Production of the Flavor Compound Benzaldehyde by Lactic Acid Bacteria: Role of Manganese and its Transport Systems in *Lactobacillus plantarum*

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Proefschrift

ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, prof. dr. ir. L. Speelman, in het openbaar te verdedigen op woensdag 19 december 2001 des ochtends om elf uur in de Aula.

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Stellingen

1. Het is onwaarschijnlijk dat de lage intracellulaire mangaan concentratie in de door Hao *et al.* (1999) geconstrueerde *Lactobacillus plantarum* mutant het gevolg is van uitschakeling van het *mntA* gen alleen.

   Hao, Z., H.R. Reiske and D.B. Wilson. 1999. Characterization of cadmium uptake in *Lactobacillus plantarum* and isolation of cadmium and manganese uptake mutants. Applied and Environmental Microbiology 65: 4741-4745.

   Dit proefschrift

2. De bijdrage van chemische stappen in bioconversies van aminozuren naar smaak componenten is in menig onderzoek wellicht ten onrechte niet bestudeerd.

   Dit proefschrift

3. Om verwarring te voorkomen, zou bij de naamgeving van genen door onderzoekers meer rekening gehouden moeten worden met reeds geintroduceerde naamgeving voor orthologen.

4. Het onschuldige imago van kruiden voor culinaire en therapeutische doeleinden is bedrieglijk bij excessief gebruik of gebrek aan taxonomische kennis.

   Craig, J. 1999. Health-promoting properties of common herbs. Am. J. Clin. Nutr. 70: 491-499.

5. De poging van Schrezenmeir en De Vrese (2001) om een definitie voor probiotica te formuleren, is gedoemd te mislukken als deze wetenschappers lactobacillen niet tot de bacteriën rekenen.

   Schrezenmeir, J., and M. de Vrese. 2001. Probiotics, prebiotics, and synbiotics - approaching a definition. Am. J. Clin. Nutr. 73 (suppl.): 361S-364S.

6. De door menigeen veronderstelde toename in aantal gevallen van hersenvliesontsteking zit tussen de oren.

   Onrust groeit na nieuw geval van meningokok. De Volkskrant, 22 augustus 2001.

7. Wie bij vloerbelasting aan de fiscus denkt, kon nog wel eens door de grond zakken.

Stellingen behorend bij het proefschrift "Production of the flavor compound benzaldehyde by lactic acid bacteria: manganese and its transport systems in *Lactobacillus plantarum*".

Masja Nierop Groot
19 december 2001

Contents

Chapter 1.

General Introduction and Outline

General Introduction

Benzaldehyde

Benzaldehyde is an aromatic flavor compound that is responsible for the taste and odor that is typical for bitter almonds. In quantity, benzaldehyde follows vanillin in application in the flavor and fragrance industries (Welsh *et al.*, 1989). The difference in price of a natural compound and its chemically synthesized counterpart can be considerable, for example synthetic benzaldehyde is selling at U.S. $ 2-2.6 kg^{-1} whereas the natural bitter oil of almonds costs U.S. $ 290-400 kg^{-1} (Clark, 1995). The world usage of benzaldehyde in 1994 was approximately 13,000 Mtons of which 100 Mtons was of natural origin (Clark, 1995). The major synthetic route to produce benzaldehyde presently proceeds via the direct oxidation of toluene using cobalt as catalyst. This process was initially designed to produce benzoic acid but the byproduct benzaldehyde was found to be worth more than the main product (Clark, 1995).

The difference in price of natural versus chemically synthesized benzaldehyde, combined with the increasing consumers demand for so called green flavors, which have a healthy image, has directed the interest of flavor and fragrance industries to flavors that can be labeled natural. Natural benzaldehyde is present as a cyanogenic glycoside (called amygdalin) in the pit meat of fruits from the Prunus family (including cherries, plums, abricots and nectarines) and can be liberated from these sources by extraction. However, hydrocyanic acid is an undesirable, toxic byproduct of the extraction of benzaldehyde from these sources. An alternative way to produce natural benzaldehyde without the risk of formation of this toxic byproduct, can be the exploitation of micro-organisms as tool for the production of benzaldehyde.

Microbial production of benzaldehyde

The amino acid phenylalanine can be used as precursor for conversion to aromatic flavor compounds such as benzaldehyde, phenylethanol and phenylacetate. This process is aided by a plentiful cheap supply of natural phenylalanine, which has become available as an intermediate of the synthesis of the high-intensity sweetener aspartame (Krings and Berger, 1998). Several pathways have been proposed for the conversion of phenylalanine to various aromatic flavor compounds (Figure 1). Phenylalanine is initially degraded to either cinnamic acid or phenylpyruvic acid. The pathway leading to cinnamic acid is catalyzed by a

phenylalanine-ammonia-lyase (PAL) while the phenylpyruvic acid can be produced by the action of an aminotransferase, dehydrogenase (Oshima *et al.*, 1991; Hummel *et al.*, 1984) or phenylalanine oxidase (Fernández-Lafuente *et al.*, 1998; Braun *et al.*, 1992; Koyama, 1982). In general, the PAL-catalyzed degradation of phenylalanine is predominantly found in fungi (Jensen *et al.*, 1994; Kawabe and Morita, 1994; Lapadatescu *et al.*, 2000) whereas bacterial conversion is initiated by the activity of an aminotransferase (Yvon *et al.*, 1997; Casey and Dobb, 1992).

Benzaldehyde production in culture medium supplemented with phenylalanine has been demonstrated for the white-rot fungi *Ischnoderma benzoinum* (see route 1 in Figure 1; Fabre *et al.*, 1996; Krings *et al.*, 1996), *Polyporus tuberaster* (Kawabe and Morita, 1994), *Phanerochaete chrysosporium* (see route 2 in Figure 1; Jensen *et al.*, 1994), *Bjerkandera adusta* (see route 3 in Figure 1; Lapadatescu *et al.*, 1999), *Dichomitus squalens* (Gallois *et al.*, 1990). Only a few reports on bacterial production of this flavour compound are available. A *Pseudomonas putida* strain (ATCC 55012) was isolated from a soil sample in enrichment experiments for growth on phenylalanine and mandelate. This strain was reported to catabolize phenylalanine through phenylpyruvic acid, phenylacetaldehyde and phenylacetate. Phenylacetate is subsequently converted to mandelate, phenylglyoxylate and finally benzaldehyde. The exploitation of this pathway for the production of benzaldehyde has been patented (see route 6 in Figure 1; Geusz and Anderson, 1992). The genes encoding the mandelate dehydrogenase and benzoylformate decarboxylase, the enzymes involved in the conversion of mandelate to benzaldehyde in *Pseudomonas putida* (ATCC 12633), have been cloned (see route 5 in Figure 1; Tsou *et al.*, 1989,1990). A combined method, using microbial conversion of phenylalanine to phenylpyruvic acid followed by an oxidation step to the aldehyde using a mild base treatment, has been reported to result in high yields of benzaldehyde (Casey and Dobb, 1992). A major drawback is that the pathogenic strain *Proteus vulgaris* was used for the formation of phenylpyruvic acid.

Phenylalanine degradation by lactic acid bacteria

Enzymatic degradation of amino acids in cheese and in other fermented foods is believed to generate compounds important for the development of flavor. Degradation products of phenylalanine, including benzaldehyde, have been found in several different types of cheese (Bosset and Gauch,

1993; Molimard and Spinnler, 1996) and may have resulted from the pathway presented in Figure 2.

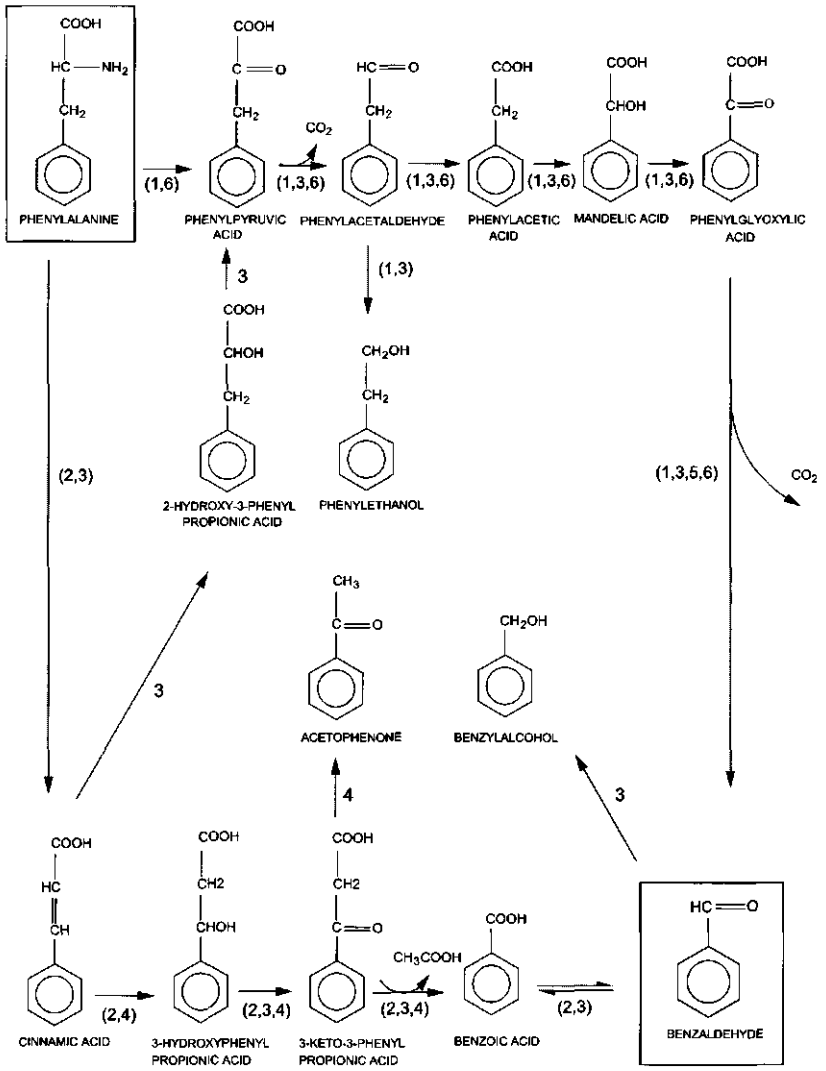

Figure 1. Overview of the pathways described in literature for the degradation of phenylalanine to various aromatic flavor compounds. The numbers refer to the following references: 1. *Ischnoderma benzoinum* (Krings *et al.*, 1996); 2. *Phanerochaete chrysosporium* (Jensen *et al.*, 1994); 3. *Bjerkandera adusta* (Lapadatescu *et al.*, 2000); 4. *Pseudomas sp.* (Hilton and Cain, 1990); 5. *Pseudomonas putida* ATCC 12633 (Tsou *et al.*, 1990). 6. *Pseudomonas putida* ATCC 55012 (Geusz and Anderson, 1992).

In addition, benzaldehyde was among the volatile metabolites formed by lactic acid bacteria (LAB) of the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Tracey and Britz, 1989). Over the last years, the pathways

for amino acid degradation by LAB involved in the production of cheese have received increasing attention as illustrated by a variety of studies on conversion of methionine (Alting *et al.*, 1995; Bruinenberg *et al.*, 1997; Fernández *et al.*, 2000), aromatic amino acids (Yvon *et al.*, 1997; Rijnen *et al.*, 1999; Gummalla and Broadbent, 2001) and branched chain amino acids (Yvon *et al.*, 1998). In *Lactococcus lactis*, a major species in cheese starter cultures, degradation of phenylalanine is strictly dependend on the aromatic aminotransferase (AraT)(Rijnen *et al.*, 1999). Besides phenylpyruvic acid, other degradation products of phenylalanine such as phenylacetate and phenyllactate (see Figure 2) are resulting from the conversion of this amino acid (Yvon *et al.*, 1997).

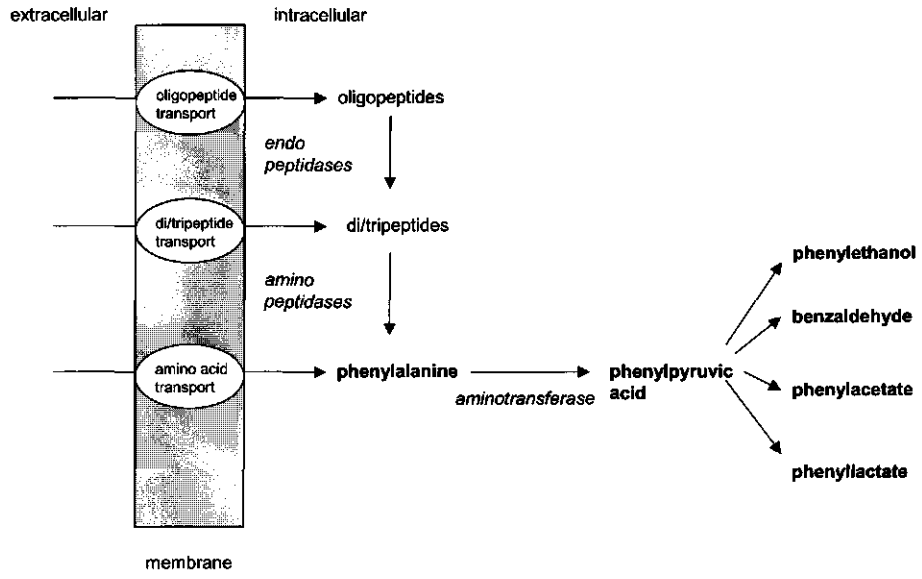

Figure 2. Possible pathway for the formation of phenylalanine-derived flavor compounds by LAB when grown on milk; the extracellular oligopeptides, di-and tripeptides and amino acids are either present in milk or formed from casein by proteolytic enzymes.

Outline of this thesis

The aim of the research described in this thesis was two-fold. Initially, the bioconversion of phenylalanine to the flavor compound benzaldehyde was investigated in *Lactobacillus plantarum* and in several other LAB. The finding that manganese ions were involved in benzaldehyde formation in *L. plantarum* led to the second aspect of this thesis: i.e. the investigation of manganese transport systems in this bacterium and in other LAB.

In **Chapter 2,** an overview is given of manganese uptake systems in bacteria. The current knowledge on the genes encoding these transport systems and their regulatory mechanisms are discussed. In **Chapter 3**, the conversion of phenylalanine to the flavor compound benzaldehyde in *L. plantarum* is reported. The results described in this chapter demonstrate that the conversion involves both an enzyme-catalyzed step and a chemical oxidation step. Manganese ions present at high levels in *L. plantarum*, and several other LAB belonging to the genera *Lactobacillus* and *Leuconostoc*, catalyze the conversion of phenylpyruvic acid to benzaldehyde. These results are described in **Chapter 4. Chapter 5**, describes the study on manganese transport systems present in *L. plantarum*. Three transport systems, that are induced upon manganese starvation, were identified and the transcription of their genes was characterized and compared to that in strains carrying mutations in one or two of these genes. In **Chapter 6**, the knockout of a fourth manganese transport system is described and the phenotype of the mutant strain is discussed. The main findings of this thesis on the role of manganese uptake for *L. plantarum* in general, and on the formation of benzaldehyde, are discussed in conjunction with the opportunities for further research in **Chapter 7**.

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Chapter 2.

Bacterial Uptake Systems for Manganese

Masja Nierop Groot,
Michiel Kleerebezem and Willem M. de Vos

Introduction

Bacteria rely on various metal ions as trace elements in biological functions. These elements can be classified as micronutrients and include iron, copper, manganese, zinc, nickel, magnesium, and cobalt. Some of these metal ions are transition metals with incompletely filled d-orbitals, that are incorporated in various redox enzymes. Among the trace elements, iron is the most abundant element in micro-organisms and is accumulated in most bacteria in concentrations of 10 to 50-fold above the level of other trace metals (Archibald, 1986). However, exceptions can be found among most lactic acid bacteria (LAB) belonging to the genera *Lactobacillus* and *Leuconostoc*. These require high amounts of intracellular manganese (millimolar levels intracellular) and show limited requirements for iron (Archibald and Fridovich, 1981; Archibald, 1983). For these LAB, the culture medium contains relatively high levels of manganese (several hundred micromolar) for optimum growth, compared to other micro-organisms. Interestingly, the natural habitat of these species is decomposing plant material, that contains high amounts of manganese and little available iron. The high intracellular manganese level at the expense of iron is a likely adaption to the natural environment but the apparent independence of iron may also have an advantage over other micro-organisms in low iron environments. An intriguing aspect is how the large amount of manganese enters the cell. Recent findings have thrown more light on the bacterial transport systems involved in manganese uptake and both on the genes encoding these systems and the regulatory proteins involved in their expression (see also a recent review by Jakubovics and Jenkinson, 2001). In this Chapter, an overview will be given about the bacterial transport systems for manganese that are identified untill now and recent advances that have been made in understanding the regulatory systems that are involved. Finally, the role of manganese on host-bacteria interaction and the perspectives of this metal ion and its transport systems are discussed.

Physiological functions of manganese

In biological systems, manganese can exist in the oxidation states Mn^{2+}, Mn^{3+}, and Mn^{4+}. However, the Mn^{2+} form is the most abundant one. Mn^{2+}

cannot donate and accept electrons in many of the reactions that involve only small free energy changes, these are generally catalyzed by iron (Archibald, 1986). The conversion of ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms of iron has a standard reduction potential of – 0.771 V and occurs already under mild conditions (Chang, 1981). However, the Mn^{2+}/Mn^{3+} standard redox potential is –1.51 V and Mn^{2+} is therefore only oxidized by highly energetic compounds such as superoxide (O_2^-). The toxicity of Mn^{2+} to cells is relatively low. For example, for *Escherichia coli* the minimal inhibitory concentration (MIC) for Mn^{2+} is 20 mM compared to ca. 1 mM for several other transition metal ions like Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (Nies, 1999).

The physiological functions of Mn^{2+} can be classified in Mn-dependend enzyme activities and in biological activities that depend on Mn^{2+}. The reported enzymes that require Mn^{2+} are numerous and are represented in most of the enzyme categories, including oxidoreductases, transferases, hydrolases, lyases, ligases and isomerases (see Archibald, 1986 for an overview). Only a few examples of a role for Mn in cell functioning will be given below.

Several enzymes involved in sugar catabolism are known to require Mn^{2+} as a cofactor. In lactic acid bacteria (LAB), pyruvate is converted to lactate by the action of the Mn-cofactored lactate dehydrogenase (LDH). The presence of Mn in this enzyme was found to control the affinity of LDH for fructose 1,6-diphosphate (FDP) in various strains of the genera *Lactobacillus* (Holland and Pritchard, 1975; Hensel *et al.*, 1977). Lactate is also resulting from the conversion of malic acid during the malolactic fermentation performed by several LAB (Lonvaud-Funel, 1999). The malolactic enzyme responsible for this conversion depends on Mn^{2+} as a cofactor. In addition, association with Mn^{2+} has been demonstrated for the glycolytic enzyme phosphoglycerate mutase in several Gram-positive endospore-forming bacteria (Chander *et al.*, 1998) and in *E. coli* (Fraser *et al.*, 1999). Mn^{2+} can replace Mg^{2+} in a variety of both structural and catalytic roles. For example, in enolase, a glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate resulting in phosphoenolpyruvate, the natural cofactor Mg^{2+}, can be substituted by Mn^{2+} (Zhang *et al.*, 1994; Vinarov and Nowak, 1999). Biosynthesis of polysaccharides in various organisms probably is dependend on Mn^{2+} since a conserved motif can be found among different families of glycosyl transferases (Wiggins and Munro, 1998; Busch *et al.*, 1998) that has affinity for Mn^{2+} (Zhang *et al.*, 2001).

In oxygenic photosynthetic organisms, such as cyanobacteria but also in algae and green plants, Mn^{2+} is required in redox reactions in the photosystem II by mediating electron transfer from water (Debus, 1992). This light-induced oxidation of water results in the evolution of oxygen. In addition, Mn^{2+} is a highly important element in the protection against damage induced by toxic oxygen derivatives such as O_2^-, H_2O_2 and $^{\cdot}OH$. In the cytoplasm of cells of bacteria, plants and animals, the enzymes *superoxide dismutase (SOD; Fridovich, 1995) and mangani-catalase* (Whittaker *et al.*, 1999) require Mn to protect DNA and protein from oxidation by reactive oxygen species. Macrophages generate reactive oxygen intermediates to attack virulent bacteria within the phagosome during infection (Tsolis *et al.*, 1995). In this process, Mn-SOD affects virulence of bacteria by serving as defense mechanism. The SOD enzyme acts as a dimer and contains generally one atom of Mn per subunit, although Fe, Cu/Zn and Ni-cofactored forms of SOD exist (Fridovich, 1995; Kim *et al.*, 1998, Benov *et al.*, 1994). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2. The hydrogen peroxide formed in this reaction, is generally degraded to water by catalase or peroxidase activity. The Mn-isoform of SOD is widespread in prokaryotes and is present in almost all mitochondria and chloroplasts (Archibald, 1986). *Lactobacillus plantarum* and several other LAB of the genera *Lactobacillus* and *Leuconostoc* are unique because these bacteria lack SOD but still are relatively oxygen tolerant (Archibald and Fridovich, 1981). Intracellular Mn^{2+} concentrations of over 30 mM can be present in *L. plantarum*. This amount of Mn^{2+} is quite extraordinary for micro-organisms and was found to have an important function in protection of the cell by scavenging of toxic oxygen species, such as the superoxide radical anion (O_2^-). The intracellularly accumulated Mn^{2+} thereby replaces the function of SOD which is present in most other oxygen-tolerant micro-organisms. The precise mechanism of the non-enzymic scavenging of O_2^- is not understood but this mechanism requires mM levels of Mn^{2+} compared to μM levels as cofactor in SOD.

Transport systems for manganese

There is a considerable amount of information on iron (see Braun *et al*, 1998 and references therein) and magnesium uptake systems (Smith and

Maguire, 1995; Hmiel *et al.*, 1989; Smith *et al.*, 1993b) in *Salmonella typhimurium* and *E. coli,* both at the biochemical and the genetic level. However, the information on Mn^{2+} uptake systems in micro-organisms has only expanded significantly over the past 5 years. Transport studies in *Salmonella typhimurium* (Snavely *et al.*, 1989; Smith *et al.*, 1998) and *E. coli* (Smith *et al.*, 1998) revealed that Mn^{2+} may enter the cell via the CorA system, which is the dominant transporter for magnesium in these species.

Table 1: Overview of genes encoding ABC-type and Nramp transport systems for Mn^{2+} based on genome blast searches or by experimental data. Genes potentially encoding homologs to CitM (*B.subtilis*), and PitA and B (*E. coli*) are shown

Species	ABC-type	Nramp affinity	PitA	CitM	SOD containing	Genome status
	high	high	low	low		
L. plantarum	***mtsCBA***	***mntH1, mntH2, mntH3***	NP	NP	no	complete
L. lactis	*mtsBCA*	*ykjB, ytjB*	NP	NP	yes	complete
B. subtilis	***mntABCD***	***mntH***	1 (*ykaB*)	***citM***	yes	complete
S. mutans	***sloABC***	1	NP	1	yes	unfinished
S. pneumoniae	***psaBCA***	NP	NP	NP	yes	complete
S. gordonii	***scaCBA***	1	NP	NP	yes	unfinished
S. pyogenes	*mtsABC*	NP	NP	NP	yes	complete
E. coli	NP	***mntH***	***pitA, pitB***	NP	yes	complete
S. epidermis	***sitABC***	1	1	NP	yes	unfinished
S. typhimurium	*sitBCDA*	***mntH***	1	NP	yes	unfinished
Y. pestis	***yfeBCDA***	1	1	NP	yes	unfinished

Blast searches were performed at the NCBI site:
(http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html)
Genes for which experimental data are available are indicated in bold: *mntABCD* and *mntH* (Que and Helmann, 2000), *sloABC* (Kitten et al., 2000), *psaBCA* (Dintilhac et al., 1998), *scaCBA* (Kolenbrander et al., 1998), *sitABC* (Cockayne et al., 1998), *yfeBCDA* (Bearden and Perry, 1999), *citM* (Krom et al., 2000, *pitA* and *pitB* (van Veen et al., 1994a,b), *mntH* in *E. coli* and *S. typhimurium* (Kehres et al., 2000).When available, gene names are given; if not, the number of homologs present are shown. NP indicates that no homolog is present in the genome sequence. However, several genome sequence projects are not completed.

However, recent findings show the presence of additional, but highly selective, Mn^{2+} transport systems in these species (Kehres *et al.*, 2000; Makui *et al.*, 2000). Moreover, a number of genes encoding high-affinity uptake systems for Mn^{2+} in both Gram-positive and -negative bacteria

have been identified. The gene products can be classified in three families of transport systems; the ATP-binding cassette (ABC) transporters, the P-type ATPases, and natural resistance-associated macrophage proteins (Nramp) (Table 1). Besides these systems, Mn^{2+} may enter the cell via a metal-citrate transporter such as CitM in *Bacillus subtilis* (Krom *et al.*, 2000) or via the phosphate-inorganic transport (Pit) systems, as has been shown in *E. coli* and *Acinetobacter johnsonii* (van Veen *et al.*, 1994a;1994b). Besides *B. subtilis*, only *Streptococcus mutans* appears to harbor a CitM system (Table 1). Expression of CitM in *B. subtilis* is induced by citrate and repressed by the presence of more easily metabolizable carbon sources (Warner *et al.*, 2000). This suggests that this transporter is only used in the absence of better growth substrates, and is not primarily involved in supplying the cell with metal ions. This system will therefore not be discussed here. Both in *E. coli* and *A. johnsonii,* the Pit-system mediates the proton motif force-dependent uptake of metal phosphate (Van Veen *et al.*, 1994a; 1994b). Transport assays demonstrated that P_i-uptake was dependent on the presence of divalent cations including Mn^{2+}. A Blast search for several available bacterial genome sequences reveals that homologs of the Pit system are present in several of the gram-positive and -negative bacteria of Table 1. However, in LAB and streptococcal species this system cannot contribute to Mn^{2+} uptake since these bacteria lack a Pit transporter (Table 1). Although it is likely that this system may have impact on the Mn^{2+} homeostasis in some bacteria, we will only focuss on the high-affinity Mn^{2+}-uptake systems.

