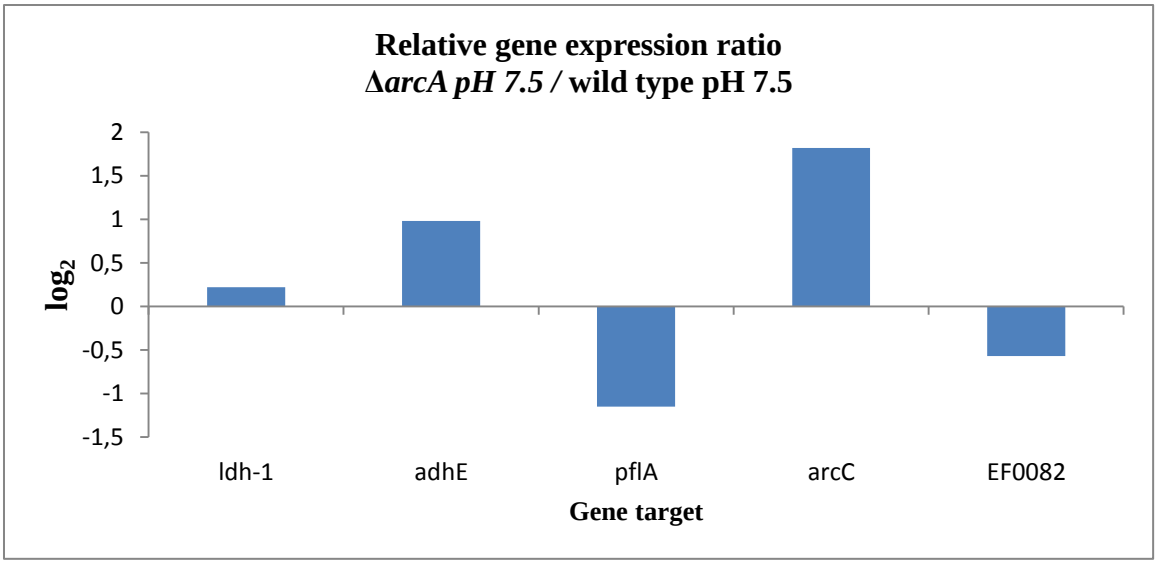


Figure 33: Relative gene expression ratio for *E. faecalis* V583Δ*arcA* cultivated in chemostat in a pH7.5 environment, compared to V583 wild type cultivated under same conditions.

An even further down regulation of *pflA*, *arcC*, *adhE* and EF0082 was observed in mutant cultivated in chemostat in a pH 6.5 environment, compared to mutant cultivated at pH 7.5, whilst an up regulation of ldh-1 was observed in the mutant cultivated at pH 6.5 (Figure 34).

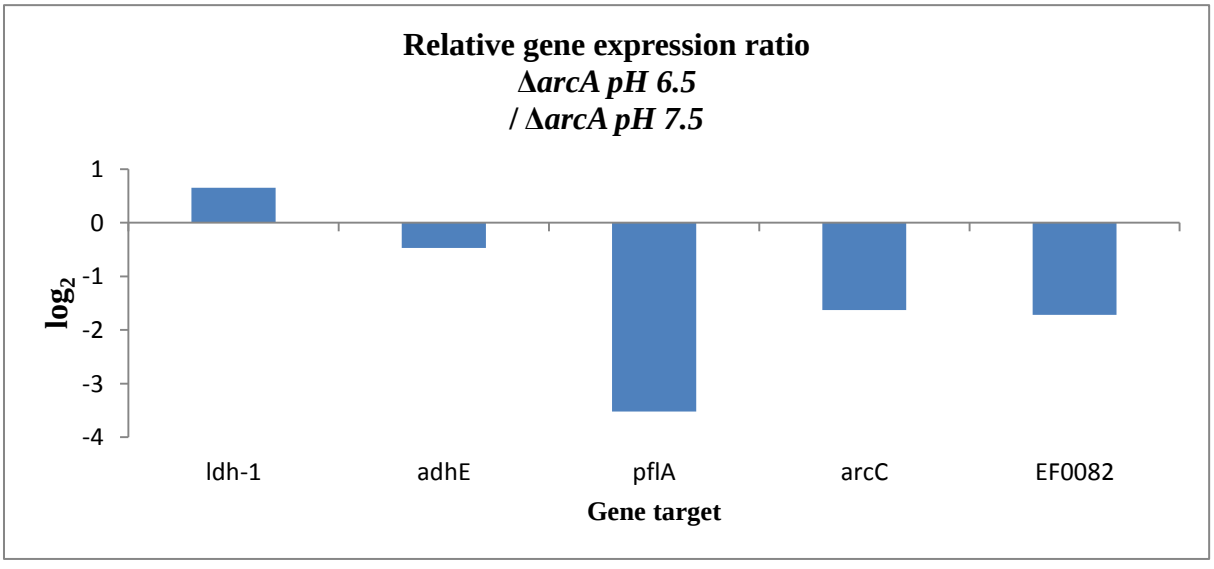


Figure 34: Relative gene expression ratio for *E. faecalis* V583Δ*arcA* cultivated in a chemostat in a pH 7.5 environment, compared to V583Δ*arcA* cultivated in a chemostat in a pH 6.5 environment.

4.5 Complementation of *E. faecalis* V583Δ*arcA*

Complementation of *E. faecalis* V583Δ*arcA* was performed to check for polar effects on the *arcABCRD operon* or if a total reversion to wild type phenotype was achievable. The complementation was done by amplifying the wild type *arcA* gene by PCR (Figure 35) using primers arcA-12, and arcA-13 resulting in *arcA* gene construct (2585 bp) which was cloned into the vector pREG via the XhoI and NotI restriction sites, before transformation into *E. coli*.

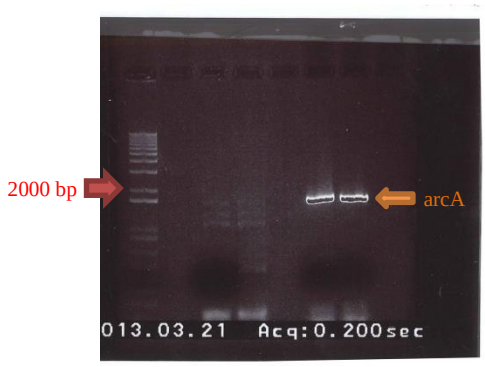


Figure 35: Photo of the *arcA* complementation construct (well 6 and 7, 2585 bp) run through a gel by electrophoresis.

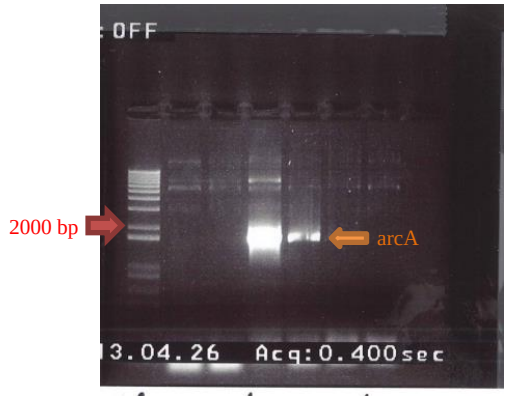


Figure 36: Photo shows gel with the *arcA* complementation construct in pREG (well 4 and 5, 2585 bp) validating the presence of the *arcA* complementation construct in pREG after pREG*arcA* transformation into *E. coli*.

Control PCR was performed to check for pREG*arcA* presence in transformants (Figure 36). After confirmation, pREG*arcA* was transformedn into competent *E. faecalis* V583Δ*arcA*.

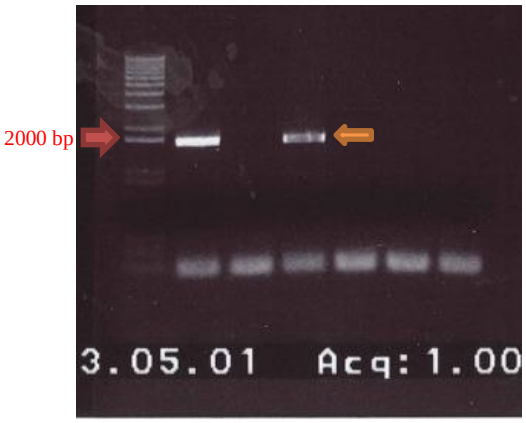


Figure 37: Photo shows gel with the *arcA* complementation construct indicated by the orange arrow (well 2 and 4, 2585 bp), validating the presence of the *arcA* complementation construct in pREG after pREG*arcA* transformation into *E. faecalis* V583.

Final control PCR of wild type *arcA* presence in *E. faecalis* V583Δ*arcA* was performed to validate complementation (Figure 37).

4.6 Construction of *E. faecalis* V583Δ*glnA*Δ*arcA*

The results from the growth experiments with batch cultures showed that the growth yield between the wild type and the Δ*arcA* mutant were not significant, results also showed that the Δ*glnA* mutant grew to a significantly lower OD_{600} than both the Δ*arcA* mutant and wild type. To investigate whether or not an *arcA* deletion could affect growth yield and growth rate for the Δ*glnA* mutant, and to further investigate the effects of both the *glnA*, and the *arcA* deletion, a Δ*glnA*Δ*arcA* double deletion mutant was constructed. In the construction of the *E. faecalis* V583Δ*glnA*Δ*arcA* double deletion mutant, *E. faecalis* V583Δ*glnA* was provided by Margrete Solheim (LMG). A 700bp sequence at the beginning of wild type *arcA* was amplified using primers arcA-sco1 and arcA-sco2 (Figure 38).

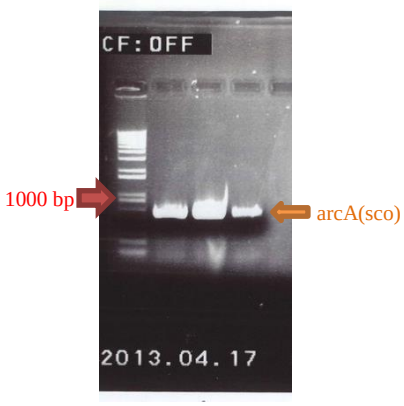


Figure 38: Photo of arcA(sco) (wells 1, 2, 3) (700 bp) product run through gel electrophoresis.

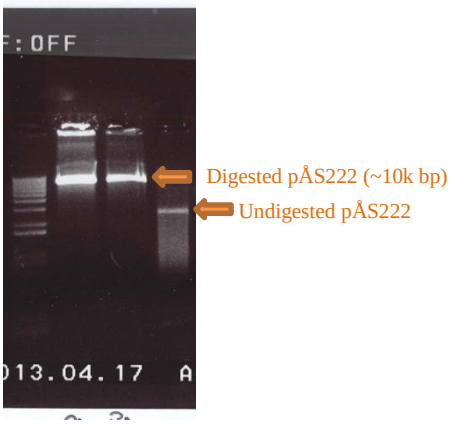


Figure 39: Photo of the cut pÅS222 vector (wells 1 and 2,~10k bp) and undigested control (well 3) run through a gel electrophoresis after fusion PCR.

The PCR product was ligated into the SnaBI site of the thermo-sensitive pÅS222 vector, and the pÅS222 vector containing the 700 bp *arcA* sco construct was transformed into *E. coli*. Control PCR using primers arcA-sco1 and arcA-sco2 was performed to validate presence of arcA sco construct in pÅS222 (shown in Figure 40). After validation the pÅS222*arcA*(sco) vector construct was then isolated from transformants and transformed into *E. faecalis* V583Δ*glnA*.

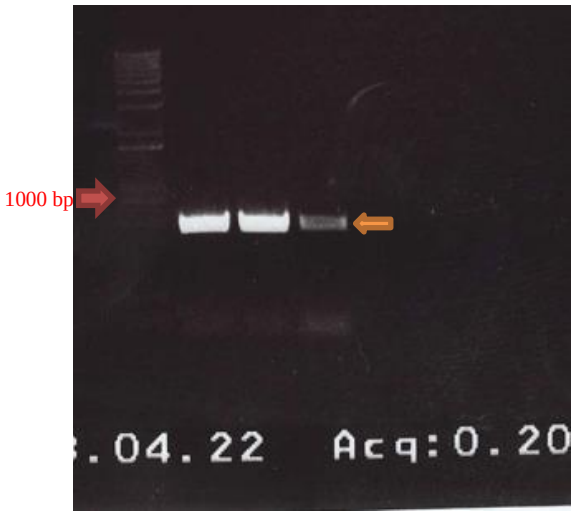


Figure 40: Photo shows gel with the arcA(sco) construct (wells 1, 2, 3, 700 bp) after control PCR, validating the presence of the arcA(sco) construct in pÅS222 after pÅS222arcA(sco) transformation into *E. coli*.