ABC-type transport system.

The ATP-binding cassette (ABC) superfamily of transporters is capable of transporting a wide variety of substrates. However, each transporter is relatively specific for a given compound. Compounds that can be transported by these systems include: amino acids, sugars, inorganic ions, polysaccharides, peptides and even proteins (Higgins, 1992, 2001). Both uptake and export of substrates are covered by ABC transporters, but a single system that can pump in both directions has sofar not been identified. The various genome sequencing projects revealed that ABC transporters are widely distributed in bacteria. In *Escherichia coli* and *Bacillus subtilis*, 57 and 78 ABC transporters were identified, respectively (Quentin *et al.*, 1999; Linton and Higgins, 1998). Members of this transporter family can be recognized by the highly conserved ATP-binding

domains, which are involved in ATP hydrolysis and generate the energy necessary to transport the substrate against a concentration gradient. A typical ABC transporter generally contains two highly hydrophobic transmembrane domains (HMP), that usually span the membrane five or six times per domain (Figure 1) resulting in a total of 10 or 12 transmembrane segments per transporter (Saurin *et al.*, 1994). However, the two transmembrane and/or the ATP-binding domains may be fused into a single polypeptide. The HMP are believed to form the pore for selective passage of the substrate. Two ATP-binding domains are localized at the cytoplasmic side of the membrane and may be anchored to the membrane via a single transmembrane helix (Tam and Saier, 1993).

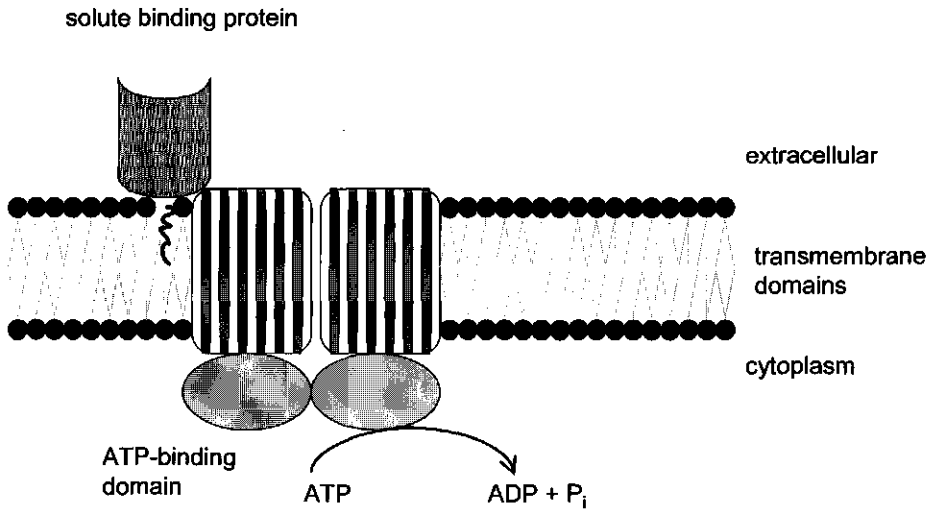

Figure 1. Structural model of a typical uptake ABC transporter of a Gram-positive bacterium. The two transmembrane domains and the two ATP-binding domains are represented here as separate polypeptides, although they can be fused as a single, large polypeptide in some cases. The two hydrophobic membrane domains are depicted here to span the membrane six times per domain. However, the number of segments can vary. The solute-binding protein in Gram-positive bacteria is usually anchored to the cell membrane via a N-terminal lipid linkage. The crystal structure of the PsaA protein of *S. pneumoniae* (Lawrence *et al.*, 1998) reveals that the solute-binding protein is embedded in the cellsurface as presented here. Modified after Higgens (1992).

Furthermore, all bacterial ABC transporters that mediate solute uptake contain an extracytoplasmic solute-binding protein (SBP). In Gram-negative bacteria, this protein is located in the periplasm, while the SBP is extracellular and bound to membrane via an N-terminal lipid anchor in Gram-positive bacteria (Tam and Saier, 1993). These binding proteins confer specifity and a high degree of affinity to the transport system

(Figure 1). However, in many bacterial exporters and in all eukaryotic systems, the extracytoplasmic solute-binding protein is absent (Fath and Kolter, 1993; Higgins, 2001).

The first molecular identification of a Mn^{2+} transporter was in the Gram-negative cyanobacterium *Synechocystis* 6803 (Bartsevich and Pakrasi, 1995). In search of photosynthesis-deficient mutants of this strain, three structural genes (*mntCBA*) encoding an ABC-type transporter complex were identified. A mutation in *mntA*, encoding the ATP-binding domain of the transporter complex, resulted in reduced growth and low oxygen evolution activity. Another mutant, carrying a deletion in *mntC*, was severly affected in growth in Mn^{2+}-deficient medium. Moreover, transport assays demonstrated a drastic reduction in Mn^{2+} uptake in the cells of a *mntC* mutant, revealing a role for this system in Mn^{2+} transport (Bartsevich and Pakrasi, 1996). In Gram-positive organisms, ABC-type transport systems for Mn^{2+} were initially found in adhesion studies of streptococcal species. Streptococci have been studied extensively because of their ability to cause life-threatening diseases to humans upon colonization of a variety of human epithelial cell surfaces. In search of factors affecting streptococcal adhesion, a gene (*psaA*) encoding a surface protein, was cloned. The *psaA*-encoded protein was initially believed to be an adhesin belonging to the LraI (lipoprotein receptor antigen I) protein family (Sampson *et al.*, 1994). Studies by Dintilhac *et al.* (1997a,b) suggested that PsaA in *Streptococcus pneumoniae* was part of a Mn^{2+} uptake system belonging to the ABC-family of transporters. Inactivation of the *psaA* gene, encoding the SBP in this strain, resulted in reduced growth in medium containing less than 0.3 μM Mn^{2+}. Besides, an effect on growth, Mn^{2+} affected the competence in this strain, as transformation of *psaA* mutant cells was completely abolished in the low- Mn^{2+} medium (Dintilhac *et al.*, 1997a). Similar binding-proteins can be found in at least nine additional species of the genus *Streptococcus* (see review by Claverys, 2001) This group of solute-binding proteins does not fit into any of the eight clusters that were defined previously (Tam and Saier, 1993). Hence a new cluster, Cluster 9, has been proposed (Dintilhac *et al.*, 1997b) that mediates the transport of metal ions. Studies in *S. gordonii* and *Y. pestis* have now provided more evidence that binding-proteins can be part of high-affinity Mn^{2+} transporters. Inactivation of *scaC* (ATP-binding protein) and *scaA* (SBP) in *S. gordonii* revealed a role of this complex in metal transport. Mutants deficient in these genes showed not only impaired growth of the cells, but also reduced uptake of Mn^{2+} in media containing < 0.5 μM Mn^{2+}.

A similar system in *Y. pestis* (the *yfe* locus) was shown to be involved in uptake of both Fe^{3+} and Mn^{2+} isotopes. Mutation of the *yfe* genes resulted in reduced transport of $^{55}Fe^{3+}$ and $^{54}Mn^{2+}$ (Bearden and Perry, 1999).

Although the Mn^{2+}-uptake studies in *S. gordonii* and in *Y. pestis* and the phenotypes of *scaA* and *psaA* mutants in *S. gordonii* and *S. pneumoniae,* respectively, clearly show a function of the corresponding ABC transporters in Mn^{2+} uptake, the nature of the metal ion bound remains to be elucidated. The X-ray crystal structure of PsaA has been determined and revealed that the metal binding site was probably occupied by Zn^{2+} as attemps to model the metal ion as Mn^{2+} failed (Lawrence *et al.*, 1998). In *S. pyogenes*, the substrate-binding protein MtsA (Janulczyk, 1999) shares over 70% sequence identity (amino acid) with PsaA, including perfect conservation of the His67, His139, Glu205, and Asp280 in PsaA that are modelled as metal binding ligands (Lawrence *et al.*, 1998). MtsA was expressed as a GST-fusion protein and used for molecular interaction studies using metal radioisotopes. Although the homology to the ScaA and PsaA counterparts was high, no interaction with Mn^{2+} was observed using proton-induced X-ray emission analysis but interaction with Zn^{2+}, Fe^{3+} and Fe^{2+} was demonstrated for the *S. pyogenes* homologue (Janulczyk *et al.*, 1998). In conclusion, the different experimental data are not in agreement with respect to the specificity of the substrate-binding protein for Mn^{2+}. However, we have to take into account that the heterologously produced protein in the latter two studies may display an altered metal-binding profile. Moreover, it is at present not known what form of Mn^{2+} is transported. In *L. plantarum*, the presence of several organic acids, including citrate, stimulates Mn^{2+} uptake in Mn^{2+} starved cells suggesting that this metal ion may be transported as a complex (Archibald and Duong, 1984).

P-type ATPase.

The P-type ATPase superfamily comprises a large family of ATP-driven pumps involved in transport of cations across the membrane. Their known cation specifities include Ca^{2+}, Na^{2+}, K^{+}, H^{+}, Cd^{2+}, Cu^{2+} and Mg^{2+} ions (Fagan and Saier, 1994). This family of transporters is represented in pro- and eukaryotes. Based on several criteria, such as ion specificity, evolutionary origin, and number of membrane-spanning segments, the ATPase superfamily can be divided into five families, each of which may be divided into two or more subfamilies (Palmgren and Axelsen, 1998). Several characteristic features are shared among all P-type ATPases.

They contain a single catalytic subunit of 70-200 kDa that is inhibited by micromolar concentrations of vanadate. Two cytoplasmic loops can be distinguished with the first cytoplasmic loop containing the phospho-aspartyl-phosphatase domain including the conserved tetrapeptide TGES (Solioz and Vulpe, 1996). The second cytoplasmic loop contains a conserved aspartyl kinase domain harboring the conserved DKTGT sequence (Möller *et al.*, 1996). Phosphorylation and dephosphorylation at the aspartate residue of the DKTGT motif during the catalytic cycle mediates cation transport.

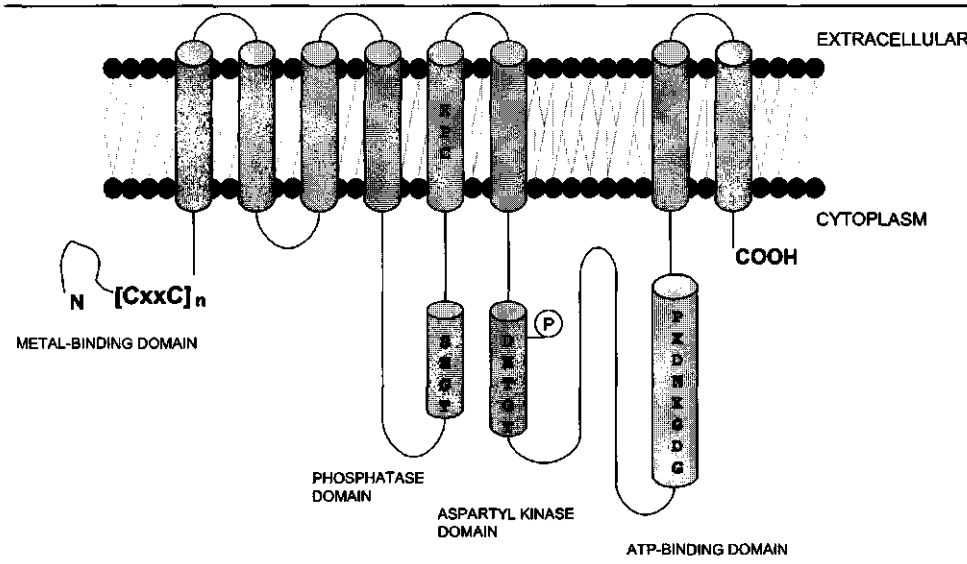

Figure 2. Generalized structure of the membrane topoloy and key features of a heavy-metal transporting ATPase. The motifs conserved among all cation-transporting ATPases are the TGES motif (phosphatase domain); the phosphorylation site DKTGT; the GDGxNDxP domain for ATP-binding. The CPx (cysteine-proline sequence) believed to be involved in ion transduction, distinguishes heavy-metal transporting ATPases from other cation-transporting ATPases;. In addition, heavy-metal transporting ATPases contain N-terminally located repeats involved in metal-binding. The most apparent feature is the repeat of the CxxC motif (one up to six repeats). However, this motif may also be replaced by methionine and histidine-rich motifs as found for CopB of *E. hirae*. Modified after Solioz and Vulpe (1996)

All known P-type ATPases contain a conserved proline residu located 43 amino acid residues N-terminal of this motif. This proline residu is located in the middle of a predicted transmembrane segment and is proposed to function in ion translocation.

A distinction can be made between heavy metal and other cation transporting ATPases. Heavy metal-transporting ATPases (see Figure 2)

generally contain 6-8 putative transmembrane segments, while other cation-transporting ATPases contain only one pair of membrane-spanning segments, located C-terminal to the ATP-binding domain (Lutsenko and Kaplan, 1995). Another distinctive feature is the presence of a conserved intramembraneous CPC, CPH or CPS motif. It has therefore been proposed to designate these heavy metal-transporting ATPases CPx-type ATPases (Solioz and Vulpe, 1996). One to six copies of a conserved repeat present in the amino terminus are thought to function as metal-binding motifs and are unique for CPx-type ATPases. The most apparent motif is two cysteine residues arranged as CxxC (Solioz and Vulpe, 1996). However, N-terminal sequences rich in methionine and histidine could also serve as metal-binding domains, as reported for instance in *Enterococcus hirae* (Odermatt *et al.*, 1993). Initially, CPx-type ATPases for heavy metal ions appeared to be restricted to bacterial resistance mechanisms, such as the CadA-catalyzed cadmium transport in *Staphylococus aureus* (Nucifora *et al.*, 1989). However, a P-type transport system for the uptake of copper was later discovered in *Enterococcus hirae* (Odermatt *et al.*, 1993) and for magnesium in *Salmonella typhimurium* (Snavely *et al.*, 1989). The topology of the CPx-type ATPases in *S. typhimurium* and *Helicobacter pylori* have experimentally been determined (Smith *et al.*, 1993a; Melchers *et al.*, 1996) (see Figure 2).

Although, cadmium efflux is known to be mediated by P-type ATPases in *Staphylococcus aureus* (Nucifora *et al.*,1989; Silver *et al.*, 1989), *Lactococcus lactis* (Liu *et al.*, 1997) and in *Listeria monocytogenes* (Lebrun *et al.*, 1994), cadmium import by such a system has sofar only been demonstrated for the MntA protein of *Lactobacillus plantarum* ATCC 14917 (Hao *et al.*, 1999b). Expression of the *mntA* gene in *E. coli* conferred increased Cd^{2+} sensitivity and increased Cd^{2+} uptake in these cells. For several Gram-positive organisms it has been demonstrated that Cd^{2+} uptake occurs via Mn^{2+} transport systems (Archibald and Duong, 1984; Laddaga *et al.*, 1985; Tynecka *et al.*, 1981). Uptake of the cadmium isotope is therefore considered to be mediated by a Mn^{2+} transport system. However, it is not conclusively shown that MntA transports Mn^{2+} in *L. plantarum* and its identification as a Mn^{2+} transporter is based on overexpression of *mntA* in *E. coli.* In addition, it can not be excluded that MntA has affinity for additional ions besides Cd^{2+} and Mn^{2+}, analogous to the P-type ATPase MgtB in *Salmonella typhimurium* which shows not only affinity for Mg^{2+} but also for other divalent metals, including Mn^{2+} (Snavely *et al.*, 1989).

The MntA protein of *L. plantarum* seems to be atypical for a heavy metal transporting ATPase. The protein contains the conserved features of P-type ATPases such as the ion transduction domain, the conserved proline residu located 43 amino acids upstream of the DKTGT motif, and the GDGxNDxP domain for ATP binding. However, the protein lacks the N-terminal CxxC motif and the CPx motif (the conserved proline is not flanked by cysteine) typical for heavy metal binding (Solioz and Vulpe, 1996). Furthermore, its predicted single transmembrane segment before the first cytoplasmic domain is different from the consensus topology (Solioz and Vulpe, 1996). The atypical features of MntA cannot be explained by the import function of this transporter since CopA of *E. hirae,* which serves a function in copper import in this species, does contain the CPC and CxxC motifs (Odermatt *et al.*, 1993). Moreover, the hydropathy profile of CopA predicts the topology to be according to the consensus topology for CPx-type ATPases.

Antibodies raised against MntA from *L. plantarum* ATCC 14017 and ATCC 8014 showed production of MntA in these strains upon Mn^{2+} starvation (Hao *et al.*, 1999b). In a mutant strain of *L. plantarum* ATCC 8014, MntA production was indeed abolished and the intracellular Mn^{2+} level in the mutant was reduced to below 1% of the level in the parental strain (Hao *et al.*, 1999b). However, this study suffers from the fact that the mutant was constructed by chemical mutagenesis. It is therefore possible that in addition to the *mntA* gene, additional genes that control Mn^{2+} homeostasis have been affected, for example genes encoding other transporters or regulatory elements. In fact, recent data show that a defined mutation of *mntA* in *L. plantarum* does not significantly reduce the intracellular Mn^{2+} level, nor affects growth under Mn^{2+} starvation (Nierop Groot *et al.*, 2001).

Nramp transport systems.

Natural resistance-associated macrophage proteins (Nramp) have initially been characterized in mammalian species and were found to control natural resistance towards pathogens such as *Salmonella typhimurium*, *Mycobacterium tuberculosis* and *Leishmania donovani* (Cellier *et al.*, 1996; Gruenheid *et al.*, 1995,1997, Vidal *et al.*, 1995b). They are also required for intestinal iron uptake (Fleming *et al.*, 1997, 1998). At least two copies of closely related genes (*nramp1* and *nramp2*) have been identified on separate chromosomes in both mice and humans (Gruenheid *et al.*, 1995; Vidal *et al.*, 1995a). Both proteins are highly hydrophobic, containing 12

predicted transmembrane (TM) domains (see Figure 3), and are extensively glycosylated in the extracellular loop; the mouse Nramp2 for example is glycosylated to over 40% of the mass (Gruenheid *et al.*, 1999). The predicted fourth cytoplasmic loop contains a consensus sequence motif that is also present in a number of prokaryotic (Kerpola and Ames, 1992) and eukaryotic (Bairoch, 1993) membrane-associated transport proteins. Evidence accumulates that mammalian Nramp proteins are divalent cation transporters of broad specificity (Gunshin *et al.*, 1997).

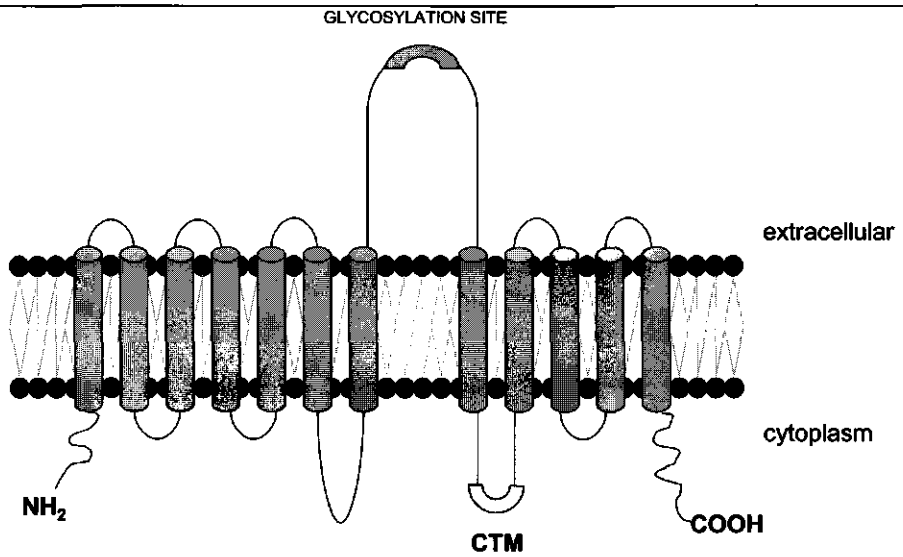

Figure 3. Structural model of the arrangement of Nramp proteins in the membrane. The fourth intracellular loop contains the consensus transport motif (CTM) that was found in several bacterial and eukaryotic Nramp transporters. The predicted glycosylated extracellular loop between transmembrane segment 8 and 9 are depicted.
Modified after Gunshin *et al.* (1997)

Bacterial Nramp homologues were recently reported for *B. subtilis*, *S. typhimurium* and *E. coli* to function as high-affinity transporters for Mn^{2+} (Que and Helmann, 2000; Kehres *et al.*, 2000; Makui *et al.*, 2000; Patzer and Hantke, 2001). Expression of the Nramp-encoding genes (*mntH*) in these bacteria was regulated by Mn^{2+}. In *S. typhimurium* and *E. coli*, Mn^{2+} is imported with apparent K_m values of 100 nM and 1 µM respectively and a transport velocity, for cells overexpressing *mntH*, of 2 nmol Mn^{2+} per OD_{600} unit of cells.min^{-1} for both strains (Kehres *et al.*, 2000). The maximum rate of transport of the *mntH* overexpressing cells was threefold increased relative to the wild-type cells. These MntH transporters are highly selective for Mn^{2+}; the divalent transition metal ions Ni^{2+}, Cu^{2+} and

Zn^{2+}, inhibit Mn^{2+} uptake only when present at a 1000-fold excess. Fe^{2+} was only a weak inhibitor of Mn^{2+} uptake by MntH at pH 7 and because of its relatively high K_m-value (25-50μM versus 0.1 μM for Mn^{2+}) at physiological pH, the extracellular Fe^{2+} concentration required for significant inhibition would not be physiologically relevant (Kehres *et al.*, 2000). The transport velocity of Mn^{2+} by this system is sensitive to pH, being higher at decreasing pH (Kehres *et al.*, 2000) in agreement with a proton-gradient driven transport. Deletion of the *mntH* gene in *B. subtilis* resulted in an increase in Mn^{2+} concentration required for optimum growth compared to the wildtype, suggesting that in *B. subtilis*, MntH is an important uptake system for this metal at low Mn^{2+} concentrations (Que and Helmann, 2000).

The role of Nramps in bacteria is not yet established. Inactivation of MntH in *B. subtilis* resulted in increased sensitivity to H_2O_2, suggesting a role in stress response to reactive oxygen (Que and Helmann, 2000). The upregulation of the Nramp transporters in *S. typhimurium* and *E. coli* in response to H_2O_2 also supports a role in oxidative stress. In analogy, putative promoter control regions matching the consensus binding site for Fur (a metalloregulatory protein that binds in response to Fe^{2+} and Mn^{2+} replete conditions) and OxyR (H_2O_2-activated transcriptional activator), as well as an inverted repeat homologous to the one found for the *sitABCD* operon, encoding an iron transport system in *S. typhimurium* (Zhou *et al.*, 1999) were present in *E. coli mntH* (Kehres *et al.*, 2000). These findings suggest that regulation of *mntH* expression involves a complex network of various regulators, and suggests an important role of MntH under various stress conditions.

Role of Mn^{2+} in host-bacteria interaction and in virulence

Evidence is accumulating that Mn^{2+} homeostasis effects virulence and adhesion properties of bacteria. A role of of the LraI family of metal-binding proteins in virulence of bacteria has been demonstrated in experimental animal models for FimA (*S. parasanguis*, Burnette-Curley *et al.*, 1995), PsaA (*S. pneumoniae*, Berry and Paton, 1996), SloC (*S. mutans*, Kitten *et al.*, 2000), EfaA (*E. faecalis*, Singh *et al.*, 1998) and Yfe (*Y. pestis*, Bearden and Perry, 1999). Although the LraI proteins were initially believed to be directly involved in virulence as adhesins, recent

findings point towards a more complex role of these proteins. Adherence studies show decreased binding capacity of *psaA* mutants of *S. pneumoniae* to type II pneumocytes (Berry and Paton, 1996) and *yfeAB* mutants of *Y. pestis* displayed a 100-fold increase in LD_{50} from a subcutaneous route of infection in mice (Bearden *et al.*, 1999). However, mutation of *scbA* in *S. crista*, encoding a SBP highly homologous to PsaA (79% identity at the amino acid level), did not alter the fibrin binding of this species (Correia *et al.*, 1996). Moreover, addition of Mn^{2+} to various mutants that were affected in the *psa* locus in *S. pneumoniae*, failed to correct the mutant phenotypes, such as defective adhesion and reduced natural transformation efficiency (Novak *et al.*, 1998). A direct role in adhesion is also not likely for SitC, a LraI homolog in *S. epidermis*, since its limited exposure on the cell surface does not favor the idea of a direct involvement of this protein in adhesion (Cockayne *et al.*, 1998). In addition, a strong argument against a direct role of PsaA in adhesion comes from its crystal structure, which suggests that PsaA is simply too small to protrude through the cell wall and its surrounding polysaccharide capsule. Consequently, PsaA would be embedded in, rather than exposed on the cell surface. A direct role of PsaA in adhesion is therefore not likely (Lawrence *et al.*, 1998). It has been suggested that Mn^{2+} import by the Psa transporter is only one element of a complex signalling pathway, affecting cellular functions such as adhesion, lysis, transformation and virulence (Novak *et al.*, 1998).

A role of Nramp transporters in protection against virulent bacteria has been established for mammalian species, where these transporters were found to control natural resistance towards pathogens such as *S. typhimurium*, *M. tuberculosis* and *L. donovani* (Cellier *et al.*, 1996; Gruenheid *et al.*, 1995,1997, Vidal *et al.*, 1995b). In mice, mutations in the *nramp1* gene, either spontaneous (Malo *et al.*, 1994) or experimentally induced (Vidal *et al.*, 1995b), resulted in decreased resistance to infection with *Mycobacterium*, *Leishmania* and *Salmonella*. In addition, human *nramp1* polymorphisms can be associated with the infectious diseases leprosis and tuberculosis in certain populations (Abel *et al.*, 1998; Bellamy *et al.*, 1998). Transport studies in *Xenopus* oocytes have demonstrated that *nramp2* codes for a proton-coupled high-affinity transporter that besides Fe^{2+}, mediates the influx of Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} ions (Gunshin *et al.*, 1997). A function of Nramp1 in Mn^{2+} transport has recently been demonstrated for mice-derived phagosomes using a divalent-cation sensing probe (Jabado *et al.*, 2000). This study

demonstrated that Nramp1 functions as a pH-dependent transporter that can extrude Mn^{2+}, but likely also other divalent cations, from the intraphagosomal space. The mechanism whereby Nramp1 contributes to the antimicrobial activity of macrophages in relation to metal transport is still poorly understood. The results of Jabado *et al.* (2000) support a role of Nramp1 in the defense against infection by the extrusion of divalent cations from the phagosomal space, thereby creating a shortage of essential cofactors required by the pathogen for protection against the reactive oxygen species produced by the host. Other studies suggest that Nramp1 transport divalent cations, especially Fe^{2+}, into rather than out of the phagosome (Zwilling *et al.*, 1999; Kuhn *et al.*, 1999) and thereby generate toxic oxygen species via the Fenton reaction.

The role of bacterial Nramp transporters in virulence is less well documented. Macrophages use Mn^{2+} and other metal ions both for the synthesis of toxic oxygen radicals, including H_2O_2 and O_2^-, and as cofactor in enzymes for self-protection. On the other hand, Mn^{2+} is required in bacteria as cofactor in an antioxidant defense against these oxygen radicals, either produced by the bacterial metabolism or by the host in response to invasion of the pathogen. In this scenario, competition between host and pathogen is centered around Mn^{2+}. The upregulation of Nramp-encoding genes in *S. typhimurium* and *E. coli* (Kehres et al., 2000) upon H_2O_2 exposure may also suggest that these transporters play a role in protection against toxic oxygen species generated by macrophages, and thereby affect virulence. Indeed, inactivation of *mntH* in *S. typhimurium* reduces virulence in a mouse model (Kehres *et al.*, 2000). However, the invasion efficiency or survival of the mutant strain in a macrophage cell line was not altered, indicating that additional factors, and probably also Mn^{2+} transporters, are involved.

Regulation of manganese uptake

For several micro-organisms, genetic and biochemical evidence has been published that at least two systems are present for Mn^{2+} uptake (Kolenbrander *et al.*, 1998; Dintilhac *et al.*, 1997; Que *et al.*, 2000). Although the individual role of the different uptake systems is not very well understood, and probably affects multiple cellular processes, it is conceivable that some form of regulation must be present in the cells to coordinate the uptake of Mn^{2+} by these systems. It has been shown for

some transporters that they are inducible by Mn^{2+} (Hao *et al.*, 1999; Jakubovics *et al.*, 2000). However, only limited information exists on the regulator(s) that could be involved in control of these systems. Only recently, several studies have been reported that indicate the involvement of a metalloregulatory protein in the uptake of Mn^{2+}.

Metalloregulatory proteins control gene expression by the reversible interaction with specific metal ions (O'Halloran *et al.*, 1993). Two of the best-characterized representatives of the metalloregulatory protein family are the transcriptional repressors of iron-regulated promoters in *E. coli* (Fur; ferric uptake repressor) and in *Corynebacterium diphtheriae* (DtxR, diphtheria toxin repressor) (Tao *et al.*, 1994 Touati *et al.*, 1995, Escolar *et al.*, 1999; Schmitt *et al.*, 1992, Braun *et al.*, 1998). DtxR, the iron-dependent toxin repressor encoded by the genome of *C. diphtheriae*, was identified over two decades ago (Murphy *et al.*, 1974). In response to the availability of iron, DtxR negatively controls at the transcriptional level the production of the diphtheria toxin in this bacterium. More recent studies in this and other species, point towards a broader physiological role of this type of repressor protein besides toxin-regulation. For example, a role of DtxR-like proteins has been demonstrated in 1. siderophore biosynthesis in *C. diphtheriae* (Tai *et al.*, 1990), *Mycobacterium smegmatis* (Dussurget *et al.*, 1996) and *Streptomyces pilosus* (Günter *et al.*, 1993); 2. control of expression of iron-uptake genes in *C. diphtheriae* (Pohl *et al.*, 1999); 3. regulation of transcription of the heme oxygenase gene in *C. diphtheriae* (Schmitt, 1997); and 4. regulation of expression of oxidative-stress response genes in *M. smegmatis* (Dussurget *et al.*, 1996). Although DtxR regulates gene expression in response to iron *in vivo*, it is not known yet what determines the iron-specifity of the regulator since *in vitro* experiments show activation of DNA-binding by several different divalent cations (Schmitt and Holms, 1993; Tao and Murphy, 1992; Pohl *et al.*, 1999).

Recent studies have shown that proteins homologous to the DtxR protein can be found in *Bacillus subtilis* (Que *et al.*, 2000), *Treponema pallidum* (Posey *et al.*, 1999), *S. gordonii* (Jakubovics *et al.*, 2000) and *E. coli* (Patzer and Hantke, 2001). Unlike DtxR, these are active as a Mn^{2+}-complex. Although the homology of these proteins to DtxR is only 26-28% (amino acid identity), most ligands for metal binding are conserved. ScaR (*S. gordonii*) and MntR (*E. coli*) act as repressors when intracellular levels of Mn^{2+} rise (see Figure 4).

Binding of the Mn^{2+}-regulator complex to the promoter of the ABC transporter operon in *S. gordonii* or the Nramp transporter in *E. coli* prevents transcription.

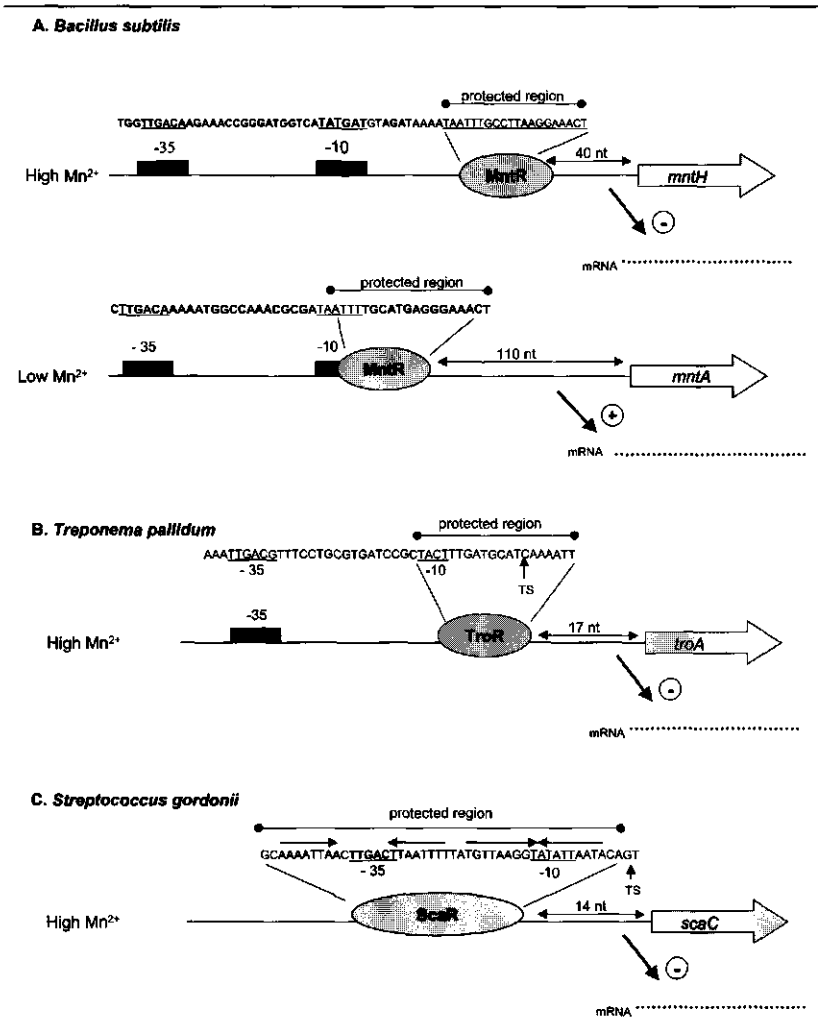

Figure 4. Schematic presentation of the various binding sites recognized by DtxR homologues that are involved in regulation of Mn^{2+} transport. In *B. subtilis* (A), MntR acts as a Mn^{2+}-dependent repressor of *mntH* under Mn^{2+} replete conditions and activates *mntABCD* under Mn^{2+} limitation. TroR from *T. pallidum* (B) functions as a Mn^{2+} dependent repressor on the *troABCD* operon by binding to the depicted region of dyad symmetry and thereby overlaps the –10 promoter region. In *S. gordonii,* the *scaCBA* operon is negatively regulated by ScaR (C) under Mn^{2+}-replete conditions. ScaR binds to a region of dyad symmetry and thereby overlaps both the –10 and –35 promoter regions.

Under Mn^{2+}-limiting conditions, the regulator is present as an apoprotein and the repression is relieved. DNA-binding studies with purified TroR

from *Treponema pallidum* (Posey *et al.*, 1999) suggest a mechanism similar to ScaR whereby the TroR-Mn^{2+} complex blocks transcription of the *tro*-operon encoding an ABC transporter. However, a role of this mechanism in Mn^{2+} homestasis has still to be demonstrated *in vivo*. A DtxR homologue in *B. subtilis*, MntR, acts as a bifunctional protein by regulating the transcription of at least two transporters in response to the availability of Mn^{2+} (see Figure 4). Transcriptional fusions of the regulatory region of *mntH* to the promoterless *lacZ* revealed that expression of this transporter is repressed by Mn^{2+} in the presence of a functional *mntR* gene. In contrast to the repressor function observed for *mntH* expression, MntR acts as an activator of the *mntABCD* operon (encoding a Mn^{2+}-specific ABC transporter) under low Mn^{2+} conditions in *B. subtilis* (Que and Helmann, 2000). A *mntR* mutant strain lost control of Mn^{2+} uptake and displayed increased sensitivity to both Mn^{2+} and Cd^{2+} supporting a role of MntR in repression of Mn^{2+} uptake (Que and Helmann, 2000). Suppression of the increased Cd^{2+}/Mn^{2+} sensitivity was obtained by a second mutation, in the *mntH* locus, encoding a homologue of the Nramp family of transport proteins. Interestingly, mutation of MntA of the ABC transporter also resulted in increased sensitivity to elevated Mn^{2+} levels, presumably because mutation of *mntA* leads to upregulation of MntH. Strikingly, the DtxR homologue of *Staphylococcus epidermis* (SirR; Hill *et al.*, 1998) acts as a Fe^{2+} or Mn^{2+}-complex. SirR binds to a control region that resembles the typical DtxR consensus sequence, suggesting that SirR represents an intermediate between the iron-sensing DtxR-like proteins and the Mn^{2+}-sensing homologues (Que and Helmann, 2000). DtxR-like proteins represent a rapidly expanding family of bacterial metalloregulatory proteins. Additonal members of this family can be found in the genomic sequence projects of *S. pneumoniae* (PsaR), *S. pyogenes* (MtsR), *E. faecalis* (EfaR) and *S. mutans* (SloR; Kitten *et al.*, 2000). A function of these proteins in metal ion homeostasis remains to be demonstrated.

In contrast to ScaR (Jakubovics *et al.*, 2000), the Mn^{2+}-sensing metalloregulatory proteins in MntR in *E. coli* (Patzer and Hantke, 2001), TroR from *T. pallidum* (Posey *et al.*, 1999) and MntR from *B. subtilis* (Que and Helmann, 2000) lack the C-terminal SH3-like domain of DtxR. The determination of the various cis-acting elements of Mn^{2+}-sensing DtxR-homologues reveals that these, apart from SirR (*S. epidermis*), do not match the DtxR consensus recognition site. The putative target sequences recognized by TroR and MntR of *T. pallidum* and *B. subtilis*, respectively, show significant sequence similarity to each other, but do not match the

interrupted palindromic consensus sequence recognized by DtxR. A difference in DNA recognition sites between MntR and DtxR is also suggested by the low sequence conservation in the HTH domain of the two proteins (Que and Hellmann, 2000). DNaseI footprint analysis of the *scaC* promoter in *S. gordonii* revealed that binding of ScaR occurs at a 46-bp region directly upstream of the transcription start site. The region bound by ScaR contains an interrupted palindromic sequence element overlapping the –35 promoter signature and, in addition, an imperfect palindromic sequence at the –10 region. The significance of the presence of these two palindromic elements in the *scaC* promoter region is at present not clear. The control region for the ScaR regulator shows no significant similarity to either the sequences recognized by MntR (*B. subtilis*), TroR, MntR (*E. coli*) nor the DtxR consensus sequence (Jakubovics *et al.*, 2000). However, the two palindromic elements are well conserved within the control regions of several metal ABC transporters in streptococcal species, suggesting a similar binding mechanism in this genus.

It is not yet known whether the Mn^{2+}-sensing DtxR proteins have global effects on transcription. In *C. diphtheriae,* at least 8 genes containing the consensus binding site are regulated by DtxR (Lee *et al.*, 1997; Schmitt, 1997; Schmitt *et al.*, 1997; Schmitt and Holmes, 1994; Tao *et al.*, 1994; Tai *et al.*, 1990). The genes encoding DtxR-like proteins in *T. pallidum*, *S. mutans* are found to cluster with genes encoding ABC transporters, however, distinct chromosomal locations for these genes were found in *S. gordonii*, *B. subtilis* and *S. epidermis*. This may reflect that these metalloregulatory proteins are not strictly related to regulation of the ABC transport system only. Indeed, bifunctional regulation has been demonstrated for *B. subtilis* (Que and Helmann, 2000) and the detection of multiple Sir-boxes in *S. aureus* (Hill *et al.*, 1998) indicates that SirR may be a pleiotropic regulator of gene expression. Future research should reveal whether the Mn^{2+}-sensing metalloregulatory proteins in bacteria serve multiple regulatory functions.

Perspectives of manganese transport systems

The analysis of both the recently discovered Mn^{2+} transport systems and the proteins involved in regulation of the expression of these transporters, provide considerably more insight in the mechanisms available for bacteria

to import this essential metal from the environment. However, the rapid developments indicate that still a large number of aspects remains to be elucidated. At present, three different transport systems have been reported for high-affinity uptake of Mn^{2+} in bacteria. Surprisingly, all Mn^{2+} transporters cloned to date appear to be high-affinity transport systems, inducible under Mn^{2+}- limiting conditions. Although uptake studies in *L. plantarum* (Hao *et al.*, 1999a) refer to the presence of a low-affinity uptake system for Mn^{2+} in these species, the gene(s) involved have not been reported. Evidence for the presence of more than one transporter for Mn^{2+} has been presented for *S. gordonii* (Kolenbrander *et al.*, 1998) and *S. pneumoniae* (Dintilhac *et al.*, 1997a). Strains carrying mutations in the genes encoding Mn^{2+} transporters were still able to grow, indicating that a second transporter is active. This transporter could be the same as the recently identified Nramp homologues in *B. subtilis* and *E. coli* (Que and Helmann, 2000; Makui *et al.*, 2000) that transport Mn^{2+} in symport with protons. Indeed, the reduced Mn^{2+}-uptake in *sca* mutants of *S. gordonii* was proton-motive force dependent (Kolenbrander *et al.*, 1998). Furthermore, growth experiments with these mutants suggest the presence of a third transporter that can handle both Zn^{2+} and Mn^{2+}. This might be the Adc-transporter (ABC transporter) characterized in *S. pneumoniae* (Dintilhac *et al.*, 1997).

For *B. subtilis*, genetic and biochemical evidence has been presented that both an ABC-type and a Nramp system are present in this bacterium (Que and Helmann, 2000). The ability of a double-mutant strain to grow in the presence of micromolar levels of Mn^{2+} suggested the presence of a third transport system that could handle Mn^{2+} in *B. subtilis*. Other transporters present in *B. subtilis* that might transport Mn^{2+} in this mutant are CitM and the Pit system (Table 1). However, the low-affinity of the CitM and the Pit system are not likely to fulfill Mn^{2+} requirements in Mn^{2+} limited medium.

Of particular interest is *L. plantarum* that harbors unusually high levels of Mn^{2+} intracellularly (Archibald and Fridovich, 1981). Because this species lacks SOD to detoxify oxygen derivatives, it is of extreme importance for this organism to keep intracellular Mn^{2+} levels high. Therefore, a highly efficient system for Mn^{2+} uptake that is tightly controlled can be expected for this bacterium. A P-type ATPase (MntA) is sofar the only uptake system reported for *L. plantarum* that is believed to transport Mn^{2+} (Hao *et al.*, 1999b) and at present, Mn^{2+} import by a P-type ATPase has only been demonstrated for this species. Differences in

transport kinetics for Mn^{2+} uptake in Mn^{2+}-starved and Mn^{2+}-sufficient cells of *L. plantarum* point towards the presence of at least two transport systems (Hao *et al.*, 1999a). Recent studies on *L. plantarum* have revealed that, in addition to the *mntA* gene, three more systems are induced under Mn^{2+} starvation and probably transport Mn^{2+} (Nierop Groot *et al.*, 2001).

Data on the regulation of Mn^{2+} transport systems has become available only very recently and will probably expand the coming years. DtxR homologues acting as a Mn-complex have been discovered in several species and with the increasing number of complete genome sequences, more homologues will become available. Experiments that show a function of these homologues in Mn^{2+}-homeostasis have been performed only for *S. gordonii* (Jakubovics *et al.*, 2000) and *B. subtilis* (Que and Helmann, 2000). Differences in the regulatory protein itself, and in their recognition sequences, suggest that the mode of action of these regulators may be different. Interestingly, a DtxR homologue is present in the *L. plantarum* genome. Further studies are necessary to unravel the Mn^{2+} regulation in bacteria. With the development of DNA micro-array and efficient proteomics technologies, powerful tools have become available to study the role of this metalloregulatory protein on a global level and to identify other factors influencing the expression of Mn^{2+}-transporters.

The increasing number of publications on Mn^{2+} uptake systems in bacteria may reflect the awareness of the potential applications of this knowledge in several fields. Since Mn^{2+} transport systems show high affinity for the toxic metal cadmium, bacterial high-affinity uptake systems for Mn^{2+}, especially from *L. plantarum*, may be explored in the bioremediation of cadmium from heavy-metal contaminated environments. *E. coli* strains have been engineerd for Hg^{2+} or Ni^{2+} bioaccumulation by introducing *merT* and *merP* (Chen and Wilson, 1997) or the *nixA* gene from *Helicobacter pylori* (Krishnaswamy and Wilson, 2000) and by simultaneously overexpressing a metallothionein. A similar approach using a high-affinity Mn^{2+} uptake system may be usefull for the bioremediation of Cd^{2+}-contaminated environments.

The recent finding that Mn^{2+} transport systems are part of a signalling pathway affecting adhesion and virulence of streptococci, pneumococci and enterococci, has resulted in a large contribution from the medical field to the current knowledge on Mn^{2+}-transport systems and their regulation. Based on the role of Mn^{2+} in adhesion, this metal ion may also be an important factor determining the host-microbe interactions in the

gastrointestinal (GI) tract. In this respect, it would be interesting to study the role of Mn^{2+} in adhesion or colonization properties of *L. plantarum* and other lactobacilli that are known inhabitants of the human GI-tract (Adlerberth *et al.*, 1996; Granato *et al.*, 1999) and are assumed to have a beneficial effect on the host (Salminen *et al.*, 1998). In addition, knowledge of the systems involved in Mn^{2+}-homeostasis of virulent bacteria can be important in the development of antimicrobial drugs. The critical role of metal ion transport systems in virulence raises the possibility that blocking transporter functions may be an effective target in preventing or controlling streptococcal or enterococcal infections.

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Chapter 3.

Conversion of Phenylalanine to Benzaldehyde initiated by an Aminotransferase in *Lactobacillus plantarum*

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Abstract

The production of benzaldehyde from phenylalanine has been studied in various microorganisms, and several metabolic pathways have been proposed in the literature for the formation of this aromatic flavor compound. In this study, we describe benzaldehyde formation from phenylalanine by using a cell extract of *Lactobacillus plantarum*. Phenylalanine was initially converted to phenylpyruvic acid by an aminotransferase in the cell extract, and the keto-acid was further transformed to benzaldehyde. However, control experiments with boiled cell extract revealed that the subsequent conversion of phenylpyruvic acid was a chemical oxidation step. It was observed that several cations could replace the extract in the conversion of phenylpyruvic acid to benzaldehyde. Addition of Cu^{2+} ions to phenylpyruvic acid not only resulted in the formation of benzaldehyde, but also in the generation of phenylacetic acid, mandelic acid and phenylglyoxylic acid. These compounds have been considered intermediates in the biological conversion of phenylalanine. The chemical conversion step of phenylpyruvic acid was dependent on temperature, pH, cation and on the presence of oxygen.

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Introduction

The increasing consumer preference for products of natural origin has directed research towards the exploitation of microbial biosynthetic pathways to produce natural flavors. Natural aromatic compounds represent a very large market in the flavor industry including benzaldehyde and vanillin. In quantity, benzaldehyde is, after vanillin, the most important of these compounds (Welsh *et al.*, 1989). The natural benzaldehyde market in 1995 was about 100 Mtons/year and is growing every year at about 5% (Clark, 1995). Natural benzaldehyde is found as a glycoside (amygdalin) in the pit of almonds and cherries and can be released by enzymatic hydrolysis. A drawback is that toxic by-products such as hydrocyanic acid may be formed.

Microbial production of benzaldehyde from phenylalanine offers an attractive alternative way to produce benzaldehyde which can be labeled 'natural'. Benzaldehyde formation in medium supplemented with phenylalanine has been reported for cultures of *Ischnoderma benzoinum* (Fabre *et al.*, 1996; Krings *et al.*, 1996), *Polyporus tuberaster* (Kawabe and Morita, 1994) and *Phanerochaete chrysosporium* (Jensen *et al.*, 1994). Immobilization of the white-rot fungus *Bjerkandera adusta* resulted in an increased production of benzaldehyde in a medium containing L-phenylalanine (Lapadatescu *et al.*, 1997). Among bacteria, benzaldehyde formation has been reported for a strain of *Pseudomonas putida*. In this strain, benzaldehyde was formed as a metabolic intermediate in the mandelic acid pathway during degradation of mandelic acid (Tsou *et al.*, 1990). Several metabolic pathways have been proposed in the literature for the formation of benzaldehyde from phenylalanine.

Benzaldehyde was found in the volatile fractions of several cheeses, and may contribute to the flavor of these products (Bosset and Gauch, 1993; Molinard and Spinnler, 1996). Additionally, benzaldehyde was formed in a complex medium when inoculated with several different strains of lactic acid bacteria (Imhof *et al.*, 1995; Tracey and Britz, 1989). However, there is no information about the contribution of lactic acid bacteria to the production of benzaldehyde.

In this study, we investigated whether a lactic acid bacterium was able to produce benzaldehyde using phenylalanine as a substrate. Incubation of a cell extract of *Lactobacillus plantarum* with phenylalanine revealed that benzaldehyde was indeed formed. More detailed studies of the mechanism involved revealed that benzaldehyde formation was

initiated by an aminotransferase, resulting in phenylpyruvic acid. The keto acid formed was subsequently subject to a chemical reaction leading to benzaldehyde. The chemical conversion of phenylpyruvic acid was demonstrated under various mild conditions.

Materials and methods

Chemicals

Phenylalanine, phenylpyruvic acid, α-ketoglutaric acid, pyridoxal 5′-phosphate (PLP), lysozyme, benzaldehyde, phenylglyoxylic acid and phenylethanol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phenylacetic acid, trans-cinnamic acid and mandelic acid were purchased from Acros Chimica (Geel, Belgium) and benzoic acid was obtained from Merck (Darmstadt, Germany).

Strain, growth medium and preparation of cell-free extract

Lactobacillus plantarum URL-LcL1 (Unilever Research Laboratory, Vlaardingen, The Netherlands) was used in this study. The organism was routinely maintained in 10% sterile litmus milk (Difco) and stored at - 80°C until use. *L. plantarum* was cultured overnight at 30°C in MRS broth (Merck). The cells were harvested (16,000 x *g*, 15 min, 4 °C) and washed twice in 50 mM sodium phosphate buffer (pH 7.0). The cells were resuspended in 50 mM triethanolamine buffer (pH 7.0) containing 20% sucrose and 0.4 mg/ml of lysozyme. After incubation for 30 min at 30°C, the suspension was centrifuged (12,000 x *g*, 30 min, 4°C) and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM PLP. The cells were disrupted by sonication for 5 periods of 30 s on ice. Cell debris was removed by centrifugation (20,000 x g, 30 min, 4°C) to give a crude cell-free extract. The protein concentration in the cell extract was determined by the method of Lowry (1951), with bovine serum albumin as the standard.

Phenylalanine catabolism by cell extract

The cell extract of *L. plantarum* was incubated with phenylalanine in sterile sealed bottles which were agitated at 37°C. The reaction mixture and the cell extract were sterilized by passing them through a sterile filter (0.2 μm pore-size, Schleicher & Schuell, Dassel, Germany). Aliquots of 1 ml were

withdrawn at various times to analyze the reaction products by high-performance liquid chromatography (HPLC). The reaction mixture contained 8 mM substrate, 2 ml of cell extract of *L. plantarum* and 0.02 mM PLP in 8 ml of 50 mM Tris-HCl (pH 8.0). For incubations with phenylalanine as a substrate, 8 mM of α-ketoglutaric acid was added to the reaction mixture. In control samples, the cell extract was boiled for 10 min.

Chemical conversion of phenylpyruvic acid to benzaldehyde

The reaction mixture for chemical conversion of phenylpyruvic acid to benzaldehyde contained 8 mM phenylpyruvic acid in 50 mM Tris-HCl (pH 8.0). Sealed bottles were filled with reaction mixture and $CuSO_4 \cdot 5H_2O$ was added from a stock solution in 50 mM Tris-HCl (pH 8.0) to a final concentration of 350 μM. Incubations were performed at either 37°, 30° or 25°C.