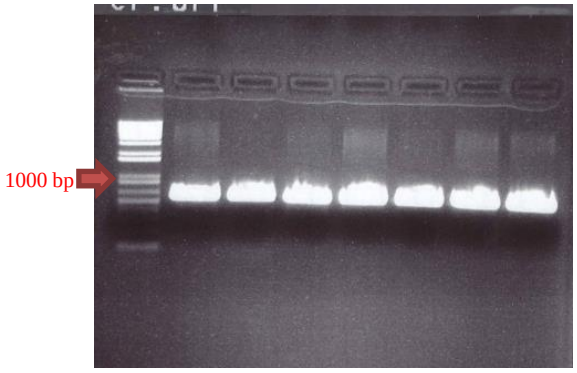


Figure 41: Photo shows gel after control PCR to check for single crossover integration. Presence of arcA(sco) construct (wells 1 to 8, 700 bp) validates the single crossover in *E. faecalis* V583.

Single crossover integration was engineered by using the thermo-sensitive qualities of pÅS222 with the tetracycline antibiotics marker also located on pÅS222. The single crossover integration into the *arcA* gene in *E. faecalis* V583Δ*glnA* is meant to disrupt the functionality of the gene product, ultimately nullifying arginine deiminase function in the cell. To validate the single crossover integration, control PCR using primers arcA-sco1 and arcA-sco2 was performed. Integration was identified in all screened colonies (Figure 41).

4.7 Acidic environment survival test

No reduction in survival capability was observed for *E. faecalis* V583Δ*arcA* in comparison to wild type ($p>0.05$).

4.8 Growth comparison of all constructed mutants

As a follow up to construction of the *E. faecalis* V583Δ*glnA*Δ*arcA* double mutant and complementing the *E. faecalis* V583Δ*arcA* mutant, a growth comparison of all constructed *E. faecalis* V583 mutants and V583 wild type was conducted (Figure 42). Because the double mutant freeze stock did not grow in 5mL ON-culture with CDM-LAB medium, all the strains were cultivated in TH-broth prior to the experiment.

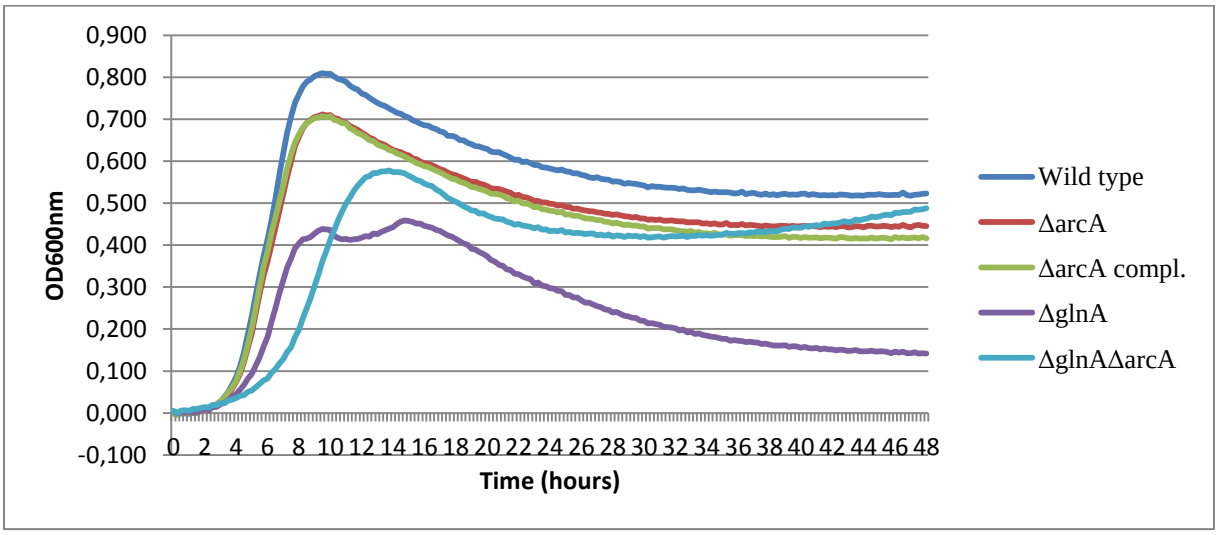


Figure 42: Growth comparison of all constructed mutants and V583 wild type in CDM-LAB medium.

Comparison of all constructed *E. faecalis* V583 mutants and V583 wild type (Figure 42) show a significantly higher OD600 for wild type compared to all the constructed mutants ($p<0.01$). No significant difference is observed between the Δ*arcA* mutant and its complemented strain.

The Δ*glnA* mutant is observed as growing to a significantly lower OD600 than the other mutants and the wild type, and the Δ*glnA*Δ*arcA* double deletion mutant is observed growing to a higher OD600 than the Δ*glnA* mutant, but significantly slower than all the other strains (Table 13).

Table 13: Calculated doubling times using data from Figure 42. Equation used: $[\log (OD_{t2}/OD_{t1})] / \log(2) = g$, doubling time (d) = (t2-t1) mins / g.

Strain	**Doubling time (d)**
WT	36
Δ*arcA*	38
Δ*arcA*+compl.	38
Δ*glnA*	52
Δ*glnA*Δ*arcA*	95

5.0 Discussion

In this study, a Δ*arcA* deletion mutant was created in *E.faecalis* V583. The *arcA* gene encodes for the enzyme arginine deiminase, involved in catabolism of the amino acid L-arginine, producing ammonia and L-citrulline. The mutant was constructed in an attempt to investigate the effect of the mutation on metabolism and gene expression of genes encoding for enzymes related to pyruvate metabolism. As cloning directly in *E. faecalis* V583 is precarious (Margrete Solheim – personal communication), cloning was first performed in *E. coli* using the commercial vector pCR™-Blunt II-TOPO®. After isolating pTOPOΔ*arcA* from *E. coli*, the original plan was to digest vector construct pTOPOΔ*arcA* and vector pÅS22 (provided by Jonsson, M.(28)) using restriction enzyme BamHI, followed by ligation of the two vectors. But as ligation of pTOPO and pÅS22 proved difficult, an alternative thermo-sensitive vector pLT06, constructed by Thurlow *et. al* (55) was used. A stable double crossover deletion mutant was achieved using a slight modification of a method described by Biswas *et. al* (6) with the thermo-sensitive qualities of the vector pLT06. The deletion was confirmed by DNA sequencing the *arcA* region in the Δ*arcA* mutant (chapter 4.2).

To study the effect of the *arcA* deletion on the metabolism of *E. faecalis* V583, growth experiments in batch culture and continuous, glucose-limited cultures (chemostats) were performed. The growth experiments (chapter 4.3) described a slight deviation of growth for the V583 Δ*arcA* mutant in comparison to the V583 wild type. The V583 Δ*arcA* mutant had a significantly lower doubling time in full CDM-LAB medium ($p<0.01$), and the Δ*arcA* mutant

reached its OD_{600} peak slower than the wild type in all batches except for the no glutamine where the Δ*arcA* mutant reached its OD_{600} peak faster than the wild type, and the low glutamine and no glycine compositions where OD_{600} peak was achieved almost simultaneously. In all composition, growth curves visually describe the Δ*arcA* mutant growing to a higher OD_{600} peak than wild type. This difference however, was not statistically significant in any of the compositions ($p>0,05$), which was surprising. It is possible that a modification of the CDM-LAB medium or use of another medium, would yield a significant result. A significant difference in OD_{600} reduction after peaking was observed, with the Δ*arcA* mutant consistently declining more rapidly and to a lower OD_{600} than the wild type ($p<0,05$ except for in serine omitted configuration ($p=0,06$). The deviations of growth observed in the V583 Δ*arcA* mutant compared to the V583 wild type could be attributed to the deletion of the *arcA* gene, but, with glucose available in the medium even after stationary phase is initiated as shown by Jönsson, et. al (28) the transcription of *arcA* should be repressed by CCR. As this repression is not absolute the differences observed in the growth experiments and the slower growth rate of the V583Δ*arcA* mutant could still be attributed to the lack of this pathway in the mutant. The tendency where the mutant grows to a higher OD_{600} peak than the wild type (albeit statistically insignificant) was observed consistently enough (tendency shown in all replicates of experiment) that it could also be an effect of the *arcA* deletion. Growth studies performed in continuous cultures by Mehmeti, *et al.* (37) showed that growth rate has an effect on *E. faecalis* V583 metabolism, and the slower growth rate of the mutant compared to wild type, could be a factor in the cells decision to transition to the stationary phase at a later time than the wild type. Studies done as part of the SysMo-LAB project describe a significant decreased survival capability in acidic environments for a Δ*arcA* mutant in *Streptococcus pyogenes* (unpublished work). Growth studies in batch culture performed by Jönsson, et. al (28) describe a final pH of ~4,5 in the batch culture inoculated with wild type. The more rapid and larger drop in OD_{600} observed in the mutant in comparison to the wild type could be because of a similar change in *E. faecalis* V583Δ*arcA,* reducing its ability to survive in the batch culture after initiating stationairy phase. An acidic environment survival test was performed to investigate this hypothesis, but results indicate no difference in survival capability in acidic environments. It might still be that the acidity of the medium after the stationary phase plays a role in the observed OD_{600} decline, but it could also have a regulatory cause, more research will have to be done in order to elaborate on this subject. In the serine omitted composition of CDM-LAB, the differences between mutant and wild type are considerably less pronounced, but the difference between the growth observed in serine

omitted CDM-LAB, and the growth observed in the other compositions, is significant. A slower growth rate with almost three times the doubling time was observed, indicating that serine, although not an essential amino acid (42), still plays a big role in cell growth. Under normal circumstances, serine can be produced from glycine by a reversible reaction catalyzed by serine hydroxymethyltransferase (*glyA),* and can be catabolized to produce pyruvate and ammonia in a reaction catalyzed by L-serine hydratase (*sdhA, sdhB*). The result that an omission of serine should reduce the differences in growth observed in the other CDM-LAB compositions, does not collaborate with the theory that the acidic environment in the batch culture is an explanation for the more rapid and larger OD_{600} drop for the mutant. In the glycine omitted configuration, growth is severely impeded and cuts off at an OD_{600} of ~0.1. Literature describes the growth inhibition in an environment with high glycine concentrations (20, 38), but does not describe the inhibition of growth from a glycine omission. Growth studies in glycine omitted medium followed by amino acid measurements by Solheim, M. (unpublished work) showed that glycine is in fact produced by the bacterium, most likely from serine as Solheim, M's results showed a similar decrease in serine to the increase of glycine in the medium. Still, the bacteria did not grow above an OD_{600} of ~0,1. Based on these findings, maybe glycine should be considered a necessary amino acid for *Enterococcus faecalis* V583. Whether it is the glycine hydroxymethyltransferase enzyme, the lack of glycine at the start of culture signaling the cell to abort growth, or something else entirely, that causes problems for *E. faecalis*, is not yet understood.

From the samples extracted from cultures in chemostat (mutant pH 6.5 and pH 7.5, and wild type pH 7.5). Supernatants were used for metabolite, ammonium, phosphate, and amino acid analysis, while the cell pellets were used for RNA isolation and transcriptional analysis. Samples were also extracted for dry weight measurements. The results from organic acid and carbohydrate metabolite measurements showed that an effect of the mutation in *arcA* is that less formate is formed in the mutant compared to wild type. Results also show that the effect of lowering the pH is that less formate and more lactate is formed at pH 6.5 than at pH 7.5. This is consistent with studies performed on *E. faecalis* V583 wild type, where the balance between homolactic fermentation and mixed acid fermentation has shown to be pH-dependant. *E. faecalis* V583 shows a skew towards mixed acid fermentation as the pH is elevated to 7.5, while displaying a more homolactic fermentation phenotype as pH is decreased to 6.5 (11). Using the metabolite results, the carbon balance was calculated for each of the samples. A carbon balance of ~82-83% was achieved, which is a value

comparable with carbon balance calculations performed in other studies (11, 31, 43). As catabolism of amino acids can disrupt the carbon balance, so can also the production of biomass and storage of metabolite intermediates (43). Another issue is that ethanol might have vaporized during in the process from extraction until analysis, which would be reflected in the formate : ethanol & acetate ratio of metabolite measurements (should be 2 formate : 1 ethanol 1 acetate). In our case the ethanol concentration is about 6-8mM lower than the acetate concentration, and vaporization could have factored into that discrepancy.

The results from amino acid measurements showed that all L-arginine was consumed by both wild type and both mutant cultures, as well as production of L-citrulline, and L-ornithine. Moreover, production of L-alanine was observed in the mutant under both conditions investigated, with the pH 6.5 mutant culture producing more L-alanine than the pH 7.5 mutant culture. The amounts of ornithine detected could be attributed other pathways such as catabolism of L-proline or L-glutamine through carbamoylphosphate, however, the observed depletion of L-arginine and production of significant amounts of L-ornithine in both the Δ*arcA* mutant cultures is surprising. Annotations in the genomic data for *E. faecalis* V583 describe no alternative *arcA* or alternative pathway for arginine catabolism (30), and no homologs for alternative pathways are inferred in any other *E. faecalis* strains either. Other bacteria such as the Gram-negative *E. coli* and *Pseudomonas aeruginosa* have alternative pathways for arginine catabolism in the arginine decarboxylase (ADC) pathway(26, 51), the arginine dehydrogenase (ADH) pathway (26), and the arginine succinyltransferase (AST) pathway, but these pathways have not been found for *E. faecalis* V583, nor in any of the other lactic acid bacteria. The amounts of ornithine detected could be attributed other pathways such as catabolism of L-proline or L-glutamine through carbamoyl phosphate. The AST pathway produces glutamate and succinate from arginine (24), the ADC pathway produces agmate from arginine (25), which is catabolized to produce ATP and ammonia through carbamoylphosphate, a reaction catalyzed by carbamate kinase (*arcC*) (36). The ADC pathway produces succinate from arginine (25), but more interesting the first step in the ADC pathway produces L-alanine and 5-guanidino-2-oxopentanoate from L-arginine and pyruvate in a reaction catalyzed by arginine pyruvate transaminase (25). This first step is interesting because of the observed production of L-alanine in the amino acid measurements. Literature so far only describes *E. faecalis* V583 with one pathway for arginine catabolism in the ADI pathway. As the results of this study indicate, it might be that this is not entirely correct. No traces of glucose or citrate were detected in the metabolite analysis, validating the glucose-

limitation of the continuous culture, but because the arginine and serine amino acids were also fully consumed (as shown in Figure 30 and Table 12), the culture was a situation where not only glucose availability was limited, but also arginine and serine availability. As arginine is an essential amino acid for *E. faecalis* V583, the arginine limitation could factor in on *E. faecalis* V583 metabolism and gene expression. In addition, lack of serine, even though it's not an essential amino acid, had a significant impact on *E. faecalis* V583 growth in batch cultures, and might be a factor in the continuous culture as well.

Ammonium measurements in the supernatants of the chemostat cultures showed no significant difference between the mutants and wild type, or the two different pH levels. As ammonium is a product of the arginine deiminase reaction, a reduction in total ammonium might have been expected. It might be that the contribution from the arginine deiminase reaction to total ammonium is marginal, or it might be that the ammonia contribution from arginine is still present in mutant supernatants, as the amino acid measurements might indicate. A similar deletion in *arcA* constructed in *S. pyogenes* M49 591 as part of the SysMO-LAB project, did show a significant reduction of ammonia production, conflicting with the results of this study. But, as no information on arginine consumption is given on *S. pyogenes* M49 591, it might be that the deletion in *arcA* was enough to prohibit catabolism of arginine in *S. pyogenes* M49 591, something the results in this study indicate was insufficient to prohibit *E. faecalis* V583's arginine catabolism.

Transcriptional analysis showed that the *arcA* deletion also resulted in changes to the gene expression of genes encoding some of the key components in pyruvate metabolism pathways of *E. faecalis* V583. The results also show a pH effect for transcriptional regulation of *pflA*, growth studies have previously shown that more homolactic fermentation occurs at pH 6.5 than at pH 7.5, but a transcriptional factor in this metabolic shift has not been previously described. Gene expression is not necessarily related to the activity of its corresponding enzyme, but in our case, the results of the transcriptional analysis coincide with the metabolite results. The transcriptional activity of *arcC* indicates that the *arcABCRD* operon is still transcribed in the absence of the *arcA* gene, and not suffering a polar effect from the deletion. If the *arcABCRD* operon is still functional with *arcA* deleted, it should be possible to for the phenotype to revert to wild type if an alternative arginine deiminase is supplied. Preliminary results from the complementation of *E. faecalis* V583Δ*arcA* indicate that this is not the case, but more research will have to be done in order to solidify that statement.

Phosphate measurements in the supernatants of samples extracted from the chemostat show a significant reduction in free phosphate levels of mutants compared to wild type, with the lowest amount of free phosphate observed in the mutant cultivated at pH 7.5. The reduction of phosphate could be attributed to a process of energy storage using chains of phosphate, termed polyphosphates (polyP). Polyphosphates are described in *E .coli* as well as *P. aeruginosa* and other organisms (3). In situations of cell starvation of nutrients, a stress response signal induces the cell to store phosphate in chains of polyphosphates, mediated by so-called polyphosphate kinases (PPK's). For *P. aeruginosa* the accumulation of phosphates has been observed during stationary phase, and in deprivation of amino acids, for *E. coli* deprivation of amino acids also induced accumulation of phosphates. Polyphosphates serve as a phosphate reserve, and can be used as an alternative to ATP in kinase reactions related to AMP, ADP, carbohydrates, nucleosides, and proteins (39). A probably inorganic polyphosphate/ATP-NAD kinase has been inferred by homology, and is at a provisional RefSeq stage for the locus EF2670 in *E. faecalis* V583 (41). It might be that the deletion in *arcA* combined with the nutrient limitations in the continuous culture has triggered the accumulation of phosphates in the *E. faecalis* V583Δ*arcA* mutant. This could explain the reduction of free phosphate observed in the phosphate measurements.

Dry weight measurements revealed a significant reduction in biomass for the *E. faecalis* V583 Δ*arcA* mutant compared to the wild type at pH 7.5. An even further reduction in biomass was observed for mutant culture in chemostat at pH 6.5 compared to mutant and wild type at pH 7.5. That the biomass would be reduced so significantly in the mutant compared to the wild type was contrary to expectations, and indicate that the deletion in *arcA* has effects on the metabolism of *E. faecalis* V583 in a way that is unfavorable for growth. In the case of an alternative catabolism of arginine, or the production of polyphosphates, a reduction of biomass could be a result of that. More research on the matter would be required to make such a conclusion.

As a follow-up to the production of *E. faecalis* V583Δ*arcA,* and the results from the growth experiments, complementation of the *arcA* gene in *E. faecalis* V583Δ*arcA* was performed to check for polar effects on the *arcABCRD* operon or if a total reversion to wild type phenotype was achievable. Unfortunately time-constraints did not allow for continuous culture using the complemented mutant, however, a preliminary growth study shows no difference between the Δ*arcA* mutant and the complemented Δ*arcA* mutant. This could indicate a polar effect, and that supplementing an alternative *arcA* is not enough for *E.*

faecalis V583 to utilize the ADI-pathway for arginine catabolism and revert to wild type phenotype.

A single crossover disruption of *arcA* was engineered in *E. faecalis* V583Δ*glnA* with the use of the thermo-sensitive pÅS222 vector. An *arcA* homologous sequence with a size of 700bp was amplified by PCR. The original idea was to perform the double deletion with an additional double crossover using the same system and materials used in constructing the *E. faecalis* V583Δ*arcA* mutant. Attempts to construct the mutant by deleting the *glnA* gene in the *E. faecalis* V583Δ*arcA* seemed to work at first, with ligation of a *glnA* omission sequence in pLT06, and subsequent transformation into *E. coli* yielding many transformants. The pLT06Δ*glnA* vector construct was isolated and successfully transformed into competent *E. faecalis* V583Δ*arcA*. Growing the transformants at 42°C to induce single crossover integration also worked, validated by a control PCR, but in attempting to engineer the double crossover all ~200 colonies screened had reverted to wild type. Suspecting that the Δ*glnA* deletion and the treatment for inducing double crossover had too severe effects on the already mutated *E. faecalis* V583Δ*arcA*; attempts to construct the mutant by deleting the *arcA* gene in *E. faecalis* V583Δ*glnA* were done, but the attempts unsuccessful. The trouble of creating a double deletion mutant by the method of double crossover selection using 4-chloro-phenylalanine and MM9YE6 agar was shared by others, and suspicions that either the thermo-sensitive integration of the pLT06 vector or the 4-chloropenylalanine treatment changes the bacteria somehow, making it more difficult to repeat the process was proposed (Dag Brede, Sabina La Rosa – personal communication). Uncovering the problems related to this, or developing alternative methods for double crossover induction, could prove to be a huge time-saver in future projects with deletion mutants. Giving up on the double deletion by double crossover entirely, attempts to disrupt expression of *glnA* in *E. faecalis* V583Δ*arcA* or expression of *arcA* in *E. faecalis* V583Δ*glnA* by single crossover integration were done using the pÅS222 vector. Many different approaches were attempted, using different vector configurations and restriction seats success at last was achieved through direct ligation of the *arcA* insert into the SnaBI site of the pÅS222 vector. The construct was blunt ligated into the SnaBI restriction site of pÅS222, and the pÅS222*arcA* vector construct was transformed into *E. coli*. The pÅS222*arcA* vector construct was isolated from *E.coli* + pÅS222*arcA* and transformed into *E. faecalis* V583Δ*glnA*. The single crossover disruption of the *arcA* gene was then engineered using the thermo-sensitive properties of pÅS222. Unfortunately, due to a time-constraint, not much time for experimentation with the *E. faecalis* V583Δ*glnA*Δ*arcA*

was available. A preliminary growth study in batch culture was set up to compare the growth of all constructed *E. faecalis* V583 mutants to each other, as well as the *E. faecalis* V583 wild type. The results of this growth study did not coincide with the previous growth studies, with the wild type growing to a significantly higher OD600 than the Δ*arcA* mutant. A significant difference in the growth of the Δ*glnA*Δ*arcA* double deletion mutant compared to all other strains but perhaps most importantly the Δ*glnA* deletion mutant, was observed. The Δ*glnA*Δ*arcA* mutant had a significantly slower growth rate than all the other strains, and reached a significantly higher OD600 than the Δ*glnA* mutant. The observations of this growth comparison of all mutants, as well as wild type should not be compared to the earlier growth studies. In the amino acid leave-out growth study, ON-cultivation of bacteria was performed in CDM-LAB. But because the Δ*glnA*Δ*arcA* did not grow at all in a primary culture using CDM-LAB, primary cultivation was performed in TH-broth. A 1000x dilution of the ON-cultures into CDM-LAB was performed. The cultivation prior to the growth experiment, could have affected the results as the results from the growth study clearly show Δ*glnA*Δ*arcA* growing in CDM-LAB, something it would not do if not for the prior cultivation in TH. In order to confirm the data of this growth comparison of all mutants, further research and growth studies will have to be done.

6.0 Conclusion and possible future work

In this study, a Δ*arcA* deletion mutant was constructed in *Enterococcus faecalis* V583 to investigate the effects of arginine deiminase on *E. faecalis* V583 growth, metabolism and gene expression. The arginine deiminase enzyme catalyzes the deamination of L-arginine in the ADI-pathway, which is according to literature, the only pathway available for L-arginine catabolism in *E. faecalis* V583. Growth in batch culture using medium with amino acid leave-out compositions showed that the availability of amino acids have a significant effect on both *E. faecalis* V583 wild type and mutant growth. Omission of serine significantly reduces growth rate and omission of glycine disrupts growth almost entirely, with the bacteria only growing to an OD of ~0.1 before initiating stationary phase. In the glucose-limited continuous culture, metabolite analysis revealed a pH-dependency on the shift between homolactic and mixed acid fermentation. A significant reduction in biomass was also observed in the mutant cultures compared to wild type, indicating a less efficient metabolism. Surprisingly, amino acid analysis showed that arginine was still broken down during glucose-limited continuous cultivation of the Δ*arcA* deletion mutant, indicating an alternative for arginine deiminase or an alternative catabolic pathway for arginine in *E. faecalis* V583. An increase in concentrations of L-alanine indicated a pathway for production of L-alanine, possibly by consumption of L-arginine through a pathway similar to the ADC-pathway described in other bacteria. A significant effect on growth and metabolism from the deletion was established, both in batch cultures, where the ADI-pathway should be repressed by CCR, and in continuous glucose-limited culture, where CCR should be close to non-existent. Transcriptional analysis also revealed a down-regulation of *pflA* in the pH 6.5 culture compared to the pH 7.5 culture, indicating a regulatory component in the pH-dependent fermentative shift. A significantly lower phosphate concentration was measured in the Δ*arcA* cultures compared to wild type culture. Provisional genomic data infer the existence of a *ppnK* gene at EF2670, which if true, would allow the bacteria to produce polyphosphates under nutritional or cell stress. The production of polyphosphates or the existence of an alternative arginine catabolic pathway might be correlated to the biomass reduction.