The effect of metal ions on the chemical conversion of phenylpyruvic acid was studied with stock solutions of $Fe_2(SO_4)_3 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, Mn(III)acetate dihydrate, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ in Tris-HCl (pH 8.0). The reaction mixtures were incubated at 37° C and samples were taken after 6 h of incubation.

The effect of pH on the chemical conversion of phenylpyruvic acid to benzaldehyde was studied using 50 mM sodium phosphate buffer (pH 6.0, 7.0 and 8.0) and 50 mM Tris-HCl buffer (pH 8.0 and 9.0). All incubation were performed at 37°C in the presence of 350 μM $CuSO_4 \cdot 5H_2O$.

HPLC analysis

Samples of the reaction mixture were diluted to appropriate concentrations with 50 mM Tris-HCl (pH 8.0). each diluted sample (900 μl) was mixed with 100 μl of 6 N HCl, and the supernatant fluid obtained after centrifugation for 5 min was analyzed for reaction products. Analyses were performed with a Hewlett-Packard (Waldbronn, Germany) HPLC Chemstation (Pascal series) equipped with an HP 1050 pumping system. Reaction products were separated onto a Chromspher5 C_{18} column (Chrompack, Bergen op Zoom, The Netherlands) using solvent A (0.115 % trifluoroacetic acid) and solvent B (0.1 % trifluoroacetic acid, 60% acetonitrile) and detected by an HP 1040 M-series II diode array detector. The products were separated by using the following linear gradient (0.5 ml

min^{-1}, 30° C): 25% B at 0 min to 65% B at 21 min followed by equilibration under initial conditions for 6 min. Detection was by UV at 260 nm for benzaldehyde, 280 nm for phenylpyruvic acid, and 216 nm for phenylacetic acid, mandelic acid and benzoic acid. Concentrations were calculated from standard curves of the pure compounds.

GC-MS analysis

For gas chromatography-mass spectrometry (GC-MS) analysis, the reaction mixture was mixed with 10% (vol/vol) of 6 N HCl. The acidified reaction mixture was then extracted with ethylacetate. The extract obtained was used for analysis on an HP5973A quadrupole MS coupled to an HP6980 gas chromatograph equipped with a fused silica capillary column (HP-5MS, 30 m by 0.25 mm inside diameter, film thickness: 0.25 µm). The following operating conditions were used: injector temperature 220° C; temperature program: 70-250°C at 7 min^{-1}, hold 10 min; injection volume: 1.0 µl; split ratio 1:50; flow rate of carrier gas (helium), 1.0 ml min^{-1}. Electron impact mass spectra were obtained at 70 eV.

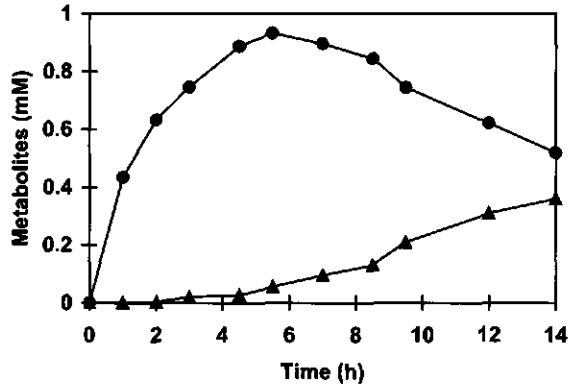

Figure 1. The formation over time of phenylpyruvic acid (●) and benzaldehyde (▲) from phenylalanine during incubation of a cell extract of *L. plantarum*. Incubations were performed at 37°C in a reaction mixture containing cell extract (8.6 mg of protein), 8 mM phenylalanine, 0.02 mM PLP and 8 mM α-ketoglutaric acid in 50 mM Tris buffer (pH 8.0). Small amounts of phenylacetic acid, mandelic acid and phenylglyoxylic acid were detected at concentrations of 0.05 mM, 0.02 mM and 0.02 mM, respectively, after 14 h of incubation.

Results

Incubation of cell extract of *L. plantarum* with phenylalanine

Incubation of the cell extract of *L. plantarum* with phenylalanine in the presence of both α-ketoglutaric acid and PLP resulted in the production of two compounds. The HPLC chromatogram showed two UV-absorbing peaks with the retention times of phenylpyruvic acid and benzaldehyde, respectively. The identity of the compounds was confirmed by GC-MS for benzaldehyde and by comparison of the UV spectrum with that of the pure compound for phenylpyruvic acid.

Figure 1 shows the formation of phenylpyruvic acid and of benzaldehyde over time. Neither phenylpyruvic acid nor benzaldehyde was formed if α-ketoglutaric acid or PLP were omitted from the reaction mixture or if boiled cell extract was used. Phenylpyruvic acid reached a maximum concentration after 5.5 h, but the benzaldehyde concentration increased continuously up to 0.36 mM benzaldehyde after 14 h of incubation.

Pathway of phenylalanine degradation

The incubation of cell extract with phenylalanine described above suggested that an aminotransferase is involved in the initial formation of phenylpyruvic acid, which is then converted into benzaldehyde. However, information on the subsequent conversion of phenylpyruvic acid to benzaldehyde was lacking. Therefore, both phenylpyruvic acid and several other compounds (proposed in the literature as intermediates in the degradation pathway of phenylalanine) were tested as the substrate for benzaldehyde production by the cell extract of *L. plantarum*. Phenylpyruvic acid was converted by the cell extract and resulted in the production of benzaldehyde at a rate six times higher than that with phenylalanine (Figure 2). Neither phenylethanol, phenylacetic acid, mandelic acid, phenylglyoxylic acid nor cinnamic acid was a substrate for the formation of benzaldehyde by the cell extract. These results strongly suggested that phenylpyruvic acid is an intermediate in the pathway leading from phenylalanine to benzaldehyde.

Conversion of phenylpyruvic acid

More detailed studies of the conversion of phenylpyruvic acid by the cell extract were performed. Surprisingly, it was observed that the rates of

benzaldehyde formation were similar in incubations containing either cell extract or boiled cell extract.

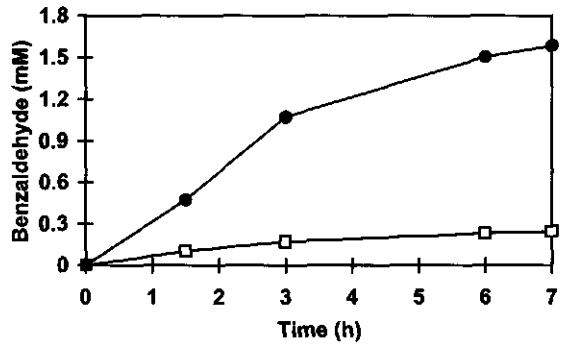

Figure 2. Benzaldehyde formation by a cell extract of *L. plantarum* using phenylalanine (❑) or phenylpyruvic acid (●) as a substrate. Incubations were performed at 37°C in 50 mM Tris buffer pH 8.0 containing 8 mM phenylalanine or phenylpyruvic acid, cell extract (11.0 mg of protein), and 0.02 mM PLP. α-Ketoglutaric acid (8 mM) was added to the mixture containing phenylalanine.

However, no benzaldehyde formation from phenylpyruvic acid was observed when the incubation systems containing either normal or boiled extract were flushed with nitrogen gas to create anoxic conditions. These observations suggested that a chemical oxidation reaction rather than an enzymatic step is involved in the conversion of phenylpyruvic acid to benzaldehyde. However, phenylpyruvic acid was not degraded if either boiled or untreated cell extract was omitted from the reaction mixture, suggesting that a component of the cell extract was essential for the conversion of phenylpyruvic acid into benzaldehyde. Several components present in the extract were tested, and it was observed that several cations could replace cell extract in the conversion of phenylpyruvic acid. Initially, the effects of Cu^{2+} ions on the conversion of phenylpyruvic acid were studied. Figure 3 demonstrates the conversion of phenylpyruvic acid into benzaldehyde in the presence of 350 μM $CuSO_4$ at 37, 30 and 25°C. The conversion of phenylpyruvic acid to benzaldehyde was temperature dependent. Decreasing the incubation temperature to 30°C or 25°C reduced the amount of phenylpyruvic acid converted after 8 h of incubation.

At 37°C, the conversion of phenylpyruvic acid was completed after 6 h of incubation, yielding 5.0 mM benzaldehyde, which corresponded to 63% conversion to benzaldehyde on a molar basis.

Besides benzaldehyde, the substrate was converted to phenylacetic acid (13% [Fig. 4C]), and mandelic acid (5.6%) and phenylglyoxylic acid (5.3%) (results not shown). The identity of these compounds was confirmed by GC-MS (phenylacetic acid) and by UV-spectra (mandelic acid and phenylglyoxylic acid).

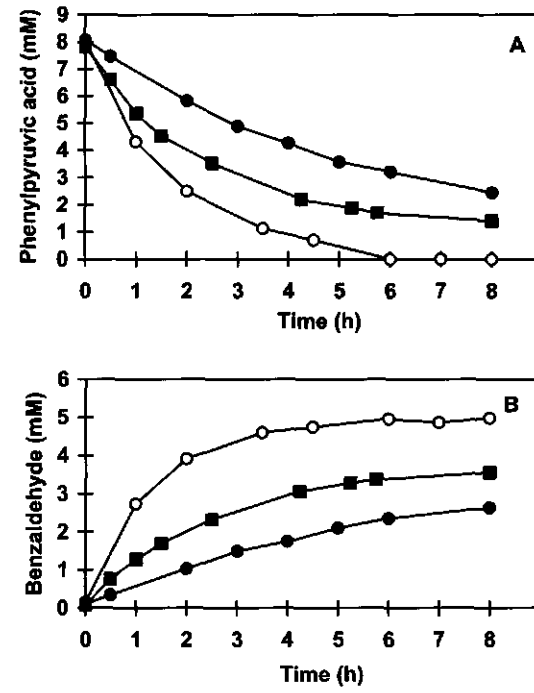

Figure 3. Chemical conversion of phenylpyruvic acid (A) to benzaldehyde (B) over time. Incubations were performed in a reaction mixture containing 8 mM phenylpyruvic acid in 50 mM Tris buffer, pH 8.0, in the presence of 350 µM $CuSO_4$ at 37 (○), 30 (■) and 25°C (●). Cell extract was omitted from the reaction mixture.

Benzoic acid was only found in trace amounts and could not account for the missing 13% of phenylpyruvic acid. Addition of $CuSO_4$ to phenylacetic acid, mandelic acid or phenylglyoxylic acid did not result in either benzaldehyde formation or degradation of these compounds.

Effect of pH on the chemical formation of benzaldehyde

Figure 4 shows the effect of pH on the chemical conversion of phenylpyruvic acid to benzaldehyde. At 37°C in the presence of $CuSO_4$, the highest conversion rate was obtained using a Tris buffer of pH 8.0. Either an increase or a decrease in pH of the reaction mixture resulted in a lower rate of phenylpyruvic acid conversion. After 8 h of incubation, 100% and 89% of the initial 8 mM of phenylpyruvic acid was converted in Tris buffer at pHs 8.0 and pH 9.0, respectively, while percentages of 65, 51 and 9 were obtained in phosphate buffer at pHs 8.0, 7.0 and 6.0,

respectively. However, increasing the pH resulted in a higher rate for phenylacetic acid formation.

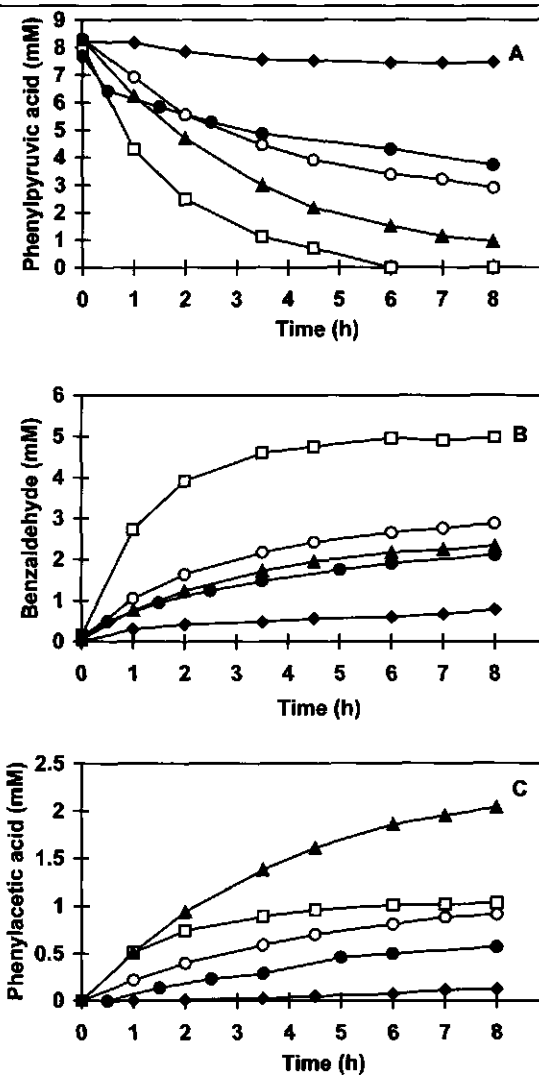

Figure 4. Chemical conversion of phenylpyruvic acid (A) and formation of benzaldehyde (B) and phenylacetic acid (C). Incubations were performed at 37°C in the presence of 350 µM $CuSO_4$, in 50 mM Tris buffer, pH 8.0 (□) and pH 9.0 (▲) or in 50 mM phosphate buffer, pH 8.0 (○), pH 7.0 (●) and pH 6.0 (◆). Cell extract was omitted from the reaction mixture.

Effect of metal ions on the chemical formation of benzaldehyde

Apart from Cu^{2+} ions, Fe^{3+}, Fe^{2+}, Mn^{2+} and Mn^{3+} ions also catalyzed the conversion of phenylpyruvic acid to benzaldehyde at different rates (Table 1). Fe^{3+} catalyzed the conversion more effectively than Fe^{2+}. Due to its poor solubility, Mn^{3+} was only tested at 10 µM and showed a slightly lower conversion of the substrate than did Mn^{2+} at this concentration. Mg^{2+} and Zn^{2+}

ions had no effect on the conversion of phenylpyruvic acid. The effect of Cu^{2+} was concentration dependent, and benzaldehyde formation was completely inhibited in the presence of the metal ion chelator EDTA or in the absence of oxygen.

Table 1. Benzaldehyde formation from phenylpyruvic acid in the presence of different metal ions

Addition to reaction mixture[a]	Benzaldehyde produced (mM)
no addition	< 0.001
350 µM Cu^{2+}	4.19
100 µM Cu^{2+}	2.13
50 µM Cu^{2+}	1.03
100 µM Cu^{2+} + 200 µM EDTA	< 0.001
100 µM Cu^{2+} flushed with N_2 gas	< 0.001
100 µM Fe^{2+}	0.22
100 µM Fe^{3+}	1.88
100 µM Mn^{2+}	1.57
10 µM Mn^{2+}	0.55
10 µM Mn^{3+}	0.38
100 µM Zn^{2+}	< 0.001
350 µM Mg^{2+}	< 0.001

[a] Each reaction mixture contained 8 mM phenylpyruvic acid in 50 mM Tris buffer, pH 8.0. Incubations were performed at 37°C. In all cases, SO_4^{2-} was the counterion of the metal ion added.

Discussion

The production of benzaldehyde from phenylalanine has been studied in various microorganisms. Fungi were investigated in most studies, but we have studied the production of benzaldehyde from phenylalanine by lactic acid bacteria because these organisms are important in the generation of flavor in dairy products.

Benzaldehyde was formed from phenylpyruvic acid, which accumulated from phenylalanine. Keto acids can be formed from amino acids by various enzymes including amino acid oxidases (Brearley *et al.*, 1994; Hemme *et al.*, 1982; Lee and Chu, 1996), aminotransferases (Hemme *et al.*, 1982; Lee *et al.*, 1985) and dehydrogenases (Asano *et al.*,

1987; Hemme *et al.*, 1982; de Boer *et al.*, 1989). In *L. plantarum*, a transaminase reaction, which depended on α-ketoglutaric acid as an amino group acceptor and on the cofactor PLP, was active. In lactic acid bacteria, very little is known about degradation of amino acids. Only recently, aminotransferases acting on several aromatic and branched-chain amino acids were purified and characterized from *Lactococcus* strains (Engels *et al.*, 1987; Yvon *et al.*, 1997).

Various research groups have worked on the biotechnological production of benzaldehyde from phenylalanine, and several metabolic pathways for benzaldehyde formation have been postulated. Krings *et al.* (1996) suggested that phenylalanine degradation is initiated by a deamination step leading to phenylpyruvic acid in the fungus *I. benzoinum*. In the next steps, phenylpyruvic acid was converted by decarboxylation, oxidation and hydroxylation reactions. Intermediates suggested in this pathway include phenylacetaldehyde, phenylacetic acid, mandelic acid, and phenylglyoxylic acid. Benzaldehyde in turn could either be oxidized to benzoic acid or reduced to benzylalcohol.

Another pathway proposed for benzaldehyde formation involves the enzyme phenylalanine-ammonia-lyase, resulting in cinnamic acid as an intermediate, as described for several fungi (Jensen *et al.*, 1994; Kawabe and Morita, 1994). Benzaldehyde formation through 2-phenylethanol was suggested by Fabre *et al.* (1996). However, intermediary molecules derived from phenylalanine consumption were not detected.

In our study, none of the intermediates in the conversion of phenylpyruvic acid to benzaldehyde suggested by the above cited authors was converted by a cell extract of *L. plantarum*. However, it was demonstrated that phenylpyruvic acid was converted to benzaldehyde in the presence of boiled cell extract, indicating that the conversion of phenylpyruvic acid in the system under study involves a chemical rather than an enzymatic step. A strong support for this conclusion is the observation that the addition of Cu^{2+}, Fe^{3+}, Fe^{2+}, Mn^{2+} or Mn^{3+} ions to phenylpyruvic acid catalyzed the formation of benzaldehyde under aerobic conditions in the absence of cell extract. Benzaldehyde formation in the presence of either untreated or boiled cell extract may therefore be due to the introduction of metal ions with the extract.

The results of the present study throw new light on the biological conversion of phenylalanine to benzaldehyde. Incubation of phenylpyruvic acid in the presence of catalyzing metal ions resulted not only in the formation of benzaldehyde but also in the generation of phenylacetic acid,

mandelic acid and phenylglyoxylic acid. These compounds were considered by Krings *et al.* (1996) to be intermediates in the route leading to benzaldehyde. These compounds did not seem to be involved in the chemical pathway leading to benzaldehyde, since no degradation of the separate compounds was observed in the presence of Cu^{2+}. They rather seem to derive from phenylpyruvic acid through a different reaction.

The role of the chemical conversion of phenylpyruvic acid to benzaldehyde in phenylalanine degradation pathways has received no attention in literature. Nevertheless, oxidation of phenylpyruvic acid to benzaldehyde and oxalic acid has been reported by Pitt (1962) as early as 1962. He observed that it was not the keto acid itself but the enol tautomer of the compound that was oxidized. The presence of bivalent metal ions can accelerate this tautomerization reaction (Bücher and Kirberger, 1952). Based on the results of the present study, a proposed enzymatic-chemical pathway for benzaldehyde formation from phenylalanine is presented in Figure 5.

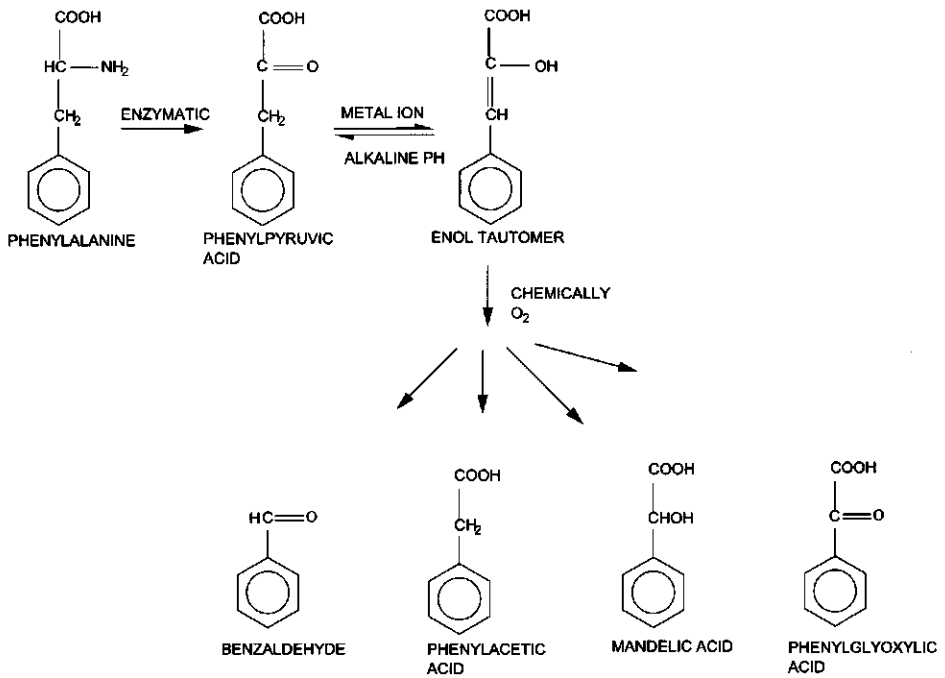

Figure 5. Proposed mechanism for benzaldehyde formation from phenylalanine by both enzymatic and chemical steps.

Phenylpyruvic acid is formed enzymatically from phenylalanine and is an unstable compound. The presence of catalyzing metal ions or alkaline conditions can enhance the enol tautomer of the keto acid, which is chemically converted to benzaldehyde, phenylacetic acid, mandelic acid and phenylglyoxylic acid in the presence of oxygen.
Benzaldehyde may be further oxidized to benzoic acid, although in our study only trace amounts of this compound were detected. More recently, Casey and Dobb (1992) converted microbially produced phenylpyruvic acid to benzaldehyde by heating the fermentation broth containing phenylpyruvic acid to 90°C at pH 9.5. The results of the present study show that in the presence of catalyzing metal ions, the conversion of phenylpyruvic acid to benzaldehyde can occur under far milder conditions.

The phosphate buffer may bind the metal ions in the reaction mix and thereby reduce the availability of the metal ion. This can cause the difference between benzaldehyde formation at pH 8.0 in phosphate buffer and that in Tris buffer. The reduced conversion rate of phenylpyruvic acid to benzaldehyde at pH 9.0 compared with that pH 8.0 in Tris buffer may be explained by a dual effect of the pH on the chemical conversion rate. The formation of the enol tautomer is induced by alkaline conditions (Pitt, 1962). This effect may be opposed by the reduced solubility of metal ions under alkaline conditions. It can be speculated that the effect of pH on the chemical conversion of phenylpyruvic acid is a combination of these two effects.

Amino acid degradation is believed to be important for flavor development in cheese. Straight-out chemical reactions are not believed to play a major role in the production of cheese flavor but rather seem to be enhanced by enzymes (Urbach, 1995). In this study, benzaldehyde formation was initiated by an aminotransferase present in the cell extract of *L. plantarum*. Aminotransferases from lactic acid bacteria have shown activity under cheese-ripening conditions (Engels *et al.*, 1997; Yvon *et al.*, 1997), but the low oxygen concentration, low ripening temperature and low pH in cheese do not favor the chemical conversion of phenylpyruvic acid to benzaldehyde. However, considering the long time involved in cheese ripening, this mechanism may still make a significant contribution.

Acknowledgments
We thank Henk Swarts for performing the GC-MS analyses.

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Chapter 4.

Involvement of Manganese in the Conversion of Phenylalanine to Benzaldehyde by Lactic Acid Bacteria

Masja N. Nierop Groot and Jan A. M. de Bont

Abstract

We examined the involvement of Mn^{2+} in the conversion of phenylalanine to benzaldehyde in cell extracts of lactic acid bacteria. Experiments performed with *Lactobacillus plantarum* demonstrated Mn^{2+}, present at high levels in this strain, is involved in benzaldehyde formation by catalyzing the conversion of phenylpyruvic acid. Experiments performed with various lactic acid bacterial strains belonging to different genera revealed that benzaldehyde formation in a strain was related to a high Mn^{2+} level.

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Degradation of amino acids by lactic acid bacteria (LAB) is important for the generation of flavor compounds during cheese ripening. We previously described that the conversion of phenylalanine to benzaldehyde in cell extracts of *Lactobacillus plantarum* (Nierop Groot and de Bont, 1998) differs from the pathways described for fungi and for *Pseudomonas putida* (Fabre *et al.*, 1996, Jensen *et al.*, 1994, Kawabe and Morita, 1994, Krings *et al.*, 1996, Lapadatescu *et al.*, 1997, Simmonds and Robinson, 1998). In *L. plantarum*, the conversion of phenylalanine to benzaldehyde involves both an enzymatic step and a chemical reaction. In the cell extract of this strain, phenylalanine is initially converted to phenylpyruvic acid by the action of an aminotransferase. In the presence of oxygen, the keto acid is then oxidized to benzaldehyde in a non-enzymatic reaction. We demonstrated that this oxidation step depended on one or more unidentified, heat-stable components from the cell extract. However, in the absence of cell extract, phenylpyruvic acid was easily converted to benzaldehyde under mild conditions after addition of several metal ions, suggesting that this chemical step, may be due to the presence of one or more metal ions in the cell extract.

Metal ions are involved in several functions in the metabolism of LAB, e.g as the catalytic centers of enzymes (for a review, see Boyaval (1989)). The so-called micronutrients, which are usually present at very low concentrations in microorganisms, include the metal ions manganese (Mn), iron (Fe), cobalt (Co) and copper (Cu) (Archibald and Fridovich, 1981). However, in several LAB, including *L. plantarum*, the intracellular level of Mn^{2+} is extremely high compared to the level of other metal ions (Archibald, 1986). This makes Mn^{2+} a possible candidate for the component in the cell extract involved in the chemical conversion of phenylpyruvic acid. The reported biological effects of Mn^{2+} are numerous and include structuring and activation of enzymes, detoxification of chemicals harmful to the bacterial cell, and stabilization of subcellular entities (Raccach, 1985). Besides contributing to the biological functions of Mn^{2+} described above, Mn^{2+} can be used by *L. plantarum* for a different purpose. Archibald and Fridovich (1981) demonstrated that *L. plantarum* can accumulate Mn^{2+} to high intracellular levels as a defense mechanism against oxygen toxicity. This strain lacks the enzyme superoxide dismutase, which is present in most aerobic and oxygen-tolerant microorganisms. In the present work, we studied the role of manganese in flavor production not only in *L. plantarum,* but also in a number of other LAB.

Addition of metal ions to dialyzed cell extracts

In our previous report, we demonstrated that several metal ions can catalyze the conversion of phenylpyruvic acid to benzaldehyde in the absence of cell extract. However, the availability of the metal ions may be reduced in a cell extract-containing system due to binding of the metal ions to components present in the cell extract. To test the catalyzing properties of the metal ions in the presence of cell extract, we compared the formation of benzaldehyde in both dialyzed and undialyzed extracts and in dialyzed extracts to which either Cu^{2+}, Mn^{2+}, Fe^{2+} or Co^{2+} was added.

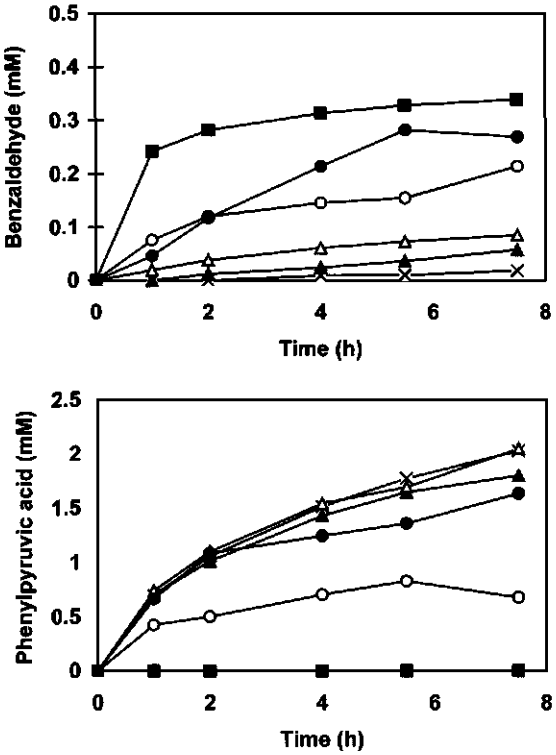

Figure 1: The formation of benzaldehyde (A) and phenylpyruvic acid (B) over time. Incubations were performed with both an undialyzed cell extract (O) and dialyzed cell extracts of *L. plantarum* LcL1 (cells grown in MRS medium) without addition of metal ions (X) and in the presence of either 350 µM Mn^{2+} (■), Fe^{2+} (●), Co^{2+} (Δ), or Cu^{2+} (▲). Incubations were performed at 37 °C in 50 mM Tris buffer (pH 8.0) containing 8 mM phenylalanine, 8 mM α-ketoglutaric acid, and 0.02 mM pyridoxal 5'-phosphate. A total of 1.70 mg of protein was present in the reaction mixture. In all cases, SO_4^{2-} was the counterion of the metal ion added. The values represent the averages of duplicate incubations and generally varied from the mean by no more than 10 %.

The metal ions were added to a final concentration of 350 µM from filter-sterilized stock solutions of either $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$ or $CoSO_4 \cdot 7H_2O$. Preparation of the cell extract from MRS (Merck)-grown cells of *L. plantarum* LcL1 and incubation of the extracts with phenylalanine was performed as described previously (Nierop Groot and de Bont, 1998). Dialysis of the cell extract was performed overnight at 4 °C against 50 mM potassium phosphate buffer (pH 7.0) containing 0.02

mM pyridoxal 5′ -phosphate. Cell extracts were stored at –20 °C for not more than 1 week until further use. Figure 1 shows that in keeping with our previous report, phenylpyruvic acid and benzaldehyde were formed upon incubation with phenylalanine in the undialyzed extract. However, no benzaldehyde was formed in the dialyzed extract; instead, phenylpyruvic acid accumulated in the cell extract over time. Addition of either Fe^{2+}, Mn^{2+}, Co^{2+} or Cu^{2+} to the dialyzed extracts restored the conversion of phenylpyruvic acid to benzaldehyde, although the rate of benzaldehyde formation was depended on the metal ion that was added to the extract. Besides benzaldehyde and phenylpyruvic acid, small amounts of mandelic acid (0.16 and 0.12 mM), phenylglyoxylic acid (0.05 and 0.07 mM) and phenylacetic acid (0.1 mM and 0.05 mM) were formed in the undialyzed cell extract and in the Mn^{2+}-supplemented cell extract, respectively. We previously observed that in the absence of cell extract, the conversion of phenylpyruvic decreased in the order $Cu^{2+} > Mn^{2+} > Fe^{2+}$. However, in the presence of dialyzed cell extract, benzaldehyde formation falls in the order $Mn^{2+} > Fe^{2+} > Cu^{2+}$. This effect may be explained by the stability constants for complex formation of the metal ions with various ligands. These constants follow the order $Mn^{2+} < Fe^{2+} < Co^{2+} < Cu^{2+}$ (Sigel and McCormick, 1970, Vallee and Coleman, 1964).

Analysis of the metals ions in the extract

We analyzed the level of Mn, Fe, Cu and Co in both the undialyzed and the dialyzed cell extracts of the MRS-grown *L. plantarum* LcL1 by inductivity-coupled plasma mass spectrometry (ICP-MS). These analyses were performed at the Department of Soil Science and Plant Nutrition of the Wageningen University (The Netherlands). The MRS medium used for cultivation of *L. plantarum* LcL1 contained Mn at 144 µM as determined by ICP-MS. Manganese was present in the undialyzed extract at 8.8 µg/mg protein which was extremely high compared to the level of Fe, Cu, and Co in the same extract. The difference in concentration of these metals was over a factor 80. If we assume that the specific internal volume is 3 µl/mg protein as reported for *L. plantarum* (Glaasker *et al.*, 1998), then it can be calculated that the intracellular Mn^{2+} concentration is as high as 53 mM. This indicates that, considering the Mn^{2+} concentration in the medium, this metal must have been transported by an active uptake system. The dialyzed extract contained only 0.05 µg of Mn/mg protein while the concentrations of the other metal ions tested were even lower. The amount of Mn present in the undialyzed cell extract of *L. plantarum* LcL1

accounts for a final concentration of 54 μM in the reaction mixture. For Fe and Cu, this value is below 1 μM, and Co is present in the nanomolar range. Therefore, the latter metal ions are not very likely to have a significant contribution to the benzaldehyde formation in the extract compared to that of Mn^{2+}.

Since Mn^{2+} seems to be important in benzaldehyde formation, we used a chemically defined medium (Kets *et al.*, 1994) for cultivation of *L. plantarum* LcL1 to study the role of this metal ion in more detail. In this medium, the concentration of Mn^{2+} could be varied while the concentration of Co^{2+}, Fe^{2+} and Cu^{2+} was kept at 0.8, 7.5 and 0.01 μM respectively. Erlenmeyer flasks of 500 ml containing 150 ml medium, supplemented with either 10, 25, 200, 300 or 500 μM of $MnSO_4$, were inoculated with cells grown overnight in medium containing the same concentrations. Cells were cultured overnight at 30 °C, and cell extracts were prepared as described previously (Nierop Groot and de Bont, 1998).

Table 1: Effect of Mn^{2+} concentration in the medium on the amount of benzaldehyde and phenylpyruvic acid formed in cell extracts of *L. plantarum* LcL1and the Mn^{2+} content of these extracts[a].

Mn^{2+} in medium (μM)	Conc. in extract[b]		
	Benzaldehyde[a] (nmol/mg protein)	Phenylpyruvic acid (nmol/mg protein)	Mn^{2+} (μg/mg protein)
10	67	1,836	1.3
25	241	391	2.4
200	683	< 300	14.5
300	766	< 300	11.1
500	767	< 300	9.6

[a] Cells were grown overnight in chemically defined medium (Kets *et al.*, 1994) containing Mn_2SO_4 at different concentrations. Incubations were performed as described in the legend to Figure 1. Each value represents the mean of duplicate measurements and varied from the mean by no more than 15%.

[b] Measured after 4 h of incubation.

The protein concentration of the cell extracts were determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. The amount of benzaldehyde formed in the cell extract after 4 h of incubation with phenylalanine increased with increasing levels of Mn^{2+} in the culture medium, up to a concentration of 200 μM (Table 1). Similarly, the Mn^{2+} content of the cell extracts increased with increasing levels of Mn^{2+} in the culture medium, up to a concentration of 200 μM. In the extracts of cells grown in medium containing 10μM or 25μM of Mn^{2+},

phenylpyruvic acid accumulated in the medium due to a limited conversion of the keto acid to benzaldehyde. In keeping with this finding, these extracts contained low levels of Mn^{2+}.

Inhibition of the aminotransferase by manganese

We observed that in the undialyzed cell extract, the amounts of phenylpyruvic acid and the metabolites formed from this compound together accounted for only 59% of the phenylpyruvic acid formed in the dialyzed extract. In the Mn^{2+}-supplemented cell extract, the proportion was only 29%. We therefore tested to see if either benzaldehyde or Mn^{2+} had a negative effect on the activity of the aminotransferase. Addition of up to 0.5 mM benzaldehyde to a dialyzed cell extract of *L. plantarum* NC8 showed no decrease in the formation of phenylpyruvic acid by the enzyme (results not shown). In order to demonstrate an effect of Mn^{2+} on the aminotransferase activity, we had to determine the formation of glutamic acid, instead of the formation of phenylpyruvic acid, over time. Glutamic acid is the transaminated product of α-ketoglutaric acid that arises when phenylalanine is converted to phenylpyruvic acid.

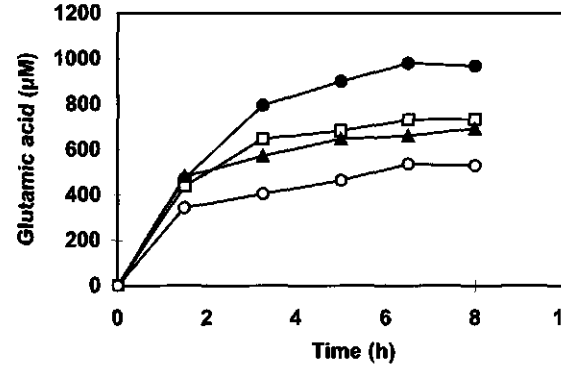

Figure 2: The formation of glutamic acid over time in a dialyzed cell extract of *L. plantarum* NC8 without the addition of Mn^{2+} (●) and in the presence of either 25 µM (□), 100 µM (▲), or 350 µM (○) Mn^{2+}. Incubations were performed under the conditions described in the legend to Figure 1. The values represent the average of duplicate incubations and varied from the means by no more than 10 per cent. Cells were grown in MRS medium. A total of 5.56 mg of protein was present in the reaction mixture.

Since Mn^{2+} ions catalyze the conversion of phenylpyruvic acid, it was not possible to study the effect of this metal ion by measuring the amount of phenylpyruvic acid formed. Glutamic acid concentrations were determined by the method of Kunte *et al.* (1993) with a Chromspher5 C18 column (Chrompack, Bergen op Zoom, The Netherlands). Figure 2 demonstrates that addition of Mn^{2+} to a dialyzed cell extract of *L. plantarum* NC8 indeed

reduces the amount of glutamic acid formed by the aminotransferase. In the dialyzed extracts supplemented with either 25, 100 or 350 μM Mn^{2+}, the amount of glutamic acid formed after 8 h of incubation was reduced by 25, 30 and 50%, respectively, compared to the amount in the manganese-free extract. The inhibition of the aminotransferase by Mn^{2+} reduces the yield in the Mn^{2+}-containing extracts compared to the dialyzed extract. There was no reduction in yield for the Co^{2+}, Fe^{2+} and Cu^{2+} supplemented extracts.

Table 2: Amounts of benzaldehyde and phenylpyruvic acid formed in dialyzed or undialyzed cell extracts of LAB and the Mn^{2+} contents of these extracts

Strain[c]	Conc. in extract[a]			
	Benzaldehyde (nmol/mg of protein), undialyzed	Phenylpyruvic acid (nmol/mg of protein)		Mn^{2+} (μg/mg of protein), undialyzed
		undialyzed	dialyzed	
L. plantarum LcL1	908	<300	2,283	11.10
L. plantarum NC8	582	<300	4,167	12.63
L. casei ssp. *casei* DSM 20011	1,196	<300	3,570	26.10
Leuc. paramesent. DSM 20288	1,266	<300	8,424	5.28
Leuc. lactis DSM 20192	<30	<300	<300	7.54
L. helveticus ATCC 15009[b]	217	<300	1,697	0.43
L. fermentum ATCC 9338	170	<300	849	6.27
L. lactis ssp. *lactis* NIZO C17	<30	3188	4,239	0.74
L. lactis ssp. *lactis* NCDO 712	74	8036	8,344	1.19
L. lactis ssp. *lactis* NIZO B26	<30	10274	12,254	0.29
L. lactis ssp. *lactis* NIZO B27	<30	6221	6,974	0.33

[a] Measured after 8 h of incubation, except for strains LcL1, B26 and B27; these values were obtained after 7 h of incubation.

[b] *L. helveticus* was grown in MRS broth.

[c] Strains were obtained from American Type Culture Collection, Rockville, Md. (ATTC); Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM); National Collection of Dairy Organisms, Reading, UK (NCDO); NIZO Food Research, Ede, The Netherlands (NIZO); Unilever Research Laboratory, Vlaardingen, The Netherlands (for LcL1); Laboratoire de Génétique Moléculaire, Université Catholique de Louvain, Louvain-la-Neuve, Belgium (for NC8).

Benzaldehyde formation in cell extracts of other LAB

We examined whether the relation between benzaldehyde formation and Mn^{2+} accumulation was restricted to *L. plantarum* or whether it could be observed for other LAB strains. Therefore, cell extracts of strains belonging to several different genera were tested for benzaldehyde formation from phenylalanine. For this experiment, cells were grown in chemically defined medium containing 300 μM of $MnSO_4$ and 10 g of glucose per liter. The *Lactobacillus* and *Leuconostoc* strains and *L. lactis*

B26 and B27 were grown in the medium described elsewhere (Kets *et al.*, 1994). The other *Lactococcus* strains were cultured in the medium described by Poolman and Konings (1988). All strains were grown at 30 °C, except for *L. helveticus* and *L. fermentum*; these strains were incubated at 37 °C. Table 2 shows that benzaldehyde was formed not only in *L. plantarum* extracts, but was also in the extracts of several other LAB. Benzaldehyde-forming strains were distributed among the genera *Lactobacillus* and *Leuconostoc*. Benzaldehyde formation by a strain correlated with high levels of Mn^{2+} in the extract. *Leuconostoc lactis* and *Lactobacillus fermentum* were exceptions, showing high Mn^{2+} contents but very poor benzaldehyde formation. In these strains, phenylpyruvic acid generation is limiting due to either low or no activity of the aminotransferase towards phenylalanine. Only minor amounts of benzaldehyde were formed in extracts of the four *Lactococcus lactis* strains. Since the production of phenylpyruvic acid in these extracts was high, the limited benzaldehyde formation can be attributed to the low level of Mn^{2+} in these extracts compared to the benzaldehyde-forming strains. Besides Mn, the levels of Co, Fe and Cu in all the extracts were determined. These values were below 0.02, 0.09 and 0.1 μg/mg protein for Co, Fe and Cu, respectively.

The work reported by Archibald and Fridovich (1981) showed that manganese accumulation in LAB provide the cells with a defense mechanism against the toxic effects of oxygen. In the present study, we clearly demonstrate that accumulation of Mn^{2+} has a surprising additional effect. We showed that in LAB that convert phenylalanine to phenylpyruvic acid, benzaldehyde is formed when these strains contain a large Mn^{2+} pool. The difference between the intracellular Mn^{2+} concentration and the concentration in the medium suggest that those strains must have a system for active uptake of Mn^{2+}. Therefore, benzaldehyde formation in LAB by the mechanism we described previously (Nierop Groot and de Bont, 1998), seems to be related to the presence of an active uptake system for Mn^{2+} in the strain. An interesting perspective of these results could be obtained by transferring the Mn^{2+} uptake system from *L. plantarum* into the more widely used *Lactococcus lactis,* thereby directing phenylpyruvic acid generation in this strain towards benzaldehyde.

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Chapter 5.

Identification and transcriptional Analysis of an ABC-Transporter and two Nramp Type Transporters that are induced upon Manganese Starvation in *Lactobacillus plantarum*

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Abstract

Manganese serves an important function in *Lactobacillus plantarum* in protection against oxidative stress and this bacterium can import Mn^{2+} up to millimolar levels intracellularly. Our knowledge on the transport systems that can potentially accomplish Mn^{2+} uptake in *L. plantarum* is very limited. The results described in this study show, that in addition to the recently described P-type ATPase, *L. plantarum* contains at least three more transport systems that are induced upon Mn^{2+} starvation. One of these systems (MtsCBA) belongs to the ABC-type transporter family for metal uptake, a group that is rapidly expanding. The *mtsCBA* genes have been cloned both from *L. plantarum* and *L. casei,* and both gene clusters appeared to be highly similar. In addition to the ABC transporter genes, *L. plantarum* harbors three genes encoding proteins belonging to the natural resistance-associated macrophage proteins (Nramp). The transcription of two of these genes (*mntH1* and *mntH2*) and the *mtsCBA* genes were induced upon Mn^{2+} starvation, suggesting a rol in the uptake of this metal ion. Strains were constructed that carry a deletion in either *mtsA*, encoding the substrate-binding protein of the ABC-transporter, or in *mntH2*. Inactivation of either the individual or both transporters did not result in an obvious mutant phenotype under the conditions tested. Nevertheless, transcriptional analysis revealed that the *mtsCBA* operon was upregulated in the *mntH2* mutant strain. Vice versa, in a *mtsA* mutant strain, a slight increase in *mntH2* transcript levels was detected. This suggest that in *L. plantarum* cross regulation occurs between MtsCBA and MntH2 transporters. No effect on *mntH1* expression was observed in the mutant strains, and combined with the fact that growth of a *mtsA-mntH2* double mutant was not affected, it can be assumed that additional transporters are involved in Mn^{2+} homeostasis. Results in this study indicate that regulation of Mn^{2+} homeostasis in *L. plantarum* is very efficient and possibly involves more transporters than have been identified to date.

Introduction

Bacteria depend on efficient uptake systems to extract essential trace metals from the environment. The Mn^{2+} ion is an important trace metal for most bacteria, either as cofactor in the catalytic centre of enzymes or in manganese-dependent biological activities in a number of metabolic functions. Previous measurements of Mn^{2+} in lactic acid bacteria (LAB) revealed that the level of this metal ion is high in *Lactobacillus plantarum* and *Lactobacillus casei,* compared to other LAB (Archibald and Fridovich, 1981a,b; Nierop Groot and de Bont, 1999). The physiological role of Mn^{2+}, and the uptake of this metal ion, has been well documented for *L. plantarum* (Archibald and Fridovich, 1981b; Archibald and Duong, 1984). In this species, superoxide dismutase (SOD) activity is lacking and therefore, Mn^{2+} is of extreme importance to tolerate oxygen stress. The high level of Mn^{2+} in the cells acts as a scavenger of toxic oxygen species, especially O_2^- and requires the presence of efficient Mn^{2+} uptake systems. The millimolar level of intracellular Mn^{2+} thereby replace the function of SOD which contains micromolar levels of Mn^{2+} as cofactor in most oxygen-tolerant micro-organisms.

While ample evidence for the presence of an active Mn^{2+} uptake system in *L. plantarum* has been provided (Archibald and Duong, 1984), only recently a gene encoding a high-affinity uptake system in *L. plantarum* with affinity for Mn^{2+} was reported (Hao *et al.*, 1999). This gene encodes a P-type ATPase (MntA), involved in Cd^{2+}- and Mn^{2+}-uptake. Expression of the *mntA* gene in *Escherichia coli* conferred increased sensitivity for Cd^{2+} and increased Cd^{2+}-uptake on these cells (Hao *et al.*, 1999). Although synthesis of MntA was demonstrated to be increased upon Mn^{2+} starvation in *L. plantarum*, the role of MntA in this bacterium remains to be determined and requires the analysis of a *L. plantarum mntA* deletion strain.

Recently, it was demonstrated that Mn^{2+} uptake is mediated by an ATP-binding cassette (ABC) family of transporters in *Synechocystis* (Bartsevich and Pakrasi, 1995) and several Gram-positive bacteria (Dintilhac *et al.*, 1997; Kolenbrander *et al.*, 1998; Bearden *et al.*, 1999). The cell-surface lipoprotein of these transporters belongs to the lipoprotein receptor antigen I (LraI) family that was initially believed to be directly involved in virulence as adhesin (Sampson *et al.*, 1994). However, similar proteins in *Streptococcus pneumoniae* (PsaA) and *S. gordonii* (ScaA) were found to constitute the extracytoplasmic substrate recognition subunit

of an ABC transporter for Mn^{2+} (Dintilhac *et al.*, 1997; Kolenbrander *et al.*, 1998) and mutation of the *scaA* gene resulted in decreased Mn^{2+}-uptake and impaired growth of *S. gordonii* in media containing low concentrations of Mn^{2+}. Additonally, *S. pneumoniae psaA* mutant cells showed increased requirements for Mn^{2+}, suggesting a role of this gene in Mn^{2+} import. Similar binding-proteins have been described in at least nine additional species of *Streptococcus* (see review by Claverys, 2001). Of these extracytoplasmic binding-proteins, a role in Fe^{2+} and/or Mn^{2+} uptake has been confirmed for *S. epidermis* (Cockayne *et al.*, 1998), *Enterococcus faecalis* (Lowe *et al.*, 1995) and *S. mutans* (Spatafora *et al.*, 2001). In addition, ABC-transporters can have specifity for multiple metal ions as was reported for *S. pyogenes* (Janulczyk *et al.*, 1999). A function of the lipoprotein of the ABC-transporter complex in virulence has been demonstrated experimentally in animal models for *S. parasanguis* (FimA, Burnette-Curley *et al.*, 1995), *S. pneumoniae* (PsaA, Berry and Paton, 1996), *S. mutans* (SloC, Kitten *et al.*, 2000) and *Enterococcus feacalis* (EfaA, Singh *et al.*, 1998).

Recently, a third type of transporter was reported to be involved in Mn^{2+} uptake in bacteria. Homologues of the mammalian Nramp (natural resistance-associated macrophage protein) transporters were identified in *Bacillus subtilis* (Que and Hellmann, 2000), *Salmonella typhimurium* (Kehres *et al.*, 2000) and *Escherichia coli* (Makui *et al.*, 2000), that conferred high-affinity uptake of Mn^{2+}. The Nramp proteins were initially characterized in mammalian species as divalent metal transporters that confer the ability to control invasion of pathogens on macrophages and other monocytes (see Govoni and Gros, 1998 for a review). At least two copies of closely related genes (*nramp1* and *nramp2*) have been identified on seperate chromosomes in both mice and humans. Studies in *Xenopus oocytes* have demonstrated that Nramp2 is a pH-dependent high-affinity Fe^{2+} uptake system with broad substrate specifity including Mn^{2+}, Zn^{2+} and Co^{2+} and Cd^{2+} (Gunshin *et al.*, 1997). A function of the mammalian Nramp1 protein in Mn^{2+}-transport has recently been demonstrated using mice-derived phagosomes (Jabado *et al.*, 2000). Interestingly, the bacterial Nramp homologues show a high degree of similarity to the mammalian proteins. Cloning, regulatory and biochemical studies of the enterobacterial (Kehres *et al.*, 2000) and the *B. subtilis* (Que and Helmann, 2000) *nramp* genes demonstrated, that they encode proton-stimulated, highly selective Mn^{2+} transporters that play a role in bacterial response to reactive oxygen species (Kehres *et al.*, 2000). It has been

suggested that, in analogy to *L. plantarum*, these transporters supply the cells with aditional, non-enzymic protection against oxidative stress (Kehres *et al.*, 2000).

Considering the importance for *L. plantarum* to maintain the intracellular concentration of Mn^{2+} at millimolar levels, a highly efficient and tightly controlled system, to fulfill this requirement is expected. At present, information on the types of transporters for Mn^{2+}, and the genes encoding these systems in *L. plantarum* is very limited. In this study, we present data on the presence of three transport systems in *L. plantarum* that are induced by Mn^{2+} starvation. Relative expression levels of *mntH1* and *mntH2* (both encoding Nramp-type transporters) and *mtsCBA* (encoding an ABC-type transporter) have been studied both in wild type and in mutants that are affected in one or two of the transport systems described.

Materials and methods

Bacterial strains and culturing conditions

Strains, plasmids and oligonucleotides used in this study are listed in Table 1a and 1b. *L. plantarum* and *L. casei* were grown in MRS broth (De Man, Rogosa and Sharpes, Oxoid), or when mentioned in the results section in a chemically defined medium (CDM, Kets *et al.*, 1994). *L. lactis* was cultured without aeration at 30 °C in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5 % (wt/vol) glucose. *E. coli* strains were grown with aeration at 37 °C in Tryptone Yeast (TY) medium (Sambrook *et al.*, 1989). Antibiotics were added to the medium when appropiate, at the following concentrations: erythromycin (Em) 10 µg/ml (*L. plantarum*) and 250 µg/ml (*E. coli*); lincomycin (Lm) 20 µg/ml (*L. plantarum*); ampicillin (Am) 100 µg/ml (*E. coli*); kanamycin (Km) 50 µg/ml (*E. coli*); chloramphenicol (Cm) 5 µg/ml (*L. lactis*).

DNA isolation, manipulations and sequence analysis

Chromosomal DNA of *L. plantarum* and *L. casei* was prepared as described previously (Bernard *et al.*, 1991). A JetStar column (Genomed GmbH, Bad Oberhausen, Germany) was used for large scale isolations of *E. coli* plasmid DNA following the instructions of the manufacturer. Isolation of small-scale plasmid DNA of *E. coli* and standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989).