A complementation of the *E. faecalis* V583Δ*arcA* mutant was performed, providing an intact *arcA* gene in trans on a plasmid. Construction of the double deletion mutant *E. faecalis* V583Δ*glnA*Δ*arcA* was also performed. Unfortunately, a time-constraint did not allow for much experiment using the complemented mutant, or the double deletion mutant. A preliminary growth study in batch culture showed no growth difference between *E. faecalis*

V583Δ*arcA* and the complemented *E. faecalis* V583Δ*arcA*. A significant difference between *E. faecalis* V583Δ*glnA* and V583Δ*glnA*Δ*arcA* was observed, with V583Δ*glnA*Δ*arcA* growing significantly slower, but reaching a higher OD_{600} before initiating stationary phase. More growth studies will have to be done to confirm this data.

The results that the *arcA* deletion shows an effect under conditions which should imply strong CCR, is noteworthy. An experiment where samples are extracted at the time of maximum OD_{600} , and the level of arginine, serine and glucose in medium is measured, would perhaps shed some light on the and effects and activity of CCR in batch cultivation. The observed effect of serine omission in batch culture reducing the differences between mutant and wild type growth, but slowing the growth rate of both is surprising. More research on serine omissions and perhaps, production of a L-serine hydratase negative mutant of with singular and double deletions (Δ*sdhA, ΔsdhB, and* Δ*sdhA*Δ*sdhB)* to investigate its effect on growth and metabolism, might return valuable data on this phenomenon. No reports exist on the inhibitory effect of the glycine omission in the CDM-LAB medium on growth, research on which concentrations of glycine are necessary, and at which time glycine would have to be added for the bacteria to grow in a more regular manner in combination with the construction of a glycine hydroxymethyltransferase (Δ*glyA*) negative mutant might yield results able to shed some light on the problem.

The consumption of L-arginine observed in the chemostat cultures, as well as the production of L-alanine, is interesting. Further studies on what alternative process is responsible for breaking down L-arginine would be fascinating, several pathways are connected to the production of carbamoylphosphate, and construction of a Δ*arcC* deletion mutant might cause a significant change in the metabolism of *E. faecalis* V583. The accumulation of phosphates for polyphosphate synthesis and the suggested polyphosphate kinase (*ppnK* / EF2670) might be a good target of investigation. Constructing a deletion in EF2670 region of the Δ*arcA* deletion mutant, one could perhaps validate the existence of *ppnK* by measuring free phosphate levels in a continuous culture with nutrient limitations.

From this study, it is clear that much is yet to be understood about the metabolism and regulatory mechanisms in *E. faecalis* V583. This study provides a good fundament for future research on *E. faecalis* V583 metabolism, transcription and genomic data.

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Appendix

Attachment 1: Zero Blunt® TOPO® PCR Cloning kit manual

Attachment 2: E.N.Z.A™ Plasmid MiniPrep manual

Attachment 3: Nucleospin® PCR Clean-up Extraction Kit manual

Attachment 4: Qiagen® Plasmid Midi Kit Quick-Start Protocol

Attachment 5: LightRun Sequencing brochure

Attachment 6: Megazyme Ammonia (Rapid) Assay Procedure manual

Attachment 7: BioVision Phosphate Colorimetric Assay Kit manual.

Attachment 8: RNA 6000 Kit manual

Attachment 9: Sequence alignment, full description

Attachment 10: CDM-medium, full description of medium.

Kit Contents and Storage

Shipping and Storage

Zero Blunt® TOPO® PCR Cloning Kits are shipped on dry ice. Kits supplied with competent cells contain a box with Zero Blunt® TOPO® PCR Cloning reagents (Box 1) and a box with One Shot® *E. coli* (Box 2).

Zero Blunt® TOPO® PCR Cloning Kit supplied with the PureLink® Quick Plasmid Miniprep Kit (Cat. no.K2800-02) is shipped with an additional box containing reagents for plasmid purification (Box 3).

Zero Blunt® TOPO® PCR Cloning® Kit (Cat. no. 450245) is shipped with only the Zero Blunt® TOPO® PCR Cloning reagents (Box 1).

Box	Store at
1	–30°C to –10°C in a non-frost-free freezer
2	–85°C to –68°C
3	Room temperature (15°C to 30°C)

Zero Blunt® TOPO® PCR Cloning Kits

Zero Blunt® TOPO® PCR Cloning Kits are available with One Shot® TOP10 Chemically Competent, One Shot® DH5α™-T1^R Chemically Competent, One Shot® Mach1™- T1^R Chemically Competent, or One Shot® TOP10 Electrocomp™ *E. coli* (see page vi for the genotypes of the strains).

Notes: Catalog no. 450245 is not supplied with competent cells.
Catalog no. K2800-02 also includes the PureLink® Quick Plasmid Miniprep Kit.

Cat. no.	Reactions	One Shot® Cells	Type of Cells
K2800-20	20	TOP10	Chemically competent
K2800-40	40	TOP10	Chemically competent
K2820-20	20	DH5α™-T1^R	Chemically competent
K2820-40	40	DH5α™-T1^R	Chemically competent
K2830-20	20	Mach1™- T1^R	Chemically competent
K2860-20	20	TOP10	Electrocompetent
K2860-40	40	TOP10	Electrocompetent
K2800-02*	20	TOP10	Chemically competent
450245	20	Not supplied	NA

*Includes PureLink® Quick Plasmid Miniprep Kit

Product Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

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Kit Contents and Storage, Continued

Zero Blunt® TOPO® PCR Cloning Reagents

Zero Blunt® TOPO® PCR Cloning reagents (Box 1) are listed below. **Note that the user must supply the proofreading polymerase.** Store Box 1 at –30°C to –10°C.

Item	Concentration	Amount
pCR™-Blunt II-TOPO®	10 ng/µL plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/mL BSA 30 µM bromophenol blue	20 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µL
M13 Forward (–20) Primer	0.1 µg/µL in TE Buffer	20 µL
M13 Reverse Primer	0.1 µg/µL in TE Buffer	20 µL
Control Template	0.1 µg/µL in TE Buffer	10 µL
Water	—	1 mL

Sequence of Primers

The following table describes the sequence and pmoles supplied of the sequencing primers included in this kit.

Primer	Sequence	pMoles Supplied
M13 Forward (–20)	5′-GTAAAACGACGGCCAG-3′	407
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′	385

PureLink® Quick Plasmid Miniprep Kit

For kit components of the PureLink® Quick Plasmid Miniprep Kit (Box 3) supplied with Cat. no. K2800-02, refer to the manual supplied with the miniprep kit.

Continued on next page

Perform the TOPO® Cloning Reaction

Introduction

After producing the desired PCR product, you are ready to TOPO® Clone it into the pCR™-Blunt II-TOPO® vector and transform the recombinant vector into competent *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transform One Shot® Competent Cells** (pages 6–8) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 12–14 in parallel with your samples.

Note

We have found that including salt (200 mM NaCl; 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (i.e. using the stock salt solution provided in the kit).

Note that you must dilute the TOPO® Cloning reaction before transforming electrocompetent cells.

- For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.
- For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ in order to prevent arcing. After performing the TOPO® Cloning reaction, and prior to electroporation, dilute the reaction 4-fold to achieve the proper salt concentration.

Continued on next page

Perform the TOPO® Cloning Reaction, Continued

Set Up the TOPO® Cloning Reaction

Use the following procedure to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown.

Note: The blue color of the pCR™II-Blunt-TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Volume
Fresh PCR product	0.5–4 µL
Salt Solution	1 µL
Water	add to a total volume of 5 µL
pCR™II-Blunt-TOPO®	1 µL
Final Volume	**6 µL**

* Store all reagents at –20°C when finished. Salt solutions and water can be stored at room temperature or 4°C.

Perform the TOPO® Cloning Reaction

1. Mix the reaction gently and incubate for **5 minutes** at room temperature (22–23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO®-cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (greater than 1 kb) or if you are TOPO®-cloning a pool of PCR products, increasing the reaction time will yield more colonies.
2. Place the reaction on ice and proceed to **Transform One Shot® Competent Cells,** on page 6.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Note

Zero Blunt® TOPO® PCR Cloning Kits are optimized to work with One Shot® Competent *E. coli* available from Life Technologies Corporation. Use of other competent cells may require further optimization.

Performing the control TOPO® Cloning reaction is recommended as this control when used with the supplied protocol will demonstrate high cloning efficiencies.

Additionally, transforming a control plasmid is highly recommended to confirm transformation efficiencies when using alternative competent cells not supplied by Life Technologies.

Transform One Shot® Competent Cells

Introduction

After performing the TOPO® Cloning reaction, transform your pCR™-Blunt II-TOPO® construct into competent *E. coli*.

Protocols to transform One Shot® chemically competent and electrocompetent *E. coli* supplied with the Zero Blunt® TOPO® PCR Cloning Kits are provided below.

To transform another competent strain, refer to the manufacturer's instructions.

Required Materials

Components required but not supplied:

- The TOPO® Cloning reaction from **Perform the TOPO® Cloning Reaction** (step 2 on page 5)
- LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic (use two plates per transformation; see page 17 for recipes)
- 15-mL snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath or electroporator with 0.1-cm cuvettes
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g. plates, spreaders)

Components supplied with the kit:

- S.O.C. medium (included with the kit)

Prepare for Transformation

For each transformation, you will need 1 vial of competent cells and 2 selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator.
- Warm the vial of S.O.C. medium to room temperature.
- Warm LB plates containing 50 µg/mL kanamycin or 25 µg/mL Zeocin™ selective antibiotic at 37°C for 30 minutes.
- Thaw *on ice* 1 vial of One Shot® cells for each transformation.

Important

If you are transforming One Shot® Mach1™-T1^R Chemically Competent *E. coli*, it is essential that you prewarm your selective plates prior to spreading for optimal growth of cells.

Continued on next page

Transform One Shot® Competent Cells, Continued

One Shot® Chemical Transformation

1. Add 2 µL of the TOPO® Cloning reaction **from Perform the TOPO® Cloning Reaction**, step 2 on page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5–30 minutes.

   Note: Longer incubations on ice do not seem to affect transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µL of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10–50 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze Transformants** on page 9).

One Shot® Electroporation

1. Add 18 µL of water to 6 µL of the TOPO® Cloning reaction from **Perform the TOPO® Cloning Reaction**, step 2 on page 5. Mix gently.

   Note: The TOPO® Cloning reaction must be diluted in this step to prevent arcing.
2. Add 2 µL of the dilute TOPO® Cloning reaction (from step 1 of this procedure) to a vial (50 µL) of One Shot® Electrocomp™ *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
3. Carefully transfer cells and DNA to a chilled 0.1-cm cuvette.
4. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see page 8.
5. Immediately add 250 µL of room temperature S.O.C. medium to the cuvette.
6. Transfer the solution to a 15-mL snap-cap tube (e.g. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
7. Spread 10–50 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies. Incubates plates over night at 37°C.
8. An efficient TOPO® Cloning reaction will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze Transformants** on page 9).

Continued on next page

Transform One Shot® Competent Cells, Continued

Diluting the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be 50–80 µL (0.1-cm cuvettes) or 100–200 µL (0.2-cm cuvettes).

If you experience arcing, try *one* of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyze Transformants

Analyze Positive Clones

1. Culture 2–6 colonies overnight in LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic. Be sure to save the original colony by patching to a fresh plate, if needed.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for 4 hours in *prewarmed* LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic before isolating the plasmid. For optimal results, we recommend inoculating as much of a single colony as possible.
2. Isolate plasmid DNA using PureLink® Quick Plasmid Miniprep Kit (supplied with Cat. no. K2800-02 or available separately, see page 19). The plasmid isolation protocol is included in the manual supplied with the PureLink® Quick Plasmid Miniprep Kit and is also available from **www.lifetechnologies.com**. Other kits for plasmid DNA purification are also suitable for use.
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequence

You may sequence your construct to confirm that your gene is cloned in the correct orientation. The M13 Forward (–20) and M13 Reverse primers are included to help you sequence your insert. Refer to the maps on page 18 for sequence surrounding the TOPO Cloning® site. For the full sequence of pCR™-Blunt II-TOPO®, refer to **www.lifetechnologies.com/support** or contact Technical Support (page 20).