L. plantarum was transformed by electroporation as described by Aukrust and Blom (1992) and *E. coli* cells were transformed by the $CaCl_2$ procedure (Sambrook *et al.*, 1989). PCR amplifications were carried out with an automated thermal cycler (Perkin-Elmer, Nieuwekerk a/d Ijssel, The Netherlands) using either *Taq* DNA polymerase or *Pwo* polymerase (Gibco/BRL Life Technologies, The Netherlands). DNA was isolated from agarose gels by using a QIAEX II gel extraction kit (Pharmacia Biotech, Uppsala, Sweden). A non-radioactive dioxygenin DNA labelling and detection kit (Roche Diagnostics Nederland BV, Almere, The Netherlands) was used to label and detect probes for Southern blotting or colony hybridization. For hybridization and detection during Southern blotting, the instructions of the manufacturer were followed. Nucleotide sequences of DNA were analysed with an ALFred automatic DNA sequencer (Pharmacia Biotech) using Cy5-labeled primers and the AutoRead sequencing kit (Pharmacia Biotech).

Cloning of *mtsCBA* from *L.casei* and *L.plantarum*

For the cloning of the *mtsCBA* genes of *L. plantarum* and *L. casei*, degenerate primers were designed on basis of conserved protein sequences derived from multiple alignment of members of the LraI protein family; ScaA (*Streptococcus gordonii,* acc. no. P42364), FimA (*S. parasanguis*, M26130), PsaA (*S. pneumoniae*, U53509), ScbA (*S. crista*, U46542) and EfaA (*Enterococcus faecalis*, U03756). Primers LPSBP2 and LPSBPR4 (see Table 1b) were based on the conserved amino acid sequences NLETGGNAW and DSYYSMM(K/N), respectively. Primers LCSBP1 and LCSBPR4 (Table 1b) were based on the amino acid stretches DPHEYEPLPE and EG(C/A)FKYFS of MtsA respectively. The degenerate primers were applied in PCR reactions using the following conditions: 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 42°C, 2 or 3 minutes at 72 °C and a 1 final incubation of 10 min at 72 °C. PCR amplifications using the primers LPSBP2 and LPSBPR4 and *L. plantarum* chromosomal DNA as a template, yielded a 620 bp fragment (PCR1), while the primers LCSBP1 and LCSBPR4 and *L. casei* chromosomal DNA resulted in 440 bp fragment (PCR2). Both products were cloned in pGEM-T (purchased from Promega Biotech, Roosendaal, The Netherlands) and the resulting plasmids were designated pGEM-TPCR1 and pGEM-TPCR2, respectively. Both cloned fragments were sequenced and appeared to encompass an internal fragment of a gene encoding a putative substrate-binding protein (SBP).

Table 1a. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic	References
Strains		
E. coli		
TG1		Sambrook *et al.*, 1989
DH5α		Hannahan,1983
L. plantarum		
NC8	silage isolate, plasmid free	Aukrust and Blom, 1992
WCFS1		WCFS collection
WCFS1Δ*mtsA*	WCFS1 derivative, Δ*mtsA*	This study
WCFS1Δ*mntH2*	WCFS1 derivative, Δ*mntH2*	This study
L. casei subsp. *casei* DSM 20011		DSM
L. lactis NZ9000	MG1363 *pepN* ::*nisRK*	Kuipers *et al.*, 1998
Plasmids		
pGEM-T	PCR cloning vector, Amp^R	Promega
pCRblunt	cloning vector for blunt PCR fragments, Km^R	Promega
pGEM-TPCR1	pGEM-T containing a 620 bp PCR fragment of *mtsA L. plantarum*	This study
pGEM-TPCR2	pGEM-T containing a 440 bp PCR fragment of *mtsA* of *L. casei*	This study
pUC18ERY	Amp^R, Ery^R	Kranenburg *et al.*, 1997
pNZ8020	expression vector carrying the *nisA* promoter, Cm^R	De Ruyter *et al.*, 1996b
pMN027	pNZ8020 derivative, Cm^R, harboring *mtsCBA* from *L. casei*	This study
pMN011	pUC18ERY derivative, Amp^R, Ery^R, knock out construct *mtsA*	This study
pMN038	pUC18ERY derivative, Amp^R, Ery^R, harboring a 649 bp fragment of *mntH2*	This study
pMN061	pMN038 derivative, Amp^R. Ery^R, knock out construct *mntH2*	This study
pMTL 22P		Chambers *et al.*, 1988
pTZ19R	Amp^R	Pharmacia
pMN070	pMTL 22P derivative containing a 2 kb *Hin*dIII fragment of *L. casei*	This study
pMN071	pTZ19R derivative containing a 2.2 kb *Eco*RI-*Kpn*I fragment of *L. casei*	This study

Table 1b. Oligonucleotides used in this study

Oligonucleotides (5′ - 3′)	Sequence
LPSBP2	AAY YTN GAR CAN GGI GGI AAY GSN TGG[1]
LPSBPR4	NTT CAT CAT ISW RTA RTA NVW RTC
LCSBP1	GAY CCI CAY GAR TAY GAR CCI YTN CCN GA
LCSBPR4	WRA ART AYT TIR RNS MNC CYT C
LCSBPF1	TGAAGCCGATGTTGTCTTCCACAATGG
LCSBPR1	ACCGCTGGCGTTCCGGAATATCAAGG
LPATPF3	TGGGCCTATAATTCGGTTGTG
LPATPR3	CGAGCCATCACCAACAATTAC
LPATPF4	ATGACGACGACGGTCGTGTTAG
LPATPR4	CCGCAAAAACATCGTGGTCAAAG
LPATPF6	TCAGCCATGGAAACGAAATTAATTGCAC
LPATPR6	ACTGTCTAGACAACCGAATTATAGGCC
LPMNTH1F	GCATTACTTTCTGTGATCCTG
LPMNTH1R	CATTGTACAAGTCAACGAACC
LPMNTH2AF	CATGCTAATGTCTGTCATCTTG
LPMNTH2AR	CATAAAGACCGAAGAATGAAGG
LPMNTH2BF	AATATCTAGACAGCAACTTGCTGCAGC
LPMNTH2BR	AAAAGTCGACGCTGTCAACATAAATAGCG
LCABCF	CGTACTGCAGGGTCACCCTAAAAATTGGAGGC
LCABCR	GCGCTCTAGAGTTCAAACGAGCGTCTTGGTGC
16SP1	GCGGCGTGCCTAATACATGC
16SP2	ATCTACGCATTTCACCGCTAC
LPABCSEQ	CTGTATGCCACGGTGAGG
LPH1SEQ	TCTTAGCGTGATCCTTCG
LPH2SEQ	GGAACCTCGATGGTACCG

[1] The codes used for the mixed bases are as follows: **Y**= C+T; **N**= A+C+G+T; **R**= A+G; **M**= A+C; **S**= C+G; **W**= A+T; **V**= A+C+G; **I**= deoxyinosine.

The homology of the corresponding amino acid sequences with ScaA and PsaA was high; 52 and 56% identity, respectively.

In order to clone the expected three genes from *L. casei*, a 352 bp PCR fragment (primers LCSBPF1 and LCSBPR1) of *mtsA* was used as probe in Southern hybridization. A 2 kb *Hin*dIII and a 2.3 kb *Eco*RI-*Kpn*I fragment of chromosomal DNA of *L. casei* hybridized with this probe. On basis of these results, enriched *Hin*dIII and *Eco*RI-*Kpn*I digested *L. casei* chromosomal libraries were constructed in either pMTL 23P (*Hin*dIII digested) or pTZ19R (*Eco*RI-*Kpn*I digested) and transformed to *E. coli*. Clones harboring the correct inserts were selected by colony hybridization using the *mtsA* internal fragment as probe. Two clones, containing either a 2 kb insert (covering *mtsB, mtsC* and the 5′ -region of *mtsA*) or a 2.3 kb insert (containing *mtsB* and *mtsA*) were selected and were designated pMN070 and pMN071, respectively. The inserts of these constructs were sequenced on both strands. The *L. plantarum*-derived DNA sequence was used to screen the *Lactobacillus plantarum* Genome Project database.

Construction of *mtsA* and *mntH2* mutants

In order to inactivate the *mtsA* and *mntH2* genes in *L. plantarum* WCFS1 by double cross-over recombination, we used the vector pUC18ERY that can not replicate in this strain. For a deletion in *mtsA*, two fragments flanking the region to be deleted were amplified by PCR. Using primers LPATPF3 and LPATPR3, a 860 bp fragment was obtained downstream of the 3′ -end of the coding region of *mtsA* (fragment B, see Figure 1). Primers LPATPF4 and LPATPR4 generated a 822 bp PCR fragment that contained 355 bp of the 5′ -end of *mtsA* and 467 bp of the 3′ -end of *mtsB* (fragment A, see Figure 1). Both fragments were cloned in pGEM-T, checked by DNA sequencing, and reisolated as a *Nco*I-*Sac*I fragment (fragment A) or as a *Nco*I-*Sal*I fragment (fragment B).

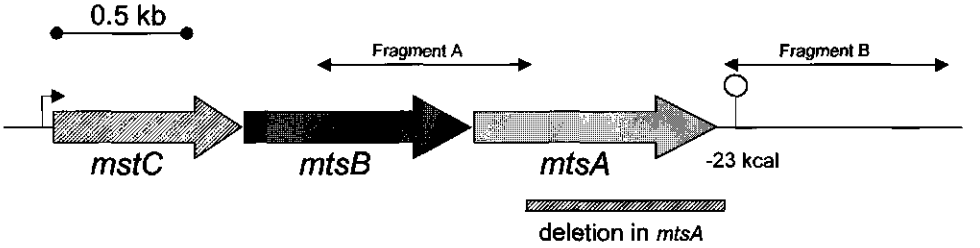

Figure 1. Organization of the *mtsCBA* genes encoding an ABC-type transporter in *Lactobacillus plantarum.* The genes *mtsC*, *mtsB* and *mtsA* in *L. plantarum* encode an ATP-binding protein, a hydrophobic membrane protein and an extracytoplasmic substrate-binding protein, respectively. The small arrow indicates the transcription start and the terminator is represented by a hairpin structure (ΔG=-23 kcal/mol) The location of fragment A and B, amplified by PCR to make the knock-out construct pMN011, are indicated above the *L. plantarum* gene organization

Cloning of these two fragments upstream of and in the opposite orientation to the *ery* gene of pUC18ERY resulted in pMN011. This construct was introduced in *L. plantarum* by electroporation and cells were subsequently cultured in MRS medium containing Em and Lm. The replicon of this plasmid is not functional in *L. plantarum* and Em (Em^R) and Lm (Lm^R) resistance can only result from integration of the plasmid in the chromosome. Several Em^R/Lm^R integrants were selected and were analysed by Southern hybridization. A single colony was selected and cultured without selective pressure to obtain an Em^S phenotype resulting from the second cross-over excission. After 100 generations, three candidate mutants (Em^S) were obtained and Southern analysis confirmed that two strains harbored the deleted version of *mtsA* (data not shown).

The *mntH2* gene was inactivated by deleting 617 bp of the gene at the 3′ -end using the double cross-over recombination strategy described above. This 617 bp deletion in *mntH2* results in loss of the conserved consensus transport motif (Kerppola and Ames, 1992) present in the fourth predicted intracellular loop between the transmembrane-associated segments (TM) TM8 and TM9. A 649 bp fragment, located at the 5′ -end of *mntH2* was amplified using primers LPMNTH2AF/LPMNTH2AR (fragment 1) and cloned in pCRblunt. The resulting plasmid was digested with *Sac*I-*Xba*I and the insert was cloned in pUC18ERY yielding pMN038. A second 714 bp fragment (fragment 2) located downstream of the stopcodon of *mntH2* was amplified using primers LPMNTH2BF/LPMNTH2BR and cloned in similarly digested pMN038 using the *Sal*I and *Xba*I sites that were introduced in the primer sequence. The resulting plasmid was designated pMN061. In order to obtain *mntH2* mutants, plasmid pMN061 was introduced in both the *L. plantarum* parental and the Δ*mtsA* strain by electroporation. Selection was made for Em^R/Lm^R colonies resulting from the first cross-over event. Single cross-over mutants in both strains were subsequently grown without selection pressure for approx. 140 generations to obtain the double cross-over mutants with a 617 bp deletion in *mntH2*. The deletion in this gene was confirmed by Southern analysis (data not shown).

Overexpression of *mtsCBA* in *L. lactis*

The nisine-inducible vector pNZ8020 (De Ruyter *et al.*, 1996a) was used to overexpress the *mtsCBA* genes from *L. casei* in *L. lactis*. Using primers LCABCF and LCABCR, the *mtsCBA* genes were amplified from *L. casei* chromosomal DNA. The resulting fragment was digested with *Pst*I and *Xba*I (sites introduced in the primers are underlined) and cloned in pNZ8020, digested with the same restriction enzymes. This construct was designated pMN027 and was introduced in *L. lactis* NZ9000 by electroporation. Upon addition of different concentrations of nisin, the MtsCBA proteins could be overproduced as was confirmed by SDS-PAGE (data not shown).

RNA isolation, Northern blotting and primer extension

Overnight-grown cells of *L. plantarum* in CDM (containing 300 μM $MnSO_4$) were washed in CDM without Mn^{2+} and diluted 1:100 in CDM with variable $MnSO_4$ concentrations (1.5 up to 300 μM) and incubated overnight at 37

°C. One ml of these cultures were used to inoculate 50 ml of CDM with the same $MnSO_4$ concentration. Cells were harvested at an OD_{600} of about 0.4-0.5 and RNA was isolated by the Macaloid methode described by Kuipers *et al.* (1993) with the following adaption. Prior to the cell disruption step, the resuspended cells were incubated with lysozyme for 5 min on ice to increase RNA yield. RNA was seperated on a 1% formaldehyde-morpholine-propanesulfonic acid (MOPS) agarose gel, blotted and hybridized as described previously (Van Rooijen and De Vos, 1990). Blots were probed with PCR-derived fragments of *mtsA* (primers LPATPF6 and LPATPR6), *mntH2* (LPMNTH2AF and LPMNTH2AR) or *mntH1* (LPMNTH1F and LPMNTH1R). For hybridization, probes were radiolabelled with [α-^{32}P]dATP by nick translation. Quantification of the transcripts in Northern blotting was performed using the Dynamics Phosphor Imaging System (Molecular Dynamics, Rochester, NY). Correction of individual values was performed by correlation of the signal to the amount of 16S rRNA, as determined by hybridization with a 700 bp probe obtained by PCR using the 16SP1 and 16SP2 primers (Table 1).

For primer extension, 20 ng of oligonucleotides were annealed to 15 µg of RNA according to the method described by Kuipers *et al.* (1993). The synthetic oligonucleotides, LPABCSEQ, LPH1SEQ and LPH2SEQ, complementary to the 5′ sequence of *mtsC*, *mntH1* and *mntH2*, respectively, are given in Table1b.

Analysis of intracellular manganese levels

To analyze the effect of *mtsCBA* overexpression on the intracellular Mn^{2+} content of *L. lactis* cells, the nisine-controlled expression system was used (De Ruyter *et al.*, 1996b; Kuipers *et al.*, 1998). *L. lactis* cells were grown in M17 medium supplemented with 200 µM $MnSO_4$ and 1% glucose (wt/vol) to an optical density at 600 nm of about 0.5, and then divided into 3 cultures to which either 1 ng/ml, 2.5 ng/ml or no nisine was added. The cultures were harvested four hours after addition of nisine, and washed 3 times with 50 mM potassium phosphate buffer. The resulting pellet was suspended in Millipore water, and cells were mechanically disrupted in the presence of zirconium beads in a FastPrep FP120 (Savant Instruments, Inc. USA). Cell drebris was removed by centrifugation and the remaining supernatant was used for Mn analysis. Analysis of Mn was performed by inductivity-coupled plasma atomic emmision spectrometry (ICP-AES). The

protein content of the cell extract was determined by the method of Bradford (1976).

DNA and deduced protein analysis
Computer analysis of DNA sequences and the deduced amino acid sequences was performed using the program Clone (version 4.0; Clone Manager). The EMBL/Genbank and ClustalW were used to search for amino acid sequence similarities.

Nucleotide sequence accession numbers
The GenBank accession numbers for the given sequences in this paper are as follows: AF416708 (*mtsCBA* from *L. plantarum* WCFS1); AF416709 (*mntH1* from *L. plantarum* WCFS1); AF416710 (*mntH2* from *L. plantarum* WCFS1). The *mtsCBA* sequence from *L. casei* subsp. *casei* DSM 20011 has been submitted to the GenBank.

Results

Sequence analysis of *mtsCBA* of *L. plantarum* WCFS1 and *L. casei* DSM20011
Previous measurements of the manganese contents of lactic acid bacteria (LAB) revealed that this metal was present at high levels in both *L. plantarum* and *L. casei,* compared to several other LAB belonging to various genera (Nierop Groot and de Bont, 1999). Therefore, we were interested in cloning the genes involved in Mn^{2+} uptake from both bacteria. Several reports have been published on the presence of ABC-type transporters in *Streptococcus sp.* that are involved in Mn^{2+} uptake. Highly conserved amino acid sequences of these Mn^{2+} transporters, and several homologues thereof, were used to find similar systems in *L. plantarum* and *L. casei*.

An internal fragment of the *mtsA* gene of *L. plantarum* NC8, encoding the putative substrate binding protein (SBP) of the ABC transporter was used to screen the *Lactobacillus plantarum* WCFS1 Genome Project database at the time it became available for up- and downstream regions. The *L. casei* genes were cloned as described in the Material and methods section. Based on homology, the *mtsCBA* genes in *L. plantarum* and *L. casei* potentially encode an ATP-binding protein (MtsC), a hydrophobic membrane protein (MtsB) and a substrate-binding protein (MtsA),

respectively (Figure 1). The amino acid sequences of the MtsC proteins include the two consensus binding sites for ATP, the Walker A and Walker B motifs (Walker *et al.*, 1982). The first nucleotide binding site starting at amino acid 34 (GPNGAGKST) is conform the Walker A consensus sequence (GxxGxGKST), while the Walker B motif (the consensus is hhhhDEPT, where h is any hydrophobic amino acid) can be found between amino acids 155 and 162 (IIIILDEPF for *L. plantarum* and IIIMDEPF for *L. casei*). MtsB is highly hydrophobic with 7 and 8 potential transmembrane helixes for *L. plantarum* and *L. casei,* respectively, as predicted by the TopPred II program (Claros and Von Heijne, 1994). Although an N-terminal signal sequence for secretion can be recognized in MtsA of both *Lactobacillus* strains, in contrast to the other substrate-binding proteins in Gram-positives, the prolipoprotein cleavage site L(S,A)(A,G)C(S,G) (Sutcliffe and Russel, 1995) was not present.

Table 2: Percentage identity of the proteins encoded by the *mts* gene cluster in *L. plantarum* with ABC transporters in other bacteria

	homology to *mts* gene product			
Species	MtsC	MtsB	MtsA	substrate*
L. casei [1]	MtsC (73%)	MtsB (77%)	MtsA (66%)	?
S. gordonii [2]	ScaC (45%)	ScaB (55%)	ScaA (47%)	**Mn^{2+}**
S. pneumoniae [3]	PsaB (43%)	PsaC (47%)	PsaA (50%)	Mn^{2+}
S. epidermis [4]	SitA (38%)	SitB (49%)	SitC (44%)	Fe^{2+} (Mn^{2+})
L. lactis [5]	MtsB (46%)	MtsC (54%)	MtsA (49%)	?
B. subtilis [6]	MntB (44%)	MntC (34%) and MntD (35%)	MntA (25%)	Mn^{2+}
Synechocystis [7]	MntA (35%)	MntB (34%)	MntC (29%)	**Mn^{2+}**
S. typhimurium [8]	SitB (33%)	SitD (37%) and SitC (33%) *	SitA (31%)	?
S. pyogenes [9]	MtsB (45%)	MtsC (55%)	MtsA (50%)	Zn^{2+}, Fe^{3+}, Cu^{2+}
Y. pestis [10]	YfeB (38%)	YfeD (37%) and YfeC (34%)*	YfeA (30%)	**Fe^{3+}, Mn^{2+}**
S. mutans [11]	SloA (45%)	SloB (55%)	SloC (50%)	Fe^{3+}

Genbank accession numbers: 1. this study. 2. P42364, P42361, P42360. 3. AF055088 4. X99127. 5. AE006363. 6. O34385, O35024, O34338. 7. S55045, Q55282, Q55281. 8. AF128999. 9. AF180520. 10. U50597. 11. AF232688.

* Substrates suggested by experimental data. Substrates determined by transport assay using radio-isotopes of the metal ions are in bold.

** Two genes encoding hydrophobic membrane proteins are present in these gene clusters.

The homology between the two *Lactobacillus* sequences is high; sequence alignment shows that 66% up to 77% of the amino acids are conserved. Table 2 shows that the proteins encoded by the two *Lactobacillus* sequences are highly homologous to the products encoded by the *sca* operon of *Streptococcus gordonii* and the *psa* operon of *Streptococcus pneumoniae*. These operons have recently been identified as Mn^{2+} transport systems (Kolenbrander *et al.* 1998; Dintilhac *et al.*, 1997). Homology was also found to the *yfe* operon of the Gram-negative *Yersinia pestis* (U50597) and the *sit* operon of *Staphylococcus epidermis* (X99127). The genes clustered in these operons encode iron/manganese (*yfe* operon) and iron (*sit* operon) transport systems.

Functional analysis of the *mtsCBA* genes

To confirm a role of *mtsCBA* from *Lactobacillus* in Mn^{2+}-uptake, the *mtsCBA* genes from *L. casei* were overexpressed in *Lactococcus lactis* NZ9000 using the nisin-inducible expression system (Kuipers *et al.*, 1998). *L. lactis* contains low levels of intracellular Mn^{2+} compared to *L. plantarum* and *L. casei* (Nierop Groot and de Bont, 1999). *L. lactis* NZ9000 was grown in M17 broth (supplemented with 200 µM of $MnSO_4$) in the presence and absence of nisin (2.5 ng/ml), and the intracellular Mn^{2+} concentration was determined by ICP-AES. Overexpression of *mtsCBA* resulted in an increase in the intracellular Mn^{2+} in *L. lactis* NZ9000 from 210 to 342 ng/mg protein (60% increase). This amount was significantly higher than the 252 ng/mg protein Mn^{2+} present in the induced control strain, harboring pNZ8020 without the *mtsCBA* genes. Overexpression of *mtsCBA* in *L. lactis* also resulted in a reduction in the growth of the cells. Although we can not exclude that the decrease in growth has an effect on the Mn^{2+} levels in *L. lactis*, this result suggest a role in Mn^{2+} uptake for these genes.

Construction of a *mtsA* deletion strain

To study the function of the *mtsCBA* genes in *L. plantarum*, the *mtsA* gene was inactivated by using a double cross-over strategy resulting in a 575 bp deletion in *mtsA* (Figure 1). The *mtsA* mutant strain was compared to the parental strain for growth at various Mn^{2+} concentrations. The mutant showed no decrease in growth rate at either 1.5 µM or 300 µM of Mn^{2+}, nor was cell density of overnight-grown cultures affected in a range of Mn^{2+}-concentrations tested (results not shown). The amount of intracellular Mn^{2+} was measured, but showed no significant difference between the parental

and the *mtsA* mutant strain (data not shown). Mutation of similar genes in *S. pneumoniae* (*psaA*) and *S. gordonii* (*scaA*) showed slow growth and reduced Mn^{2+}-transport at low Mn^{2+} concentrations (Dintilhac *et al.*, 1997; Kolenbrander *et al.*, 1998). The absence of a *L. plantarum mtsA* mutant phenotype was unexpected, and suggests that either additonal systems in *L. plantarum* can compensate for Mn^{2+} uptake, or that the *mtsCBA* genes do not encode a Mn^{2+} transporter.

Transcriptional analysis of the *mtsCBA* operon in *L. plantarum*

The expression of *mtsCBA* in *L. plantarum* was analysed to substantiate the postulated role of these genes in Mn^{2+} uptake. Cells were grown in CDM which allows variation of the Mn^{2+} concentration without changing other components.

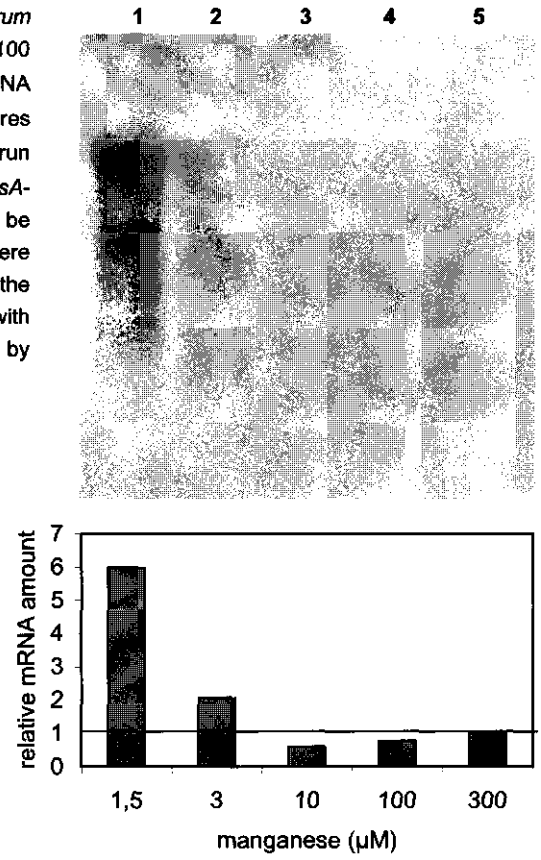

Figure 2. mRNA levels of *mtsA* in *L. plantarum* grown in CDM containing either 1.5, 3, 10, 100 or 300 µM of Mn^{2+} (lane 1-5 respectively). RNA was isolated from exponentially growing cultures ($OD_{600} \approx 0.5$). Equal amounts of RNA were run on a 1% formaldehyde gel. Using a *mtsA*-specific probe, a transcript of 2.6 kb can be detected. Relative mRNA amounts were calculated from the radioactivity measured in the transcript bands at each Mn^{2+} concentration with respect to the background signal (indicated by the horizontal line)

A probe specific for the complete *mtsA* gene was synthesized by PCR using primers LPATF6 and LPATPR6. Using this probe, a single transcript of 2.6 kb was detected in cells grown at 1.5 µM and at 3 µM, albeit that the

signal intensity was significantly lower in the latter sample (Figure 2). The transcript size corresponds to the length required to encompass the three *mts* genes. For cells grown at Mn^{2+} concentrations above 3 µM, no significant transcripts were detected. Transcript quantification using a Phosphor Imaging System demonstrated a 6-fold increase of *mts*-RNA values in cells grown at 1.5 µM compared to the signal intensity detected for 300 µM-grown cells. At Mn^{2+} concentrations above 1.5 µM, the signal intensity dropped rapidly. Regulation of *mtsCBA* expression by either the growth phase, or by the level of aeration was not observed (results not shown).

Primer extension was performed on total RNA, isolated from cells grown at 1.5 µM of Mn^{2+}, to determine the transcription initiation start sites of the *mtsCBA* gene cluster. Two transcription start sites, a guanosine-residue 34 nucleotides, and an adenosine-residue 39 nucleotides upstream of the ATG start codon of the *mtsCBA* genes were identified (Figure 3).

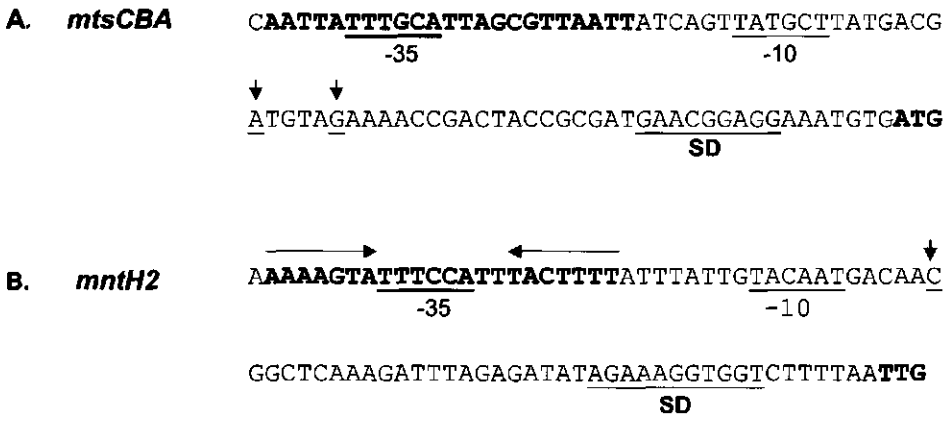

Figure 3. Transcription initiation sites of the *mtsCBA* (panel A) and *mntH2* (panel B) genes as determined by primer extension analysis. Trancription start sites are underlined and indicated with a vertical arrow. Proposed –35 and –10 promoter regions are indicated. Shine-Dalgarno sequences (SD) are indicated. DNA sequences resembling metalloregulator target regions are indicated in bold.

The proposed transcription start of the three *mts* genes is preceded by a sequence that has similarity to the –10 box consensus sequence (TNTGNTATAAT) of the σ^A promoters from Gram-positive bacteria (Helmann, 1995). These results demonstrate that *mtsC,B* and *A* are organized in an operon. Transcription of the *mts* operon starts from a promoter upstream of *mtsC*, that is activated upon Mn^{2+} starvation. This

supports the hypothesis that MtsCBA indeed is involved in Mn^{2+} transport, and that the failure to demonstrate a *mtsA* mutant phenotype in *L. plantarum* is probably due to the presence of alternative transporters for Mn^{2+}, that compensate for the inactivity of the Mts system.

Manganese induction of *mntH1* and *mntH2*

In the *L. plantarum* genome sequence, four genes were found with homology to putative manganese transporters. One gene was recently described by Hao *et al.*, (1999) and encodes a protein that belongs to the family of P-type cation translocating ATPases. Three other genes were found, that encode proteins with homology to the Nramp family of transporters. Recently, these type of transporters have also been identified in *E. coli* and *S. typhimurium* (Kehres *et al.*, 2000) and in *B. subtilis* (Que and Helmann, 2000) as highly selective Mn^{2+} transporters. We therefore focused on the expression of the Nramp-type transporters in *L. plantarum*.

The three genes that encode proteins of the Nramp family in the *L. plantarum* genome sequence were designated *mntH1*, *mntH2* and *mntH3*. Table 3 shows the homology of the proteins encoded by these *mntH* genes to the recently identified Nramp transporters for Mn^{2+} in *B. subtilis* (Que and Hellman, 2000), *Salmonella typhimurium* (Kehres *et al.*, 2000) and *E. coli* (Makui *et al.*, 2000).

Similar systems are encoded in the *S. gordonii* (Jakubovics and Jenkinson, 2001), *S. mutans* (http://www.genome.ou.edu/smutans) genome, but no Nramp homologues are present in the *S. pneumoniae* (Tettelin *et al.*, 2001) and *S. pyogenes* (Ferreti *et al.*, 2001) genomes. Whereas in most bacteria one or sometimes two Nramp proteins are present, the *L. plantarum* genome harbors 3 paralogs. The MntH sequences from *L. plantarum* share between 26 to 76% identity to the other Gram-positive and -negative MntH proteins. In particular, homology was high to the recently described HitA protein from *Lactobacillus brevis* (Hayashi *et al.*, 2001).

Table 3: Comparison of *L. plantarum* MntH proteins with several bacterial Nramp transporters

Species	Gene	No. of aa	Homology to *L. plantarum* (percentage identity)			Experimental data	Reference
			MntH1	MntH2	MntH3		
L. plantarum	*mntH1*	464	100%	55%	45%	Induced by Mn^{2+} starvation	This study
	mntH2	530	55%	100%	42%	Induced by Mn^{2+} starvation	This study
	mntH3	441	45%	42%	100%		This study
B. subtilis	*mntH*	425	36%	34%	30%	Mn^{2+} transport	Que and Helmann (2000)
L. brevis	*mntH*	464	76%	53%	44%	Hop induced	Hayashi *et al.* (2001)
E. coli	*mntH (yfeP)*	412	34%	32%	31%	Mn^{2+} transport	Kehres *et al.* (2000)
L. lactis	*ykjB*	314	54%	76%	38%		http://spock.jouy.inra.fr/
	ytjB[1]	419	30%	26%	28%		http://spock.jouy.inra.fr/
P. auruginosa	*mntH1*	391	45%	39%	40%		Kehres *et al.* (2000)
	mntH2	438	46%	41%	39%		Kehres *et al.* (2000)
S. typhimurium	*mntH*	413	33%	31%	31%	Mn^{2+} transport	Kehres *et al.* (2000)
S. epidermis	*mntH*	453	56%	49%	43%		http://www.tigr.org[2]
Y. pestis	*mntH*	409	32%	29%	30%		www.sanger.ac.uk
S. mutans	*mntH*	446	58%	54%	45%		www.genome.ou.edu/smutans

[1] This gene may contain a frameshift mutation.
[2] preliminary sequence data was obtained from The Institute of Genomic Research.

To test whether these putative transporters were expressed in *L. plantarum*, RNA was isolated from cells grown at various Mn^{2+} concentrations. Using *mntH1*- and *mntH2*-specific probes, single transcripts of 1.5 kb and 2.1 kb respectively, were detected. For both genes, the mRNA levels remained unchanged in the range of 10 up to 300 μM Mn^{2+}. However, the levels of mRNA were found to increase 13- and 6-fold for *mntH2* and *mntH1*, respectively, when the Mn^{2+} concentration was reduced from 10 to 1.5 μM (Figure 4). The presence of a single transcription initiation start upstream of *mntH2* was identified by primer extension analysis. Transcription of *mntH2* starts at the cytosine-residue located 39 nucleotides upstream of the translational start codon TTG (Figure 3). Despite several attempts, we did not succeed in obtaining a primer extension product for the *mntH1* gene.

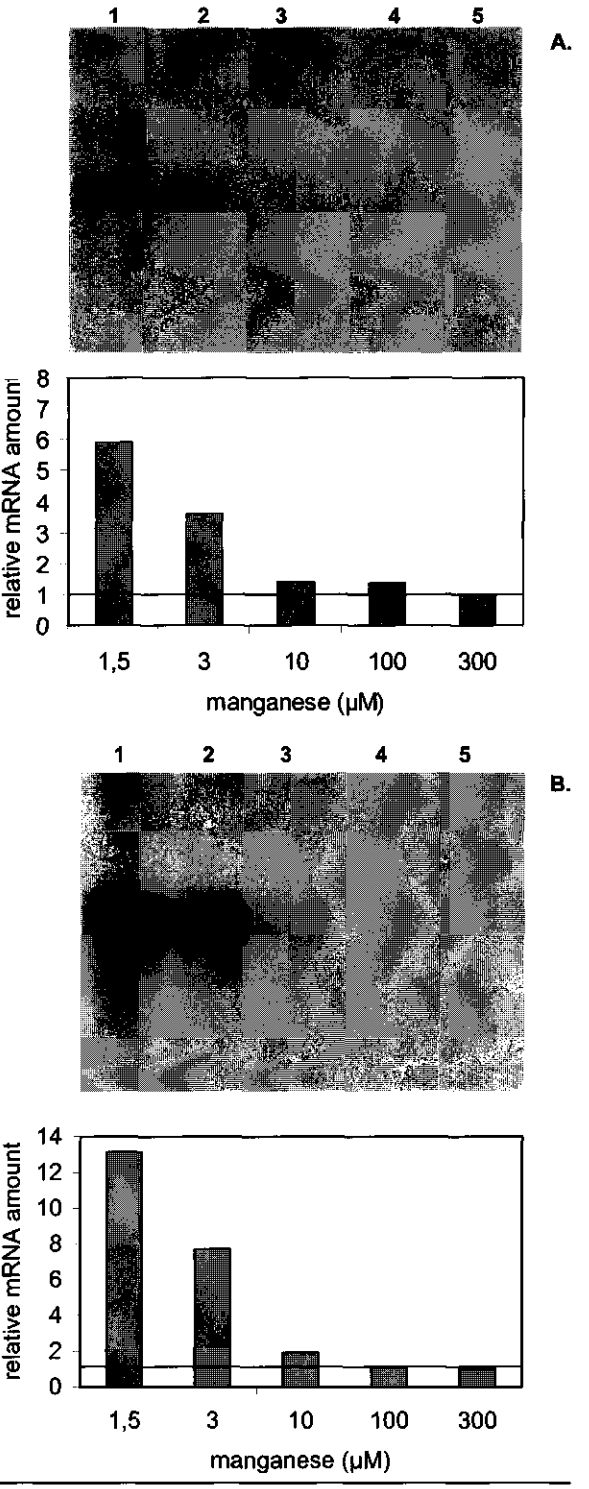

Figure 4. Expression of *mntH1* (4A) and *mntH2* (4B) mRNAs upon manganese starvation in *L. plantarum*. mRNA was isolated from exponentially growing cultures in CDM containing either 1.5, 3, 10, 100 or 300 µM of Mn^{2+} (lane 1-5 respectively). The transcript size is 1.5 kb for the *mntH1*-specific probe and 2.1 kb for the *mntH2*-specific probe. Relative mRNA amounts were calculated from the radioactivity measured in the transcript bands at each Mn^{2+} concentration with respect to the background signal (indicated by the horizontal line).

The RNA levels for this gene were quite low, and the presence of a small inverted repeat in the region of the ATG start codon presumably prevented a succesful primer extension reaction. The transcript length of 2.1 kb, detected with the *mntH2*-specific probe, corresponds to the size of *mntH2* plus a downstream located open reading frame (*orf2*). Orf2 has no significant similarity to any sequence from the database. There is no strong inverted repeat present between *mntH2* and *orf2*, suggesting a bicistronic transcript starting from the promoter upstream of *mntH2*. Although the transcription initiation site for *mntH1* could not be determined, the transcript length of 1.5 kb for *mntH1* suggest a monocistronic mRNA. To a limited extent, cross-hybridization occured using *mntH1*- and *mntH2*-specific probes, however the difference in transcript length allowed us to discriminate between the two transcripts. No transcript was detected using the *mntH3*-specific probe under the conditions tested, suggesting that this transporter is not involved in transport of Mn^{2+} and its function remains unknown. These results clearly show, that beside the ABC-transporter, at least two more putative transporters are induced upon Mn^{2+} starvation.

Construction of a deletion in *mntH2*

A deletion was introduced in the *mntH2* gene in both the parental, and in the *ΔmtstA* strain. The 617 bp deletion made in *mntH2* results in loss of a conserved transport motif (Kerppola and Ames, 1992; Vidal *et al.*, 1993) present in the intracellular loop between the transmembrane associated segments TM8 and TM9. The growth rate of the *mntH2* mutant and the double mutant (*mntH2-mtsA*) was tested under aerobic conditions in medium containing 1.5 or 300 μM of Mn^{2+}, however both strains were not affected in growth rate compared to the parental strain. RNA was isolated from both the wildtype, the *mtsA* mutant, the *mntH2* mutant and the double mutant (*mtsA-mntH2*) grown at 1.5 μM of Mn^{2+}, and analyzed. Transcript quantification revealed a 45% increase in *mtsCBA*-transcript level in the *mntH2* mutant strain at 1.5 μM of Mn^{2+} compared to the parental strain (Figure 5), suggesting that the *mntH2* mutation is compensated via upregulation of the ABC transporter. Vice versa, in the *mtsA* mutant strain, we could detect an increase in the *mntH2* transcript level of 35% at 1.5 μM compared to the wildtype. The transcription level of the third candidate gene, *mntH1*, was not affected in either the *mtsA*, *mntH2* or in the double-mutant.

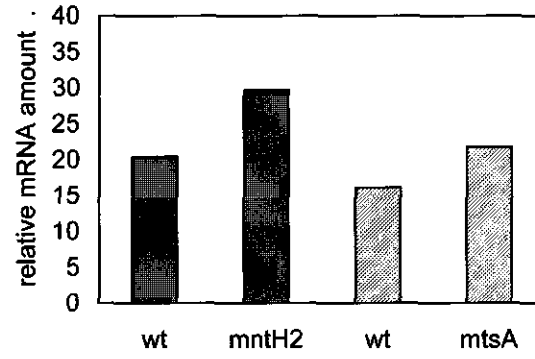

Figure 5. Expression of *mtsCBA* in the wildtype and in the *ΔmntH2* strain (grey bars) and *mntH2* expression in the wildtype and the *ΔmtsA* strain (hatched bars). Transcipts of *mtsCBA* and *mntH2* were detected with a *mtsA* and *mntH2*-specific probe, respectively. Relative mRNA amounts were calculated from the radioactivity measured in the transcript bands at 1.5 µM Mn^{2+} with respect to the background signal. The values indicated are based on two independent experiments.

Analysis of promoter regions of *mtsCBA, mntH1,* and *mntH2*

Recently, several proteins of the DtxR family of iron-repressors have been reported that, in contrast to DtxR, respond to Mn^{2+} in *S. gordonii* (ScaR; Jakubovics *et al.*, 2000), *E. coli* (MntR; Patzer and Hantke, 2001), *Treponema pallidum* (TroR; Posey *et al.*, 1999) and in *B. subtilis* (MntR; Que and Helmann, 2000). In the *L. plantarum* genome, a gene encoding a protein homologous to a metalloregulatory protein of this DtxR family, is present. To locate sequences that could potentially serve as binding sites for this protein, DNA sequences upstream of *mtsCBA*, *mntH1* and *mntH2* of *L. plantarum* were compared to sequences known to be recognized by the above-mentioned Mn^{2+}-dependent regulators. Potential binding sites, similar to the control regions of MntR of *B. subtilis* and *E. coli* can be recognized in the –35 regions of *mtsCBA*, *mntH2* and the putative – 35 region of *mntH1* of *L. plantarum* (Figure 6A). This consensus sequence was also identified in the region between –10 and the ribosome-binding site of *hitA* of *L. brevis*, encoding a Nramp protein highly homologous to *mntH1* of *L. plantarum*. The conservation in the potential target region of *mntH2* and *mtsC* is high; 17 out of 23 bases (74%) are conserved. The potential target region of *mntH1* is less conserved to *mntH2* and *mtsC*, having 10 and 11 bases out of 23 identical, respectively. Remarkably, the *mntH2* promoter region in *L. plantarum* (Figure 6B) shared also similarity to the conserved palindromic sequences present in the control region of *scaC* in *S. gordonii* that reponds to Mn^{2+} (Jakubovics *et al.*, 2000). The interrupted palindromic sequence overlapping the –35 promoter element, and the imperfect non-interrupted palindrome at the –10 element are conserved within control regions of corresponding transporters in streptococci. For the –35 element, *L. plantarum mntH2* shared 15 out of 22

bases with *scaC* of *S. gordonii*. The homology in the –10 region was lower with 8 out of 15 bases identical. This potential recognition site is not present in the control regions of *mtsCBA* and *mntH1*.

A.

B. subtilis mntA	ATAATTTTGCATGAGGGAAACTT -109-ATG
B. subtilis mntH	AATAATTTGCCTTAAGGAAACTC - 39-ATG
L. plantarum mtsC	AATTATTTGCATTAGCGTTAATT - 59-ATG
L. plantarum mntH2	AAGTATTTCCATTTACTTTTATT - 56-TTG
L. plantarum mntH1	TAATAGTTGACTAATAGGTTAAT -115-ATG
L. brevis hitA	TGGGAATTGATTGAGTGCTCACT - 44-ATG
E. coli mntH	AAAACATAGCCTTTGCTATGTTT -195-ATG
T. pallidum troA	GCTACTTTGATGCATCAAAATTC - 17-TTG

B.

L. plantarum mntH2	CTAAAAAAGTATTTCCATTTACTTTTATT..TATTGTACAATGACAAC-39-TTG
S. gordonii scaC	TTGCAAAATTAACTTGACTTAATTTTTATGTTAAGGTATATTAATACA-16-ATG
S. mutants sloA	TATAAAAATTAACTTGACTTAATTTTTATATTAGGTTATATTACTTAT-13-ATG
S. pneumoniae psaB	TTCAAAAATTAACTTGACTTAATTTTTTTTTTAATGTATATTAAGAGA-18-ATG
	**** ** * * *** **** * ** ** * *

Figure 6. Alignment of potential binding sites for MntR in *L. plantarum* with similar target sequences recognized by homologs. Panel A: the putative control regions of *mtsC, mntH2 and mntH1* in *L. plantarum*, aligned with experimentally determined binding sites in the *mntH* and *mntA* control region of *B. subtilis* (Que and Helmann, 2000), the *troA* promoter region of *T. pallidum* (Posey *et al.*, 1999), *mntH* of *E. coli* (Patzer and Hantke, 2001) and a potential binding site in the *hitA* promoter region of *L. brevis* (Hayashi *et al.*, 2001). Bases identical in at least 4 sequences are in bold. Bases common in *mtsC* and *mntH2* are underlined. Panel B: Comparison of the potential binding site for MntR in *L. plantarum* in the control region of *mntH2* with the experimentally determined ScaR binding site in *S. gordonii* of the *scaC* promoter region (Jakubovics *et al.*, 2000), and similar promoter regions of *sloA* in *S. mutants* (Kitten *et al.*, 2000), and *psaB* in *S. pneumoniae* (Tettelin *et al.*, 2001). Bases identical in at least 3 sequences in the palindromes are bold; bases common to all sequences are marked by asterisks.

Discussion

The high manganese requirement of *L. plantarum* has been established (Archibald and Fridovich, 1981b) but data on manganese uptake systems at the molecular level in this bacterium has only been presented recently with the report of the Mn^{2+} and Cd^{2+} specific P-type ATPase (Hao *et al.*, 1999). We now provide evidence that in addition to the P-type ATPase, at least three more transport systems, that are expressed under Mn^{2+} starvation, are present in *L. plantarum*.

The ABC-transporter identified in the present study in both *L. plantarum* and *L. casei* is highly homologous to the ABC-transporters involved in Mn^{2+}-uptake that were reported for *S. pneumoniae* and *S. gordonii* (Dintilhac *et al.*, 1997; Kolenbrander *et al.*, 1998). The arrangement of the genes in the clusters of the two *Lactobacillus* strains is typical for previously characterized operons in Gram-positive organisms; *i.e.* the ATP-binding gene is at the 5′ -end and the substrate-binding gene at the 3′ -end of the operon. A similar system can also be found in the *Lactococcus lactis* IL1403 genome sequence (Bolotin *et al.*, 2001), suggesting that this type of metal transporter represents a common system in lactic acid bacteria. In addition, MtsA is homologous to substrate-binding proteins of several iron transporters such as SloC (FimA) in *S. mutans* (Spatafora *et al.*, 2001) and SitC in *S. epidermis* (Cockayne *et al.*, 1998). A three dimensional structure of PsaA (*S. pneumoniae*) is available and reveals that a metal binding site is formed by the sidechains of His67, His139, Glu205 and Asp280 (Lawrence *et al.*, 1998). These four amino acid residues are perfectly conserved in MtsA from both *L. plantarum* and *L. casei*. Remarkably, an N-terminal, cysteine residue containing, prolipoprotein signal sequence (Sutcliffe and Russell, 1995) common for gram-positive bacteria is absent in the MtsA protein of both *Lactobacillus* sp. Based on the presence of a signal sequence, characteristic for export through the cytoplasmic membrane, in MtsA of both *Lactobacillus* sp., it is reasonable to assume that this protein is located extracellularly. However, the structure by which MtsA is tethered to the cell wall remains unclear, since common modes of anchoring of surface proteins, found in Gram-positives (Navarre and Schneewind, 1999) could not be identified.

Two other transporters in *L. plantarum* are expressed under Mn^{2+} starvation and belong to the Nramp family. Both MntH1 and MntH2 are highly hydrophobic proteins with 11 and 12 putative transmembrane domains respectively, typical for bacterial Nramp proteins. Both proteins contain a conserved transport motif (CTM) in the fourth intracellular loop, as found in several bacterial and eukaryotic Nramp transporters (Cellier *et al.*, 1994). Genomic sequence data reveal that this type of transporter is widespread, but not common in bacteria since this transporter is not present in the completed genome sequences of *S. pyogenes* and *S. pneumoniae*. Sofar, *L. plantarum* is the only bacterium known to harbor three genes encoding Nramp-type transporters, although two copies are

present in *P. auruginosa* (Kehres *et al.*, 2000) and in *L. lactis* (Bolotin *et al.*, 2001). In analogy to homologs reported in *B. subtilis* (Que and Helmann, 2000), *E. coli* and *S. typhimurium* (Kehres *et al.*, 2000; Makui *et al.*, 2000), the increased expression of *mntH1* and *mntH2* upon Mn^{2+} starvation in *L. plantarum* supports a role for these genes in Mn^{2+} transport,. In contrast to *mntH1* and *mntH2*, we could not detect expression of *mntH3*. The presence of a gene, potentially encoding a ferrochelatase, upstream of *mntH3* suggests a role for MntH3 in iron uptake rather than Mn^{2+}. The homology of MntH1 to HitA from *L. brevis* (Hayashi *et al.*, 2001) was very high (76% amino acid identity) compared to the scores of MntH2 (50%) and MntH3 (43%) from *L. plantarum*. The *hitA* gene in this beer spoilage isolate of *L. brevis* was highly expressed upon addition of iso-α-acids, that are bitter hop compounds. No expression of *hitA* was detected when cells were grown in MRS broth without added $MnSO_4$. However, in this medium the Mn^{2+} concentration is presumably above the inducing Mn^{2+} concentration, and thus transcripts were possibly not detectable as demonstrated in the present study. It has been postulated that *L. brevis* expresses the *hitA* gene as a stress response to the bitter compounds in hop. One of these hop compounds is known to exchange extracellular protons for intracellular Mn^{2+}, thereby lowering the intracellular pH (Simpson, 1993) that possibly coincides with a shortage for Mn^{2+}. Both the results from Hayashi *et al.* (2001) and the present study demonstrate a link between the homologs *mntH1* and *hitA* in *Lactobacillus* sp. to Mn^{2+}. Both the Northern analysis and the expression of the *Lactobacillus mtsCBA* genes support a role for this transport system in Mn^{2+} transport. The upregulation of the *mts*-operon in *L. plantarum* under low-Mn^{2+} conditions is in agreement with the Mn^{2+}-regulated expression of the *sca* and the *psa* operon in *S. gordonii* (Kolenbrander *et al.*, 1998) and *S. pneumoniae* (Dintilhac *et al.*, 1997).

Sofar, Mn^{2+} transport systems have been studied in bacteria that contain SOD, and therefore require only micromolar concentrations of Mn^{2+} as cofactor for this and several other enzymes. *L. plantarum* is quite different from these bacteria in its extreme high level of Mn^{2+} intracellularly, that exceeds by far the micromolar levels necessary for cofactor functions. Considering the important role of Mn^{2+} in *L. plantarum*, a highly efficient system to fulfill this requirement is expected. Therefore, it is not surprising that *L. plantarum* is highly adaptive to inactivation of the *mtsA* and *mntH2* genes. The fact that mutation of *mtsA* in *L. plantarum* did not result in an obvious phenotype, is therefore not comparable to the

reported phenotype for mutants in the orthologous genes *scaA* and *psaA* in *S. gordonii* (Kolenbrander *et al.*, 1998) and in *S. pneumoniae* (Dintilhac *et al.*, 1997), respectively. Similar findings have recently been reported for, inactivation of the *fimA* gene (encoding the SBP of the ABC transporter) in *S. mutants* (Spatafora *et al.*, 2001) and *mntH* in *E. coli* (Makui *et al.*, 2000). Disruption of these genes did not result in either a reduction in growth (*fimA* and *mntH*) or metal uptake (*fimA*), suggesting the presence of additional systems for metal uptake in this species.