Continued on next page

Plasmid Mini Spin Protocol

Plasmid Mini Spin Protocol

All centrifugation steps used are preformed at room temperature. unless otherwise noted. For low copy plasmids refer to page 16. This protocol is designed to isolate plasmid from E. coli grown in a 1-5 mL LB culture.

User Supplied Equipment:

- 96-100% Ethanol (Do not use denatured alcohol)
- Microcentrifuge capable of 15,000 x g
- 1.5 ml or 2 ml Centrifuge Tubes
- Appropriate Centrifuge Tube for step 1

Things to do before starting:

- Preheat Elution Buffer to 70°C if Plasmid DNA is >10kb
- Prepare DNA Wash Buffer and Solution 1 according to directions on page 6

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~ 12-16 hr at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 ml culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.

2. Decant or Pellet bacterial cells by centrifugation at 10,000 x g for 1 min at room temperature.

3. Resuspend the bacterial pellet by adding 250 µl of Solution I/RNase A solution, and vortexing (or pipetting up and down). Complete re-suspension (no visible cell clumps) of cell pellet is vital for obtaining good yields. Transfer suspension into a new 1.5 ml microcentrifuge tube.

4. **Add 250 µl of Solution II** and gently mix by inverting and rotating tube six (6) times to obtain a clear lysate. A 2-3 minute incubation may be necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.

   Note: ***Do not allow the lysis reaction to proceed more than 5 min.***
   (Store Solution II tightly capped when not in use to avoid acidification of Solution II from CO_2 in the air.)

5. **Add 350 µl of Solution III** and mix immediately by inverting six (6) times until a flocculent white precipitate forms.

   Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.

Plasmid Mini Spin Protocol

6. Centrifuge at 13,000 x g for 10 min at room temperature. A compact white pellet will form. Promptly proceed to the next step.

7. Prepare a **HiBind DNA Mini Column** by placing into a 2 mL collection tube. **Add 100 µl of Equilibration Buffer.** Centrifuge at 13,000 x g for 30-60 seconds. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

8. **Add the cleared supernatant from step 6** by CAREFULLY aspirating it into the HiBind DNA Mini Column. Ensure that the pellet is not disturbed and that no cellular debris has carried over into the HiBind DNA Mini Column. Centrifuge at 13,000 x g for 1 min at room temperature to completely pass lysate through the HiBind DNA Miniprep Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

9. **Add 500 µl of Buffer HB** and Centrifuge at 13,000 x g for 30 to 60 seconds at room temperature to wash the HiBind DNA Mini Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

This step ensures that residual protein contaminations are removed, thus ensuring high quality DNA that will be suitable for downstream applications.

10. **Add 700 µl of DNA Wash Buffer** (diluted with absolute ethanol) and Centrifuge at 13,000 x g for 30 to 60 seconds at room temperature to wash the HiBind DNA Miniprep Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

NOTE: DNA Wash Buffer Concentrate must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room temperature before use.

11. **OPTIONAL:** Repeat wash step 10 with another 700 µl of DNA Wash Buffer (diluted with absolute ethanol).

12. Centrifuge the empty HiBind Mini Column at 13,000 x g for 2 min to dry the column

IMPORTANT: *Do not skip this step - it is critical for good yields*

13. Place the HiBind DNA Mini Column into a new/clean 1.5 ml microcentrifuge tube(not supplied). Depending on desire concentration of final product, **add 50-100 µl of Elution Buffer** (10 mM Tris-HCl, pH 8.5) or sterile deionized water directly onto the center of the column matrix. Incubate at room temperature for 1 minute. Centrifuge for at 13,000 x g for 1 min to elute DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

5 Protocols

5.1 PCR clean-up

The following protocol is suitable for PCR clean-up as well as DNA concentration and removal of salts, enzymes, etc. from enzymatic reactions (SDS < 0.1 %).

Before starting the preparation:

- Check if Wash Buffer NT3 was prepared according to section 3.

1 Adjust DNA binding condition

For very small sample volumes < 30 µL adjust the volume of the reaction mixture to 50–100 µL with water.
It is not necessary to remove mineral oil.

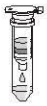
Mix **1 volume of sample** with **2 volumes** of **Buffer NTI** (e.g., mix 100 µL PCR reaction and 200 µL Buffer NTI).

Note: For removal of small fragments like primer dimers dilutions of Buffer NTI can be used instead of 100 % Buffer NTI. Please refer to section 2.3.

+ 2 vol NTI per 1 vol sample

2 Bind DNA

Place a **NucleoSpin® Gel and PCR Clean-up Column** into a Collection Tube (2 mL) and load up to 700 µL sample.



Load sample

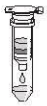
Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

11,000 x *g* 30 s

Load remaining sample if necessary and repeat the centrifugation step.

3 Wash silica membrane

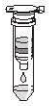
Add **700 µL Buffer NT3** to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.



+ 700 µL NT3

11,000 x *g* 30 s

Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and improve A_{260}/A_{230} values (see section 2.7 for detailed information).

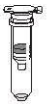


+ 700 µL NT3

11,000 x *g*
30 s

4 Dry silica membrane

Centrifuge for **1 min** at **11,000 x *g*** to remove **Buffer NT3** completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.



Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

11,000 x *g*
1 min

5 Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a **new** 1.5 mL microcentrifuge tube (not provided). Add **15–30 µL Buffer NE** and incubate at **room temperature** (18–25 °C) for **1 min**. Centrifuge for **1 min** at **11,000 x *g***.

+ 15–30 µL NE

RT
1 min

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. See section 2.6 for detailed information.

11,000 x *g*
1 min

5.2 DNA extraction from agarose gels

Before starting the preparation:

- Check if Wash Buffer NT3 was prepared according to section 3.

1 Excise DNA fragment/solubilize gel slice

Note: Minimize UV exposure time to avoid damaging the DNA. Refer to section 2.5 for more tips on agarose gel extraction.

Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess agarose.

Determine the weight of the gel slice and transfer it to a clean tube.

For each **100 mg of agarose gel < 2%** add **200 µL Buffer NTI**.

+ 200 µL NTI per 100 mg gel

For gels containing > 2 % agarose, double the volume of Buffer NTI.

Incubate sample for **5–10 min** at **50 °C**. Vortex the sample briefly every 2–3 min until the gel slice is **completely** dissolved!

50 °C
5–10 min

2 Bind DNA

Place a **NucleoSpin® Gel and PCR Clean-up Column** into a Collection Tube (2 mL) and load up to 700 µL sample.

Load sample

Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

11,000 x *g*
30 s

Load remaining sample if necessary and repeat the centrifugation step.

3 Wash silica membrane

+ 700 µL NT3

Add **700 µL Buffer NT3** to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

11,000 x *g*
30 s

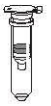
Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and low A_{260}/A_{230} (see section 2.7 for detailed information).

+ 700 µL NT3

11,000 x *g*
30 s

4 Dry silica membrane

Centrifuge for **1 min** at **11,000 x *g*** to remove **Buffer NT3** completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.



Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

11,000 x *g*
1 min

5 Elute DNA

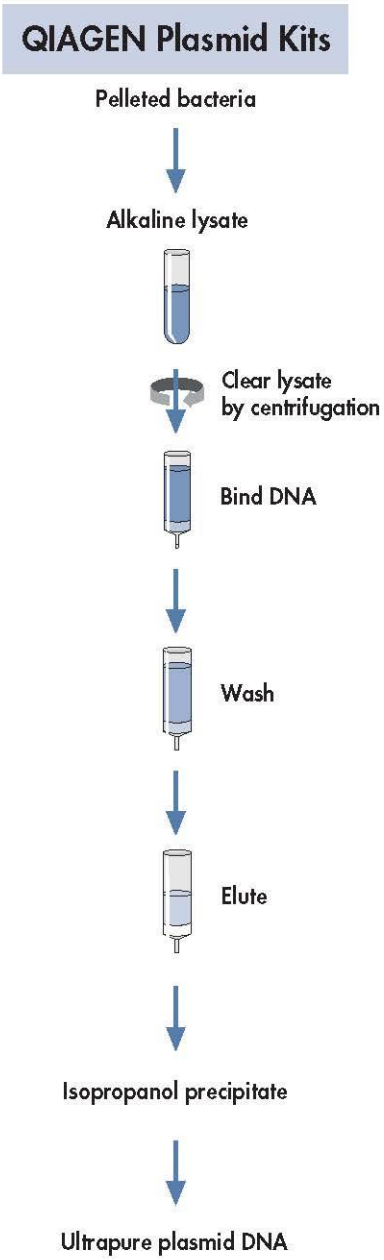
Place the NucleoSpin® Gel and PCR Clean-up Column into a **new** 1.5 mL microcentrifuge tube (not provided). Add **15–30 µL Buffer NE** and incubate at **room temperature** (18–25 °C) for **1 min**. Centrifuge for **1 min** at **11,000 x *g***.

+ 15–30 µL NE

RT
1 min

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. See section 2.6 for detailed information.

11,000 x *g*
1 min


QIAGEN Plasmid Kits
Pelleted bacteria
Alkaline lysate
Clear lysate
by centrifugation
Bind DNA
Wash
Elute
Isopropanol precipitate
Ultrapure plasmid DNA

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For all protocols:

- Standard microbiological equipment for growing and harvesting bacteria (e.g., inoculating loop, culture tubes and flasks, 37°C shaking incubator, and centrifuge with rotor and tubes or bottles for harvesting cells)
- QIArack or equivalent holder (see "Setup of QIAGEN-tips", page 13)
- Ice
- Isopropanol
- 70% ethanol
- Plasmid resuspension buffer (e.g., TE buffer, pH 8.0, or Tris·Cl, pH 8.5)

For QIAGEN Plasmid Mini Kit protocol:

- Microcentrifuge
- 1.5 ml or 2 ml microcentrifuge tubes

For QIAGEN Plasmid Midi, Maxi, Mega, and Giga Kit protocols:

- Centrifugation tubes or vessels with suitable capacity for the volumes specified in the appropriate protocol.
- Refrigerated centrifuge capable of ≥20,000 x *g* with rotor for the appropriate centrifuge tubes or bottles

Table 1. Origins of Replication and Copy Numbers of Various Plasmids and Cosmids

DNA construct	Origin of replication	Copy number	Classification
Plasmids			
pUC vectors	pMB1*	500–700	high copy
pBluescript® vectors	ColE1	300–500	high copy
pGEM® vectors	pMB1*	300–400	high copy
pTZ vectors	pMB1*	>1000	high copy
pBR322 and derivatives	pMB1*	15–20	low copy
pACYC and derivatives	p15A	10–12	low copy
pSC101 and derivatives	pSC101	~5	very low copy
Cosmids			
SuperCos	pMB1	10–20	low copy
pWE15	ColE1	10–20	low copy

* The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The high-copy plasmids listed here contain mutated versions of this origin.

Culture media

QIAGEN plasmid purification protocols are optimized for use with cultures grown in standard Luria Bertani (LB) medium to a cell density of approximately 3–4 x 10^9 cells/ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium. Please note that a number of slightly different LB culture broths, containing different concentrations of NaCl, are commonly used. We recommend growing cultures in LB medium containing 10 g NaCl per liter (Table 2) to obtain the highest plasmid yields.

Rich media are not recommended for plasmid preparation with QIAGEN-tips. If rich media must be used, growth time must be optimized, and culture volumes reduced. For more details, visit our plasmid resource page at www.qiagen.com/goto/plasmidinfo and click on the link "General Considerations for Optimal Results".

Table 2. Composition of Luria Bertani Medium

Contents	per liter
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Please refer to Appendix B on page 44 for preparation of LB medium.

Protocol: Plasmid or Cosmid DNA Purification Using QIAGEN Plasmid Midi and Maxi Kits

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAGEN Plasmid Midi Kit, or up to 500 µg using the QIAGEN Plasmid Maxi Kit. For additional protocols, such as for purification of very low-copy plasmids or cosmids of less than 10 copies per cell, see page 29 or visit www.qiagen.com/goto/plasmidinfo .

Low-copy plasmids that have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Maximum Recommended Culture Volumes*

	QIAGEN-tip 100	QIAGEN-tip 500
High-copy plasmids	25 ml	100 ml
Low-copy plasmids	100 ml	500 ml

* For the QIAGEN-tip 100, the expected yields are 75–100 µg for high-copy plasmids and 20–100 µg for low-copy plasmids. For the QIAGEN-tip 500, the expected yields are 300–500 µg for high-copy plasmids and 100–500 µg for low-copy plasmids.

Important points before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo .
- If working with low-copy vectors, it may be beneficial to increase the lysis buffer volumes in order to increase the efficiency of alkaline lysis, and thereby the DNA yield. In case additional Buffers P1, P2, and P3 are needed, their compositions are provided in Appendix B: Composition of Buffers, on page 44. Alternatively, the buffers may be purchased separately (see page 49).
- Optional: Remove samples at the steps indicated with the symbol ☞ in order to monitor the procedure on an analytical gel (see page 41).
- Blue (marked with a ▲) denotes values for QIAGEN-tip 100 using the QIAGEN Plasmid Midi Kit; red (marked with a ●) denotes values for QIAGEN-tip 500 using the QIAGEN Plasmid Maxi Kit.

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (centrifuge briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (centrifuge briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate ▲ 25 ml or ● 100 ml medium with ▲ 25–50 µl or ● 100–200 µl of starter culture. For low-copy plasmids, inoculate ▲ 100 ml or ● 500 ml medium with ▲ 100–200 µl or ● 250–500 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in ▲ 4 ml or ● 10 ml Buffer P1.**

   For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add ▲ 4 ml or ● 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. **Add ▲ 4 ml or ● 10 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for ▲ 15 min or ● 20 min.**

   Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

   Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

   Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Kits or Cartridges (see www.qiagen.com/products/plasmid/LargeScaleKits).

8. **Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

   This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

   ☞ Remove a ▲ 240 µl or ● 120 µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

9. **Equilibrate a ▲ QIAGEN-tip 100 or ● QIAGEN-tip 500 by applying ▲ 4 ml or ● 10 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. **Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.**

    The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

    ☞ Remove a ▲ 240 µl or ● 120 µl sample from the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

11. **Wash the QIAGEN-tip with ▲ 2 x 10 ml or ● 2 x 30 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    ☞ Remove a ▲ 400 µl or ● 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with ▲ 5 ml or ● 15 ml Buffer QF.**

    Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

    Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

    ☞ Remove a ▲ 100 µl or ● 60 µl sample of the eluate and save for an analytical gel (sample 4).

    ⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding ▲ 3.5 ml or ● 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with ▲ 2 ml or ● 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred (see page 41).

LIGHTrun

Jochen Schäfer, Head of Single Read Sequencing Services, GATC Biotech

NEW sequencing service:

- barcode order only
- no sequencing order
- free sample shipment

Data delivered from 8 am – 1 pm next day!

Any questions about *LIGHTrun*?

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- Test *LIGHTrun* sequencing service
- Request free test barcodes now!

www.gatc-biotech.com/lightrun GATC BIOTECH WWW.GATC-BIOTECH.COM **www.gatc-biotech.com/lightrun**

Sample requirements

Purified plasmid DNA or purified PCR fragments with primer premixed in tubes

1. Template DNA of **5 µl** with either of following concentrations:
   - Plasmid DNA (purified): 80 – 100 ng/µl
   - PCR product (purified): 20 – 80 ng/µl
2. Add **5 µl** of primer with following concentration:
   - 5µM (5pmol/µl)

Please send total sample amount of **10 µl** in 1,5 ml tubes.

Primer characteristics

- The melting temperature (T_M) of the primer should be between 52° and 58°C and the length should be between 17 – 19bp. Ideally, the GC content of an 17mer should be 10 G+C; for an 18mer 8 – 9 G+C and for a 19mer 7 – 9 G+C.
- G or C should be at the 3' end, but not more than 3 Gs or Cs.
- The primer sequence should be a good mix of all 4 nucleotides with no more than 4 identical bases in a row (AAAA or GGGG).

To ensure the best possible quality of sequence data, the total volume of the sample cannot be less than 10 µl. We recommend measuring the DNA concentration on an agarose gel. Sequencing reactions cannot be repeated and all reactions have to be paid. Your DNA material cannot be stored. Thank you for your understanding.

Sample shipment

1. GATC Collection Box
There are many GATC Collection Boxes installed throughout Europe for free sample shipment. Look for the one near you.

2. FREE shipment by UPS
If there is no GATC Collection Box near you, we will provide you with UPS labels for shipment free of charge (depending on the size of the barcode batch ordered).

3. By post
If there is no GATC Collection Box near you, and you only have a few samples to send, please ship them by post. Use stable, padded envelopes or falcon tubes to ensure safe transport. Please then send your samples to:

GATC Biotech AG
European Custom Sequencing Centre
Gottfried-Hagen-Straße 20

51105 Köln

LIGHTrun in 5 easy steps:

Order *LIGHTrun* sequencing barcodes

Go to www.gatc-biotech.com/lightrun and click on "Order *LIGHTrun* barcodes". Choose service type and order your *LIGHTrun* sequencing barcodes. They will be sent to you without delay.

Premix template and primer

Add to your tube:
5 µl template + 5 µl primer (according to sample requirements).

Label tubes with barcodes

Attach one part of the barcode to the tube and keep the other part for your lab record.

Send your samples

Send by post, drop them at a GATC Collection Box or send by UPS (depending on order volume).

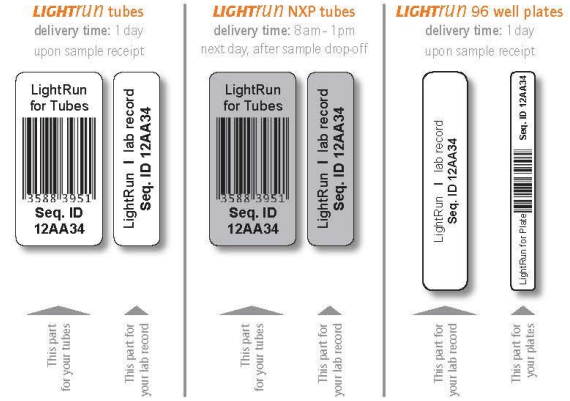
Download results

Once your data are ready, you'll receive an email. Go to www.gatc-biotech.com/lightrun and click on "Download results".

Affix the barcode in the front of the tube

Please don't use parafilm to seal your tubes!

LIGHTrun barcode types



LIGHTrun service types

Single read sequencing of purified plasmids or PCR fragments

LIGHTrun tubes
up to 1,100 nt
delivery time: 1 day

LIGHTrun NXP tubes
up to 1,100 nt
delivery time: 8 am - 1 pm the next day

LIGHTrun 96 well plates
up to 1,000 nt
delivery time: 1 day

For tubes: each *LIGHTrun* barcode label corresponds to one single sequencing reaction
For 96 well plates: each *LIGHTrun* barcode label corresponds to one entire plate.

Next day data delivery 8 am - 1 pm depends on logistics available at your location.

INTRODUCTION:

Ammonia is a widely occurring natural compound, often produced as a consequence of microbial protein catabolism, and thus serves as a quality indicator of fruit juice, milk, cheese, meat, seafood and bakery products. Unlike some other kits, this kit benefits from the use of a glutamate dehydrogenase that is not inhibited by tannins found in, for example, grape juice and wine. K-AMIAR can be used to determine ammonia manually (see page 4, "A") or in auto-analyser format (see page 6, "B"). In the wine industry, ammonia determination is important in the calculation of yeast available nitrogen (YAN). YAN comprises three highly variable components, free ammonium ions, primary amino nitrogen (from free amino acids), and the contribution from the side chain of L-arginine[1]. Thus, for the most accurate determination of YAN, all three components should be quantified, and this is possible using Megazyme's L-Arginine/Urea/Ammonia kit (K-LARGE) and NOPA kit (K-PANOPA).

PRINCIPLE:

In the presence of glutamate dehydrogenase (GlDH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), ammonia (as ammonium ions; NH_4^+) reacts with 2-oxoglutarate to form L-glutamic acid and $NADP^+$ (1).

(1) 2-Oxoglutarate + NADPH + NH_4^+ $\xrightarrow{\text{(GIDH)}}$ L-glutamic acid + $NADP^+$ + H_2O

The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia. It is NADPH consumption which is measured by the decrease in absorbance at 340 nm[2].

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for ammonia. In the analysis of reagent grade ammonium sulphate, results of approx. 100% can be expected.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.018 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.071 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.2 to 7 µg of ammonia per assay (Figure 2). In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to an ammonia concentration of approx. 0.018 to 0.035 mg/L of sample solution. If the sample is diluted during sample preparation, the result is

multiplied by the dilution factor, F. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of ammonia has been completed within the time specified in the assay (approx. 3 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding ammonia (approx. 4 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding ammonia to the sample in the initial extraction steps.

In alkaline buffer solution, protein fragments may slowly release ammonia which can lead to a slow creep reaction. This is not a problem with this kit, because the reaction is completed so quickly.

Tannins in fruit juice can lead to significant inhibition of GlDH from beef liver, the enzyme employed in Ammonia and Urea/Ammonia kits supplied by others. However, the enzyme used in this kit does not suffer from this limitation (Figure 3).

SAFETY:

The reagents used in the determination of ammonia are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02% w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 96 assays in manual format (or 960 assays in auto-analyser format or 960 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (36 mL, pH 8.0) plus 2-oxoglutarate and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x 2) NADPH.
Stable for > 5 years at -20°C.

Bottle 3: Glutamate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C.

Bottle 4: Ammonia standard solution (5 mL, 0.04 mg/mL) in 0.02% (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years at -20°C (to avoid repetitive freeze / thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3 & 4. Use the contents of bottles 3 and 4 as supplied. Store the bottles in an upright position. **Swirl bottle 3 to mix contents before use.** Stable for > 2 years at 4°C.

NOTE: The ammonia standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of ammonia is determined directly from the extinction coefficient of NADPH (page 5).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 12.5 mL Combitip® [to dispense 0.5 mL aliquots of NADPH buffer (solution 2)].
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No.1 (9 cm) filter papers.

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for ammonia can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of ammonia **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.262 mL
Linearity: 0.1-0.7 µg of ammonia per well (in 0.01-0.2 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.030 mL	0.030 mL	0.030 mL
solution 2 (NADPH)	0.020 mL	0.020 mL	0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 3 (GlDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 min**.			

* for example using microplate shaker, shake function on a microplate reader, or repeated aspiration (e.g. using a pipettor set at 50 - 100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

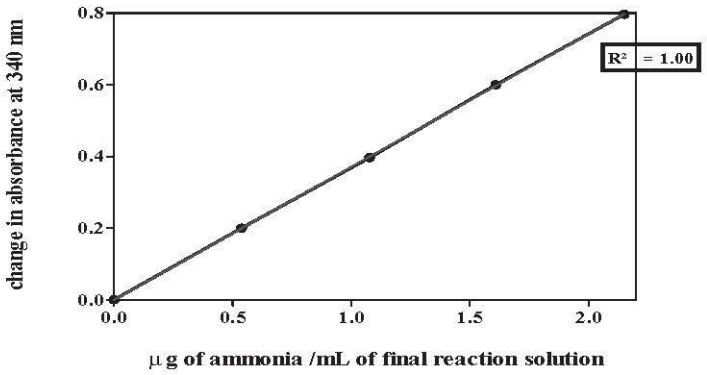


Figure 1. Calibration curve demonstrating the linearity of K-AMIAR. The reactions used to generate this calibration curve were performed at 37°C for 5 min, using a 10 mm path-length cuvette.

SAMPLE PREPARATION (Manual Format, A):

1. Sample dilution.

The amount of ammonia present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.2 and 7 µg. The sample solution must therefore be diluted sufficiently to yield an ammonia concentration between 0.01 and 0.07 g/L.

Dilution Table

Estimated concentration of ammonia (g/L)	Dilution with water	Dilution factor (F)
< 0.07	No dilution required	1
0.07-0.7	1 + 9	10
0.7-7.0	1 + 99	100

If the value of $\Delta A_{ammonia}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification:

Carrez reagents cannot be used for deproteinisation as their use results in significantly reduced recoveries. Perchloric or trichloroacetic acid are used as alternatives (see specific examples).

BioVision

rev. 2/13

For research use only

Phosphate Colorimetric Assay Kit

(Catalog #K410-500; 500 assays; Store Kit at Room Temp.)

I. Introduction:

Phosphate is one of the most important of the inorganic ions in biological systems. It functions in a variety of roles. One of the most important roles is as a molecular switch, turning enzyme activity on and off through the mediation of the various protein kinases and phosphatases in biological systems. Phosphate is also of great importance in mineralization processes and is a primary stimulus of algal blooms frequently found in bodies of fresh water, due to run-off from areas of high fertilizer use. The newly designed Phosphate Colorimetric Assay Kit provides an easy, quick and sensitive means of assessing phosphate over a wide range of concentrations. The assay utilizes a proprietary formulation of malachite green and ammonium molybdate which forms a chromogenic complex with phosphate ion giving an intense absorption band around 650 nm. Phosphate concentrations between 1 µM and 1 mM, with a lower limit of detection of approximately 0.1 nmol, can be directly determined. The Phosphate Colorimetric Assay Kit provides 500 assays using microtiter plates or 100 assays using 1 ml cuvettes.

II. Kit Contents:

Components	K410-500	Cap Code	Part Number
Phosphate Reagent	15 ml	WM	K410-500-1
Phosphate Standard (10 mM)	0.5 ml	Yellow	K410-500-2

III. Reconstitution of Reagents:

Phosphate Reagent: Ready to use as supplied and may be kept at room temperature. There may be a small amount of precipitate visible which doesn't affect the assay.

IV. Assay Protocol:

1. **Phosphate Standard Curve:** Dilute 10 µl of the 10 mM Phosphate Standard to 990 µl dH_2O, mix well to generate 100 µM working Phosphate Standard. Add 0, 10, 20, 30, 40, 50 µl of the 100 µM working Phosphate Standard to individual wells. Adjust the volume to 200 µl with dH_2O to generate 0, 1, 2, 3, 4, 5 nmol of Phosphate standard.
2. **Preparation of sample:** No sample pretreatment is necessary. Add between 0-200 µl of sample for the assays and bring the well volume to 200 µl with distilled water. If the approximate phosphate concentration is not known, we recommend widely different sample volumes (1, 10, 100 µl) be tested.

   The absorbance of samples should be in the linear range of the standard curve (0-5 nmol/well). If they fall outside of this range, samples should be diluted and rerun or smaller sample volumes be used. The detection limit of the assay is approximately 0.1 nmol per well (1 µM) of Phosphate.

3. **Reaction:**
   1) Add 30 µl Phosphate Reagent to all standard and sample wells, mix well.
   2) Cover the plate and incubate at room temperature for 30 min.
   3) Read the absorbance at 650 nm using a plate reader. The color is stable for several hrs.
4. **Protocol for using 1.0 ml cuvettes:** Increase all reaction components 5X when using 1 ml cuvettes. The 1 ml total reaction mixture will contain 0-25 nmol phosphate (0-500 µl), 150 µl of Phosphate Reagent and made up to 1.0 ml with distilled water. Incubate at room temperature for 30 min then read at 650 nm.

5. Calculations:

1) Plot standard curve: Plot absorbance at 650 nm as a function of Phosphate concentration.

2) Determine sample Phosphate concentration:

$$\text{Phosphate concentration} = \frac{\text{(sample absorbance-blank absorbance)}}{\text{(slope of standard curve) x (µl of sample)}}$$

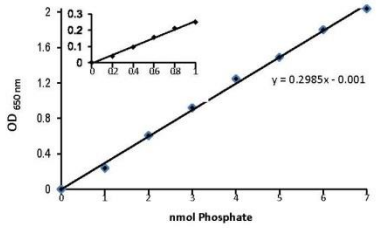
OR

$$Sa/Sv = \text{nmol/µl or mM Phosphate}$$

Where Sa is the sample amount (in nmoles) read from the standard curve.

Sv is the sample volume (undiluted) added to the wells.

Caution: Many laboratory detergents contain high amounts of phosphates which can adhere to cleaned glassware. It is highly recommended to use disposable plastic labware for all samples, standards and reagents to avoid contamination.



Phosphate Standard Curve: Assay is performed following the kit protocol.

RELATED PRODUCTS:

Apoptosis Detection Kits & Reagents	Cell Fractionation Kits
Glucose and Sucrose Assay Kits	Cholesterol, LDL/HDL Assay Kit
Glutathione Assay Kits	Ethanol and Uric Acid Assay Kits
NAD/NADH and NADP/NADPH Assay Kits	Lactate Assay Kits
Pyruvate Assay Kits	Total Antioxidant Assay Kit
Triglyceride, Fatty Acid Assay Kits	cAMP/cGMP Kits
Inorganic ions (Na, K, Ca, Cu, Fe, Mg, Mn)	Phosphatase/Kinase Assays

FOR RESEARCH USE ONLY! Not to be used on humans.

BioVision Incorporated
155 S. Milpitas Boulevard, Milpitas, CA 95035 USA

Tel: 408-493-1800 | Fax: 408-493-1801
www.biovision.com | tech@biovision.com

Preparing the Gel-Dye Mix

WARNING

Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

⇒Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples.

⇒Handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

1 Allow all reagents to equilibrate to room temperature for 30 minutes before use. Protect the dye concentrate from light while bringing it to room temperature.
2 Vortex RNA 6000 Nano dye concentrate (blue ●) for 10 seconds and spin down.
3 Add 1 µl of RNA 6000 Nano dye concentrate (blue ●) to a 65 µl aliquot of filtered gel (prepared as described in "Preparing the Gel" on page 14).
4 Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye. Store the dye concentrate at 4 °C in the dark again.
5 Spin tube for 10 minutes at room temperature at 13000 g (for Eppendorf microcentrifuge, this corresponds to 14000 rpm). Use prepared gel-dye mix within one day.

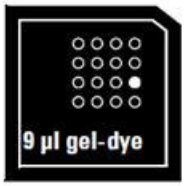
NOTE

A larger volume of gel-dye mix can be prepared in multiples of the 65+1 ratio, if more than one chip will be used within one day. Always re-spin the gel-dye mix at 13000 g for 10 minutes before each use.

Loading the Gel-Dye Mix

NOTE Before loading the gel-dye mix, make sure that the base plate of the chip priming station is in position (C) and the adjustable clip is set to the top position. Refer to "Setting up the Chip Priming Station" on page 7 for details.

1 Allow the gel-dye mix to equilibrate to room temperature for 30 minutes before use and protect the gel-dye mix from light during this time.
2 Take a new RNA Nano chip out of its sealed bag .
3 Place the chip on the chip priming station.
4 Pipette 9.0 µl of the gel-dye mix at the bottom of the well marked G and dispense the gel-dye mix.



NOTE When pipetting the gel-dye mix, make sure not to draw up particles that may sit at the bottom of the gel-dye mix vial. Insert the tip of the pipette to the bottom of the chip well when dispensing. This prevents a large air bubble forming under the gel-dye mix. Placing the pipette at the edge of the well may lead to poor results.

5 Set the timer to 30 seconds, make sure that the plunger is positioned at 1 ml and then close the chip priming station. The lock of the latch will click when the Priming Station is closed correctly.

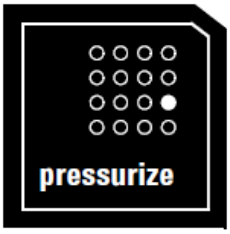
Appendix: Attachment 8, Page 3.

6 Press the plunger of the syringe down until it is held by the clip.

7 Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.

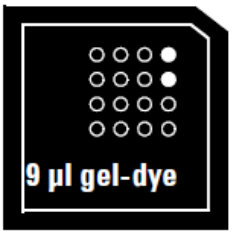
8 Visually inspect that the plunger moves back at least to the 0.3 ml mark.

9 Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.



10 Open the chip priming station.

11 Pipette 9.0 µl of the gel-dye mix in each of the wells marked.



NOTE Please discard the remaining vial with gel-dye mix.

Loading the RNA 6000 Nano Marker

1 Pipette 5 µl of the RNA 6000 Nano marker (green ●) into the well marked with the ladder symbol and each of the 12 sample wells.

NOTE Do not leave any wells empty or the chip will not run properly. Unused wells must be filled with 5 µl of the RNA 6000 Nano marker (green ●) plus 1 µl of the buffer in which the samples are diluted.

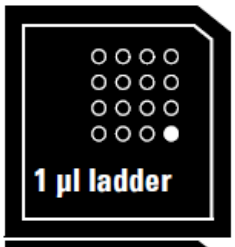
Loading the Ladder and Samples

NOTE Always use RNase-free microfuge tubes, pipette tips and water.

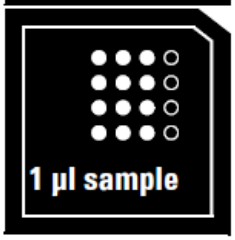
1 Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process)

2 To minimize secondary structure, heat denature (70 °C, 2 minutes) the samples before loading on the chip.

3 Pipette 1 µl of the RNA ladder into the well marked with the ladder symbol .



4 Pipette 1 µl of each sample into each of the 12 sample wells.



CAUTION *Wrong vortexing speed*

If the vortexing speed is too high, liquid spill that disturbs the analysis may occur .

⇒Reduce vortexing speed to 2000 rpm!

5 Set the timer to 60 seconds.

6 Place the chip horizontally in the adapter of the IKA vortex mixer and make sure not to damage the buldge that fixes the chip during vortexing.

If there is liquid spill at the top of the chip, carefully remove it with a tissue.

7 Vortex for 60 seconds at 2400 rpm.

8 Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

NOTE Depending on the RNA isolation protocol, varying results can be expected. Known dependencies include: salt content, cell fixation method and tissue stain. Best results are achieved for samples which are dissolved in deionized and RNase-free water. Avoid genomic DNA contamination by including DNase treatment in the preparation protocol.

Inserting a Chip in the Agilent 2100 Bioanalyzer

1 Open the lid of the Agilent 2100 bioanalyzer.

2 Check that the electrode cartridge is inserted properly and the chip selector is in position (1). Refer to "Setting up the Bioanalyzer" on page 8 for details.

3 Place the chip carefully into the receptacle. The chip fits only one way.

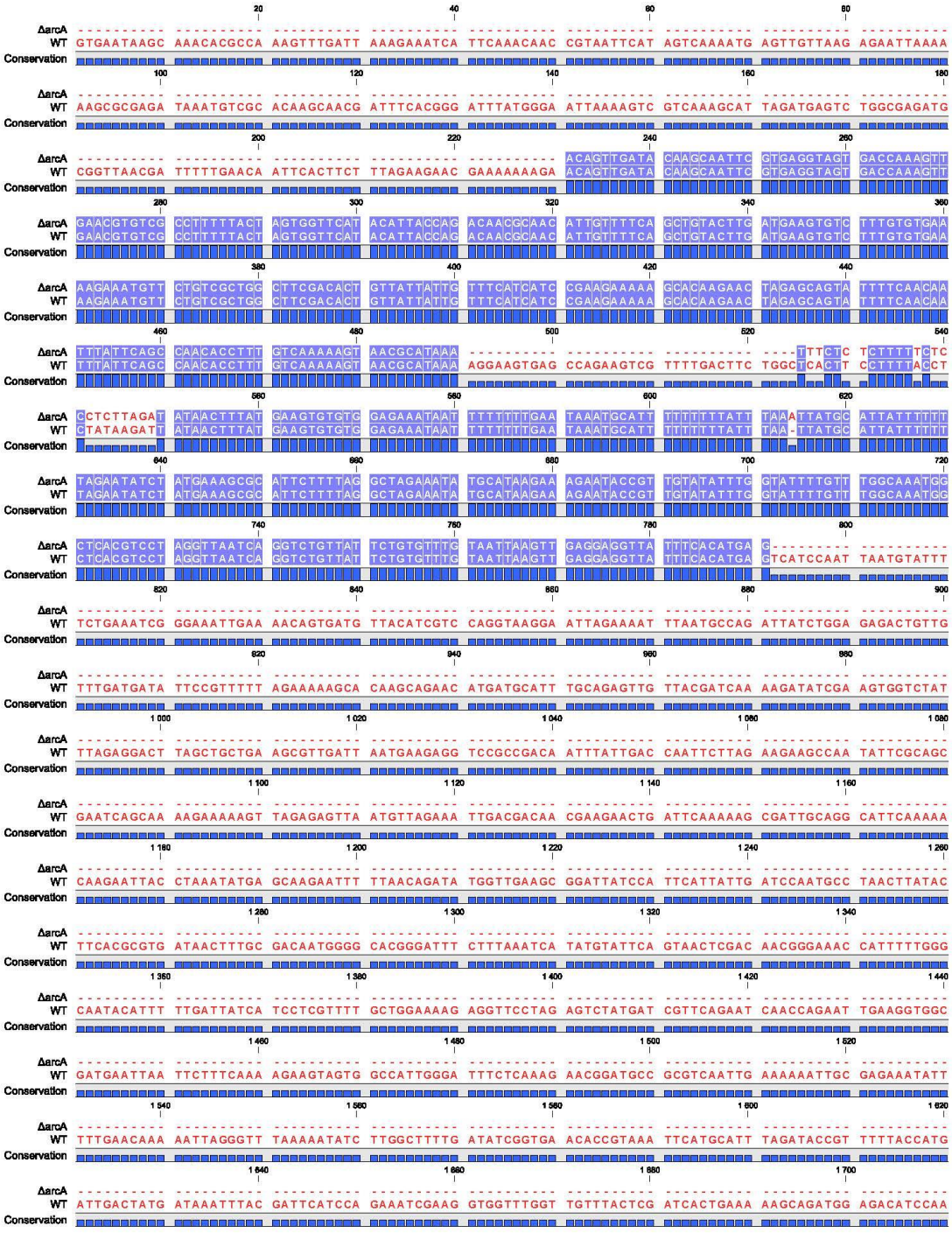
CAUTION *Sensitive electrodes and liquid spills*

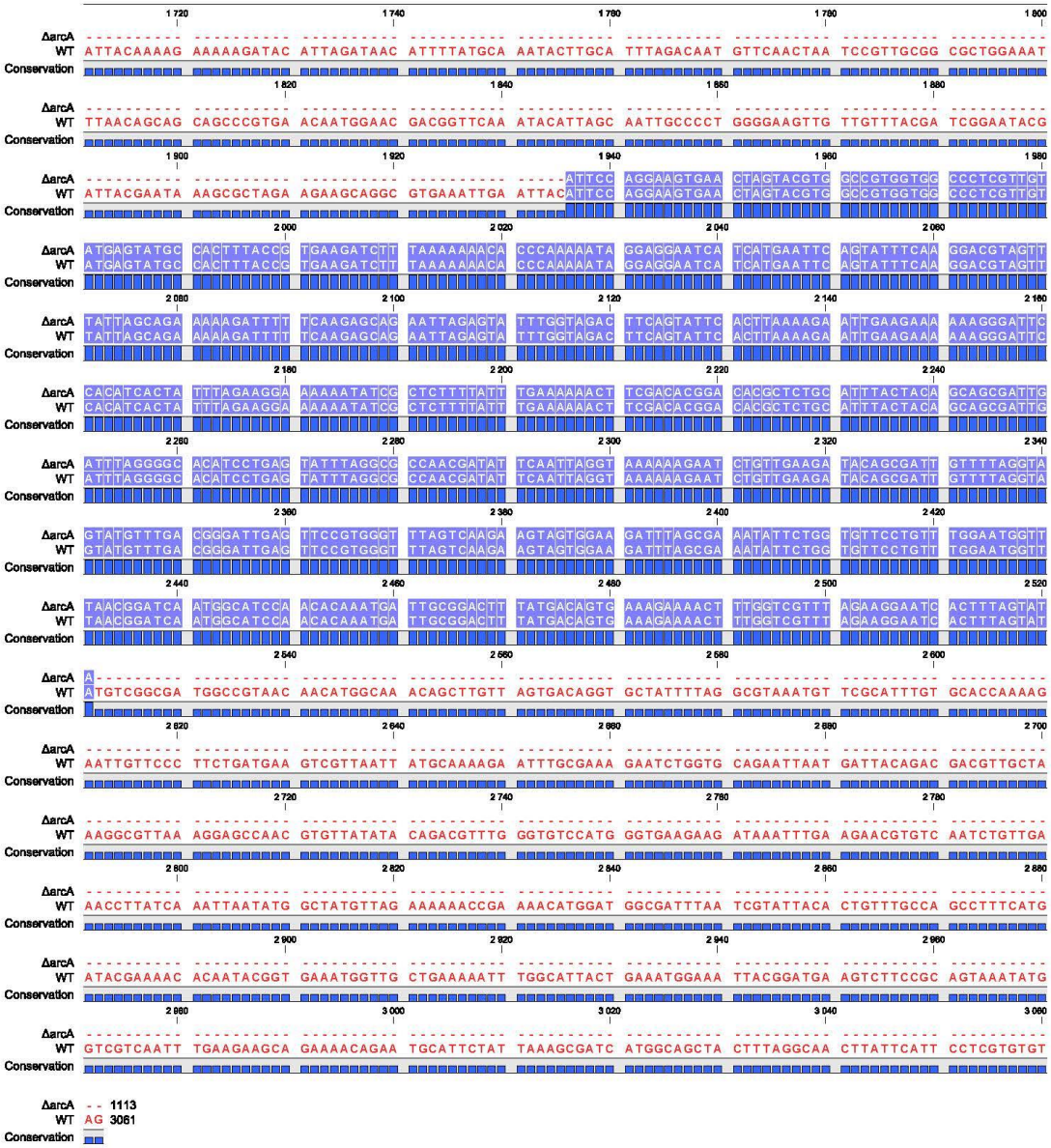
Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results.

⇒Do not use force to close the lid and do not drop the lid onto the inserted chip.

4 Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.

5 The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the *Instrument* context.


ΔarcA
WT
Conservation


ΔarcA
WT
Conservation
1113
3061

			volume of stock	1	*liter*								
conc	dim	stock	effective chemical	stock gram	stock g/L	mg/L	g per 20 L	final mM	chemical # UR	MW	formula / syn	note	chemical # Ams
100	x	acgu	adenine	3,85	3,85	38,5	0,77	**0,285**	A9126	135	adenine		Sigma A-2786
100	x	acgu	cystine	5	5	50	1	**0,2**	fluka 30200	240			
100	x	acgu	guanine	2,75	2,75	27,5	0,55	**0,15**	Aldrich 51030	188	Guanine HCl		Sigma G-6263
100	x	acgu	uracil	2,2	2,2	22	0,44	**0,2**	U0750	112			Sigma U-1128
20	x	aa19	alanine	4,8	4,8	240	4,8	**2,7**	Fluka 05129; A7627	89			Sigma A-7627
20	x	aa19	arginine	10	10	500	10,0	**2,9**	roth 1655.1; Sig 11039	174	arg H2O		Sigma A-5006
20	x	aa19	asparagine	2	2	100	2,0	**0,76**	fl 11150; Sigma 11149	132			
20	x	aa19	aspartate	8,4	8,4	420	8,4	**3,2**	A8949	133			Sigma A-9256
20	x	aa19	glutamate	10	10	500	10,0	**3,4**	Fluka 49601	147			Sigma G-1251
20	x	aa19	glutamine	4	4	200	4,0	**1,4**	Fluka 49419	146			
20	x	aa19	glycine	3,5	3,5	175	3,5	**2,3**	Roth 3908.2	75			Sigma G-7126
20	x	aa19	histidine	3	3	150	3,0	**1,0**	H8000	155			Sigma H-8000
20	x	aa19	isoleucine	4,2	4,2	210	4,2	**1,6**	I2752	131			Sigma I-2752
20	x	aa19	leucine	9,5	9,5	475	9,5	**3,6**	Sigma 61819	131			Sigma L-8000
20	x	aa19	lysine	8,8	8,8	440	8,8	**2,7**	Merck 1.12233	164	lysine H2O		Sigma L-5626
20	x	aa19	methionine	2,5	2,5	125	2,5	**0,8**	M9625	149			Sigma M-9625
20	x	aa19	phenylalanine	5,5	5,5	275	5,5	**1,7**	Fluka 78020	165			Sigma P-2126
20	x	aa19	proline	13,5	13,5	675	13,5	**5,9**	P0380	115			Sigma P-0380
20	x	aa19	serine	6,8	6,8	340	6,8	**3,2**	S4500	105			Sigma S-4500
20	x	aa19	threonine	4,5	4,5	225	4,5	**1,9**	T8625	119			Sigma T-8625
20	x	aa19	tryptophane	1	1	50	1,0	**0,25**	T0254	204			Sigma T-0254
20	x	aa19	tyrosine	5	5	250	5	**1,4**	Fluka 93830	181			Sigma T-3754
20	x	aa19	valine	6,5	6,5	325	6,5	**2,8**	V0500	117			Sigma V-0500
100	x	metal	Mo	0,25	0,25	2,5	0,05	**0,002**	Merck 1.01182	1236	(NH4)6Mo7O24		BDH 10028
100	x	metal	Ca	5	5	50	1	**0,340**	Merck 1.02382.1, (1.02379)	147	CaCl2 × 2 H2O		Merck 102382
100	x	metal	Co	0,25	0,25	2,5	0,05	**0,009**	Merck 1.02556	281	CoSO4 × 7 H2O		Merck 2546
100	x	metal	Cu	0,25	0,25	2,5	0,05	**0,010**	Merck 1.02390.0250	250	CuSO4 × 5 H2O		BDH 10091
100	x	metal	Fe2+	0,5	0,5	5	0,1	**0,025**	Merck 1.03861.0250	199	FeCl2 × 4 H2O		Sigma F-2130
100	x	metal	Fe3+	0,3	0,3	3	0,06	**0,011**	Merck 1.03943.0250	270	FeCl3 × 6 H2O		Sigma F-2877
100	x	metal	Mg	20	20	200	4	**0,984**	Serva 28305/500 (Fl 63063)	203	MgCl2 × 6 H2O		BDH 10149
100	x	metal	Mn	1,6	1,6	16	0,32	**0,081**	Merck 5927.0100	198	MnCl2 × 4 H2O		BDH 10152
100	x	metal	Zn	0,5	0,5	5	0,1	**0,017**	Merck 1.08883.0100	288	ZnSO4 × 7 H2O		BDH 10299
40	x	PCA buffer	NH3 citrate	24	24	600	12	**2,5**	Sigma A-1332	243	NH3 citrate tribasic		Sigma A-1332
40	x	PCA buffer	K2HPO4	40	40	1000	20	**5,7**	Roth P749	174			Merck 1.05104
40	x	PCA buffer	KH2PO4	200	200	5000	100	**36,7**	Merck 1.04873	136			Merck 1.04873
40	x	PCA buffer	Na-acetate	40	40	1000	20	**12,2**	Merck 1.06268	82			Merck 1.06268
100	x	vitamin	pantothenate, B5	0,1	0,1	1	0,02	**0,004**	fluka 21210	238	pantothenate hemi-Ca		Sigma P-6045
100	x	vitamin	biotin	0,25	0,25	2,5	0,05	**0,010**	Sigma B-4501	244			Sigma B-4501
100	x	vitamin	inosine	0,5	0,5	5	0,1	**0,019**	Sigma I-4125	268			Sigma I-4125
100	x	vitamin	lipoic acid	0,25	0,25	2,5	0,05	**0,012**	Sigma T-5625	206	plus alpha lipoic, thioctic		Sigma T-5625
100	x	vitamin	nicotinic acid	0,1	0,1	1	0,02	**0,008**	Sigma N-0765	123	B3, niacin		Sigma N-0765
100	x	vitamin	orotic acid	0,5	0,5	5	0,1	**0,032**	sigma 01756	156			Sigma O-2625
100	x	vitamin	aminobenzoic	1	1	10	0,2	**0,073**	fluka 06930	137	4-Aminobenzoic acid, PABA		Sigma A-0129
100	x	vitamin	pyridoxamine	0,5	0,5	5	0,1	**0,021**	Sigma P-9380	241	pyridoxamine 2HCl	min 20C	Sigma P-9380
100	x	vitamin	pyridoxine	0,2	0,2	2	0,04	**0,010**	Sigma P-9755	206	pyridoxine HCl, B6		Sigma P-9755
100	x	vitamin	riboflavin	0,1	0,1	1	0,02	**0,0027**	Sigma R-4500	376	B2, (–)-Riboflavin		Sigma R-4500
100	x	vitamin	thiamine	0,1	0,1	1	0,02	**0,003**	fluka 95160	337	thiamine HCl		Sigma T-4625
100	x	vitamin	thymidine	0,5	0,5	5	0,1	**0,021**	sigma T1895	242			Sigma T-5018
100	x	vitamin	B12	0,1	0,1	1	0,02	**0,00074**	Sigma V-2876	1355	Cyanocobalamin		Sigma V-2876
40	g/l	xanthine	xanthine	40	40	10	0,2	**0,066**	Sigma X-7375	152			
1			cysteine			130	2,6	**0,74**	C7352; Fl 30200; Fl 30129	176			
			ascorbate			500	10	**2,8**	A5960	176			
			glucose			11000	220	**61**		180			
			Na bicarbonate			2500		**30**	Merck 1.06329	84	Na H carbonate		