In this study, we demonstrate that inactivation of *mtsA* and *mntH2* results in upregulation of *mntH2* and *mtsCBA*, respectively. The increased *mtsCBA* transcript levels in the *mntH2* mutant, and vice versa, suggests that cross-regulation occurs in *L. plantarum.* This phenomenon can explain the absence of a phenotype in the *mntH2* and *mtsA* mutants. However, inactivation of both *mntH2* and *mtsCBA* apparently had no affect on growth of *L. plantarum*. Upregulation of other Mn^{2+} transporters presumably occurs in the *mtsA-mntH2* mutant of *L. plantarum*. MntH1 can be excluded since the transcript levels of *mntH1* remained unchanged in the mutant strains. Another candidate gene in *L. plantarum* for upregulation in the *mtsA-mntH2* mutant, is *mntA,* encoding a P-type ATPase (Hao *et al.*, 1999). This P-type ATPase is induced in *L. plantarum* in Mn^{2+}-limited medium and is shown to transport Cd^{2+}/Mn^{2+}. We did not succeed in detecting *mntA* transcripts by Northern analysis, although expression of this transport system in *L. plantarum* has been clearly demonstrated by immunoblotting (Hao *et al.*, 1999). In *B. subtilis*, Mn^{2+} may enter the cell via the metal-citrate transporter (CitM) (Krom *et al*, 2000) and the phosphate-inorganic transport system (Pit) in *E. coli* and *Acinetobacter johnsonii* (van Veen *et al.*, 1994a; 1994b) is known to mediate Mn^{2+} uptake as well. However, homologs of these systems can not be found in the *L. plantarum* genome suggesting the presence of other, presently unknown, transporters with affinity for Mn^{2+} in this bacterium.

Based on the results in this study we can assume that maintenance of Mn^{2+} homeostasis in *L. plantarum* is highly efficient and presumably requires strict regulation. Indeed, a gene (designated *mntR*) encoding a protein homologous to the recently identified metalloregulatory proteins of Mn^{2+}-uptake systems in *S. gordonii* (ScaR; Jakubovics et al.,2000), *T. pallidum* (TroR; Posey *et al.*, 1999), and *B. subtilis* (MntR; Que and Helmann, 2000) is present in *L. plantarum*. The metalloregulatory protein of *L. plantarum* is related to SloR (*S. mutants*, Kitten *et al.*, 2000) and

ScaR having 41% and 38% amino acids identical, respectively. Homology to MntR (*B. subtilis*, 23%), TroR (*T. pallidum*, 27%) and MntR (*E. coli*, 20%, Silke and Patzer, 2001) is lower. Several attemps to inactivate this *mntR* gene in *L. plantarum* failed, which suggest that this gene is essential in this bacterium. Involvement of this protein in Mn^{2+} homeostasis may also be suggested by the presence of a conserved sequence similar to the sequences bound by the MntR of *B. subtilis* (Que and Helmann, 2000) and *E. coli* (Patzer and Hantke, 2001), in the –35 promoter region of *mntH2* and *mtsCBA* of *L. plantarum*. Binding of the regulator to this sequence was demonstrated to repress *mntH* in both *E. coli* and in *B. subtilis* and the *tro*-operon in *T. pallidum*. However, binding of the same protein at the –10 region of the *mnt*-operon (*B. subtilis*) activates transcription. The target region for binding of this regulator overlaps the –10 promoter region of the *tro*-operon in *T. pallidum* and the *mnt*-operon in *B. subtilis* with TroR acting as a repressor and MntR as an activator. In *L. plantarum*, this sequence is located at the –35 promoter region and hence the mode of action the putative regulator in *L. plantarum* requires more experimental data. Remarkably, a second potential target region can be recognized in the –35 region of *mntH2* of *L. plantarum* that resembles the palindromic elements recognized in *S. gordonii* and is present in other streptococci (Jakubovics *et al.*, 2000). A role of the *L. plantarum* homolog in Mn^{2+} homeostasis is possible, but prediction of the mode of action would be highly speculative and therefore requires further analysis. In conclusion, we have identified three transport systems that are induced upon Mn^{2+} starvation in *L. plantarum*. For the first time, mutants carrying directed mutations in the various Mn^{2+} transport systems have been analysed in this species. The results reveal a highly adaptive behaviour of *L. plantarum* to mutations affecting genes involved in Mn^{2+} homeostasis. *L. plantarum* is therefore an interesting organism for future research that is focused on the transport- and regulation systems for Mn^{2+} homeostasis in bacteria.

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Chapter 6.

Inactivation of *mntA*, encoding a Manganese-inducible P-type ATPase Transporter in *Lactobacillus plantarum*

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Abstract

The *mntA* gene encoding a P-type ATPase in *Lactobacillus plantarum* was disrupted resulting in a growth defect and clumping of the mutant cells. The mutant strain showed no significant change in intracellular Mn^{2+} concentration, implying that this gene is not crucial for Mn^{2+} homeostasis in *L. plantarum*.

Introduction

It has been well documented that Mn^{2+} is of extreme importance to *Lactobacillus plantarum* for protection against reactive oxygen species such as O_2^- (Archibald and Fridovich, 1981a,b). Whereas in other micro-organisms this metal ion is sufficient at nanomolar levels in the growth medium, *L. plantarum* requires micromolar levels for optimal growth. This results in intracellular accumulation of Mn^{2+} at the millimolar level implying that this metal ion is imported by one or more active transport systems. Previously, we reported the presence of an ABC-type transporter (MtsCBA) and two Nramp-type transporters (MntH1 and MntH2) that are induced upon Mn^{2+} starvation in *L. plantarum* WCFS1 (Nierop Groot *et al.*, 2001). Strains of *L. plantarum* were constructed carrying deletions in either *mtsA*, encoding the substrate-binding protein of an ABC-type transporter (MtsCBA), or in *mntH2*, encoding a Nramp type transporter (MntH2). Individual or simultaneous inactivation of these transporters did not result in an obvious reduction in growth rate or decrease in intracellular Mn^{2+} concentration. The absence of a phenotypic difference in these mutants may be the result of upregulation of other transport systems. This was indeed demonstrated for the *mntH2* and *mtsA* deletion mutants, in which *mtsCBA* and *mntH2* transcript levels were raised, respectively (Nierop Groot *et al.*, 2001). However, in the *mtsA-mntH2* double mutant, we could not detect higher transcription levels of *mntH1* (encoding an additional Nramp-type transporter that is induced upon Mn^{2+} starvation). These findings suggest that *L. plantarum* harbors additional transporter(s) that are involved in uptake of Mn^{2+}. A candidate transporter is one of the P-type family of cation-transporting ATPases (MntA), that was recently described for *L. plantarum* (Hao *et al.*, 1999b). Expression of this gene in *Escherichia coli* conferred both increased Cd^{2+}-sensitivity and increased energy-dependent Cd^{2+} uptake on these cells. Immunoblotting of MntA in *L. plantarum* demonstrated that this protein was only expressed under Mn^{2+} starvation (Hao *et al.*, 1999b). Moreover, a mutant of this bacterium obtained via chemical mutagenesis was severely disabled and required 5,000 times increased Mn^{2+} levels in the medium for growth. In addition, a severe reduction in Cd^{2+}-uptake, compared to the parental strain, was demonstrated for this mutant (Hao *et al.*, 1999a). The MntA transport protein could not be detected by immunoblotting in this mutant (Hao *et al.*, 1999b) indicating that at least the expression of this gene was affected by the mutation(s). However, a directed mutation in the *mntA* gene was not

constructed by these authors, leaving the impact of the P-type ATPase transporter on Mn^{2+}-levels in *L. plantarum* to be elucidated.

In an attempt to understand Mn^{2+} homeostasis in *L. plantarum*, we constructed a mutation in *mntA* and compared the growth rate and intracellular Mn^{2+} levels of this mutant strain to the wild-type. The mutant showed impaired growth at 300 µM of Mn^{2+} and cells formed aggregates in liquid medium, but the Mn^{2+}-levels were not significantly reduced in the mutant strain. Complementation of the mutant with a plasmid-encoded copy of *mntA* did not restore growth nor cell-clumping, indicating that the mutant phenotype probably resulted from a polar effect on downstream located genes. The results in this study indicate that MntA alone is not crucial for Mn^{2+} homeostasis in *L. plantarum*. The previously reported phenotype of the chemically constructed mutants is likely to be a result of multiple mutations in genes encoding either additional transporters, or genes involved in regulation thereof, rather than a single mutation in *mntA*.

Materials en methods

Bacterial strains, plasmids and culture conditions

Lactobacillus plantarum WCFS1 or NC8 (Aukrust and Blom, 1992) were cultured in either MRS broth (De Man, Rogosa and Sharpes, Oxoid), or in chemically defined medium (CDM, Kets *et al.*, 1994) at 37 °C. *Escherichia coli* DH5α (Hannahan, 1983) was cultured in Tryptone Yeast (TY) medium at 37 °C (Sambrook *et al.*, 1989).

Transformation and DNA isolation

Chromosomal DNA of *L. plantarum* was isolated as described elsewhere (Bernard *et al.*, 1991). For large scale isolation of *E. coli* plasmid DNA, a JetStar column (Genomed) was used following the instructions of the manufacturer. *L. plantarum* was transformed by electroporation as described by Aukrust and Blom (1992). Cells were plated on MRS agar containing erythromycin (10 µg/ml) and lincomycin (20 µg/ml) and were incubated at 37 °C in a jar under an atmosphere of 85 % N_2, 5 % CO_2, and 10% H_2. Competent cells of *E. coli* were prepared according to the $CaCl_2$ procedure described by Sambrook *et al.* (1989).

Construction of plasmids and Southern hybridization

To inactivate the *mntA* gene by single cross-over recombination, a 1143 bp internal fragment of *mntA* was amplified by PCR using the primers LPATP3 (GCCTGAATTCTGCAGGCACAATGGTGGTAGC) and LPATPR3 (CGGAGGATCCCTTCGTTTGGCTAAATCAGTGG)(indicated in Figure 1). This fragment was cloned in pUC18ERY (van Kranenburg *et al.*, 1997) using *Eco*RI and *Bam*HI (restriction sites underlined in the primer sequences) to obtain pMN035. Plasmid pMN035 was transformed to *L. plantarum* by electroporation and integrants were selected based on erythromycin and lincomycin resistance. Integration of pMN035 in the chromosome of *L. plantarum* was confirmed by both PCR using primers LPATP1 (TGGCTTTATTGCGTTAATGCTGGTCTTTGC) and EMFORW (GTACCGTTACTTATGAGCAAG) and Southern hybridization. The vector pMN034 was used to complement the *mntA* mutation in *L. plantarum*. Primers LPMNATPF (TACACCATGGTTGAAGATTTAACGGAG) and LPMNATPR (AATATCTAGAATATCACCGTTAATCAGC) were used to amplify *mntA* from *L. plantarum* chromosomal DNA. This PCR fragment was cloned in pNZ8048 (Kuipers *et al.*, 1998) using *Nco*I and *Xba*I (sites underlined in primers). The sequence of the cloned PCR fragment was verified by sequence analysis. A fragment containing the *mntA* gene and the *nisA* ribosome binding site present on pNZ8048 was isolated by *Bst*BI and *Sac*I digestion. After Klenow fill-in of the *Bst*BI site, this fragment was cloned in pNZ278 (Platteeuw *et al.*, 1994), digested with *Eco*RI (blunted by Klenow) and *Sac*I, to obtain pMN034.

Southern blots were hybridized at 68 °C with a homologous DNA probe. A non-radioactive dioxygen DNA labelling and detection kit (Roch Diagnostics Nederland BV, Almere, The Netherlands) was used to label and detect probes for Southern blotting according to instructions of the manufacturer.

Growth experiment

Overnight-grown cells in MRS broth (De Man, Rogosa and Sharpes, Oxoid) were washed in CDM without Mn^{2+} and diluted 1/100 in CDM with either 1.5 or 300 μM of Mn^{2+} and incubated statically at 37 °C. Two hundreds μl of these cultures were used to inoculate test tubes containing 10 ml of CDM with the same Mn^{2+} concentration. Cell were incubated at 37 °C in a Gyrotory waterbath (model G76, New Brunswick Scientific Co. Inc., USA) and shaken at speed 6. At regular time intervals samples were

taken in triplicate for OD measurement at 600 nm. Before removing the sample, test tubes were vortexed vigorously to seperate the aggregates.

Analysis of manganese

Overnight-grown cells in CDM (300 μM Mn^{2+}) were diluted 1/100 in 50 ml CDM (300 μM Mn^{2+}) and cultured overnight. These cells were harvested by centrifugation and washed 3 times with CDM (without manganese). The resulting pellet was suspended in Millipore water and cells were mechanically disrupted in the presence of zirconium beads in a FastPrep FP120 (Savant Instruments, Inc. USA). Cell debris was removed by centrifugation and the remaining supernatant was diluted in MilliQ for analysis of the Mn content. Analysis of Mn was performed by Inductivity-Coupled Plasma Atomic Emmision Spectrometry (ICP-AES). The given values are averages of two independent experiments. The protein content of the cell extract was determined by the method of Bradford (1976) and used to express Mn levels in ng per mg of protein.

Results and discussion

Chromosomal organization of *mntA*.

The sequence encoding the P-type ATPase in *L. plantarum* ATCC14977 is deposited as *mntA* under accession number AF136521 (Hao *et al.*, 1999b). Using primers LPMNATPF and LPMNATPR, this gene was amplified from *L. plantarum* NC8, cloned in pNZ8048 and sequenced. Sequence analysis revealed that this gene is highly identical (99% at the nucleotide level) to the *mntA* gene in *L. plantarum* ATCC14977. In the *L. plantarum* WCFS1 genome sequence, a *mntA* homologue is present (99% of the nucleotides identical), indicating that *mntA* is a common and highly conserved gene in *L. plantarum*. We searched the *L. plantarum* WCFS1 genome sequence for regions flanking the *mntA* coding sequence (Figure 1). Upstream of *mntA* in *L. plantarum* WCFS1, an open reading frame (ORF) is located, encoding a protein homologous to both bacterial and eukaryotic enolases (Figure 1). No apparent inverted repeat was present between this *orf1* and *mntA*. Downstream of *mntA*, two ORFs were present with *orf2* encoding a protein that shares 23% identical amino acids to a putative alcohol dehydrogenase from *Aquifex aeolicus* (acc. no. C70418) and 20% to a putative oxidoreductase in *Agrobacterium*

tumefaciens (AAK86631). This *orf2*-encoded protein contains a motif typical for a zinc-binding domain in dehydrogenases (Pfam00107). This organization is similar to the one reported for *L. plantarum* ATCC14977 (Hao *et al.*, 1999b). An additonal ORF (*orf3*) without similarity to any sequence in the databases is located downstream of *orf2*.

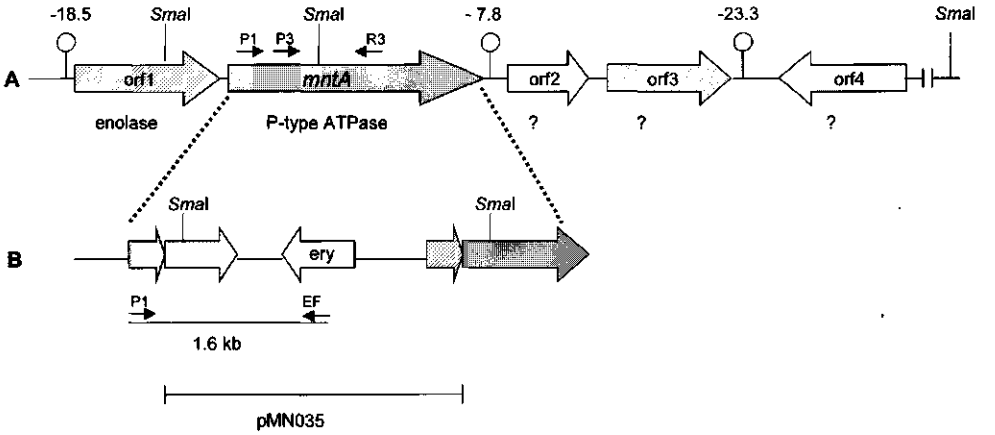

Figure 1. Chromosomal organization of *mntA* and flanking open reading frames (indicated by an arrow in panel A). Putative functions based on sequence similarity are given below the open reading frames. Inverted repeats are indicated by a hairpin structure (values shown are in kcal). Primers used for the amplification of various PCR fragments as described in Materials and methods are indicated by P1 (LPATP1), P3 (LPATP3), R3 (LPATPR3) and EF (EMFORW). In panel B, disruption of *mntA* by integration of pMN035 is depicted.

Inactivation of *mntA*

We inactivated the chromosomal located *mntA* gene in *L. plantarum* WCFS1 by disruption via single cross-over recombination. Therefore, electrocompetent cells were transformed with pMN035 and subsequently plated on MRS-agar plates containing erythromycin (Em, 10 µg/ml) and lincomycin (Lm, 20 µg/ml) to select for plasmid integration. Plates were incubated at 37 °C, anaerobically because of the expected oxygen sensitivity of mutants (Hao *et al.*, 1999a). One single colony was obtained that showed the expected integration since a 1.6 kb PCR fragment was obtained using primers LPATP1 and EMFORW (see Figure 1). Chromosomal DNA was isolated from this candidate mutant to verify the integration of pMN035 in *mntA*. Southern blotting of the *Sma*I-digested chromosomal DNA revealed that for this mutant, recombination indeed had occured in the *mntA*-gene (see Figure 2). When grown in liquid

medium, the *mntA*-disrupted strain was severely stressed, which was indicated by the aggregate-formation of the cells (see Figure 3).

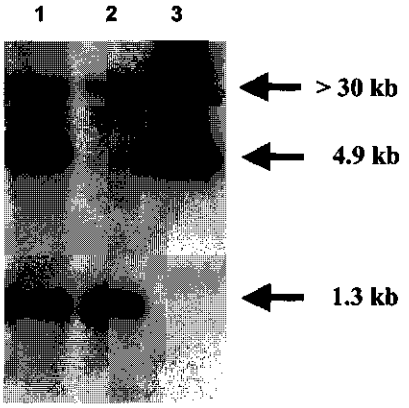

Figure 2 Southern hybridization of chromosomal DNA of the *mntA*-disrupted strain (lane 1) and wild-type (lane 2) of *L. plantarum*, and pMN035 (lane 3) digested with *Sma*I and using the internal fragment of pMN035 as probe. Two fragments (1.3 kb and > 30kb) present in both the wild-type and the mntA mutant strainare indicated. The 4.9 kb fragment corresponding exactly to the size of plasmid pMN035 can only result from integration in *mntA*.

The aggregate formation of the mutant strain occured irrespective whether cells were grown aerobically or anaerobically. The growth rate of the mutant was reduced in medium containing 300 μM, but not significantly in medium with 1.5 μM of Mn^{2+} (Table 1).

Table 1. Growth rates of the *L. plantarum* wild-type and the *mntA* mutant strain in CDM with either 300 or 1.5 μM of $MnSO_4$. Cells were cultivated in a shaken waterbath at 37 °C.

Strain	Aggregate forming	Growth rate (h^{-1})	
		300 μM Mn^{2+}	1.5 μM Mn^{2+}
wild-type	-	0.65 ±0.01[a]	0.36 ±0.01
mntA mutant	+	0.48 ±0.02	0.33 ±0.04
mntA mutant + pMN034	+	0.33 ±0.01	0.24 ±0.01

[a] Standard deviation (n=3)

Measurement of manganese contents

The observed growth inhibition and aggregate-forming of the *mntA* mutant strain was expected to result from a shortage of Mn^{2+}. Therefore, the amount of manganese present in both the wild-type strain and the *mntA* mutant strain was analysed. Cells were grown overnight in CDM containing 300 μM of manganese and cell extracts were prepared as decribed in the Materials and methods section. The ICP-AES analysis revealed that the manganese level in the *mntA* mutant strain (7.0 ± 1.4 μg/mg protein) was not significantly reduced compared to the wild-type cells (9.1 ±0.8 in μg/mg protein), suggesting that the observed phenotype

of the *mntA* mutant strain is not a direct result of an intracellular Mn^{2+} shortage.

Complementation of the *mntA* mutant

Because of the absence of a strong transcriptional terminator between *mntA* and *orf2* (see Figure 1), the observed growth defect may result from a polar effect on one or more genes downstream of *mntA*, rather than from inactivation of *mntA* itself. The *mntA* mutant strain was therefore complemented with a plasmid (pMN034) containing a copy of *mntA* under control of the bacteriophage (ϕSK11G) promoter and a chloramphenicol cassette for selection. The ϕSK11G promoter allows expression in *L. plantarum* (Platteeuw *et al.*, 1994). This construct was introduced in the *mntA* mutant strain by electroporation.

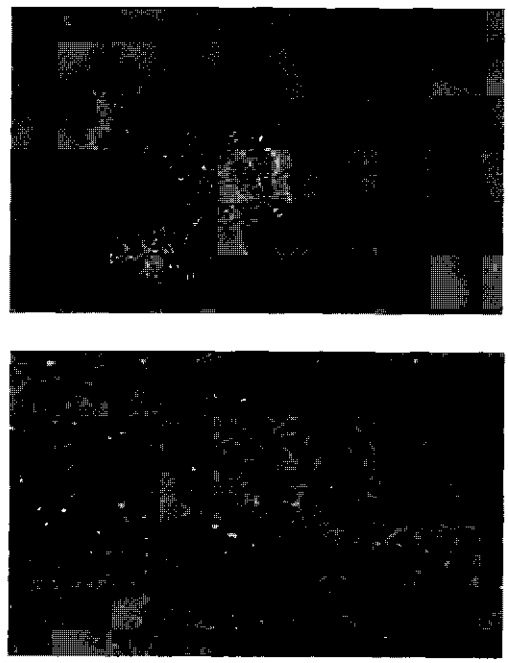

Figure 3. Cells of the *mntA*-disrupted (panel A) and the wild-type (panel B) *L. plantarum* WCFS1 grown in MRS medium.

Plasmid DNA was isolated from five Cm^R/Em^R colonies and all five were found to harbor the pMN034 construct. However, the plasmid-encoded copy of *mntA* did not relieve the clumping of the mutant strain. The growth rate of the pMN034 containing mutant strain was even reduced compared to the non-complemented mutant (Table 1). This reduction in growth rate may be a consequence of the high copy number of the plasmid and possibly overexpression of the *mntA* gene. A high level of MntA may disturb the membrane. Preliminary experiments indicate that

overexpression of the *mntA* gene under control of the nisin-inducible promoter in *L. lactis* severly affects the growth rate (data not shown). These findings suggest, that the observed growth defect of the mutant strain is the result of a polar effect. The *orf2*-encoded protein downstream of *mntA* contains a motif typical for a zinc-binding domain in dehydrogenases (Pfam00107) and has similarity to alcohol dehydrogenase/oxidoreductases but is 100 amino acids shorter. No putative function can be given for the *orf3*-encoded protein.

Although the nature of the growth defect has not been elucidated in this study, we can draw an important conclusion about the function of *mntA* in *L. plantarum*. The inactivation *mntA* in *L. plantarum* does not result in a significant reduction in the intracellular Mn^{2+} level. The absence of growth recovery in the pMN034-complemented mutant suggests that the observed reduction in growth rate does not result from *mntA* mutation, but is probably caused by a polar effect on *orf2* and/or *orf3*. The *L. plantarum* mutants constructed by chemical mutagenesis by Hao *et al.* (1999a,b), contained less than 1% of intracellular Mn^{2+} levels compared to the wild-type cells and showed extreme high Mn^{2+} requirements. Although the MntA transporter was apparently not expressed in this mutant strain, the *mntA* mutant strain constructed in the present study demonstrates that the observed phenotype can not arise from a single mutation in *mntA*. However, it is more likely that a selection has been made for one or more additional mutations in genes encoding transporters for Mn^{2+} or in other structural or regulatory genes affecting Mn^{2+} homeostasis in *L. plantarum*. The *mntA* mutant strain constructed in the present study allows to determine the contribution of the P-type ATPase to the overall import of Mn^{2+} or other cations in *L. plantarum* in transport studies.

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Chapter 7.

Summary and Concluding Remarks

Summary and concluding remarks

One of the aims of the research described in this thesis (Chapter 1 and 2) was to investigate the conversion of phenylalanine to the aromatic flavor compound benzaldehyde in lactic acid bacteria (LAB) (Chapter 3). *Lactobacillus plantarum* was used as the model organism to study phenylalanine degradation in LAB. This bacterium was selected in an initial screening for phenylalanine degradation since it produced higher levels of benzaldehyde than other LAB (Chapter 4). In addition, the availability of genetic tools for this bacterium was an advantage for the second aim of this thesis: the identification and characterization of Mn^{2+} transport systems in *L. plantarum* (described in Chapter 5 and 6).

Benzaldehyde

Amino acid degradation is important for the formation of flavor compounds such as aldehydes, thiols and keto acids in fermented dairy products (Urbach, 1995). Hence, considerable attention has focused on the role of starter LAB in the production of amino acids and their proteolytic systems. Except for the sulphur-containing amino acids (Alting *et al.*, 1995; Fernández *et al.*, 2000; Bruinenberg *et al.*, 1997), and the identification of aromatic aminotransferases in *Lactococcus lactis* (Yvon *et al.*, 1997; Gao *et al.*, 1997), little information is available on the contribution of LAB to the degradation of amino acids. In the study presented here, research was focused on the conversion of phenylalanine and it was demonstrated that LAB can contribute to the formation of aromatic flavors by conversion of this amino acid to benzaldehyde (Chapter 3).

Benzaldehyde formation in *L. plantarum* occurs via a pathway different from those proposed for several fungi and for *P. putida* (Chapter 1). In *L. plantarum*, the conversion of phenylalanine to benzaldehyde involves only a single enzymatic step. The phenylpyruvic acid, formed by the activity of the aminotransferase of *L. plantarum*, is an unstable compound that is chemically converted to benzaldehyde mainly. In addition, small amounts of phenylacetic acid, mandelic acid, and phenylglyoxylic acid are derived from phenylpyruvic acid (Figure 1). In Chapter 3, we demonstrated that, in the presence of the metal ions Fe^{2+}, Fe^{3+}, Co^{2+}, or Mn^{2+}, this chemical conversion of phenylpyruvic acid was accelerated with increasing pH and temperatures, in an oxygen-dependent reaction. The physical parameters of Gouda cheese, such as the low pH (pH 5.3) and the relatively low temperature used for maturation of the

cheese (approximately 13° C), are not in favor of this chemical reaction. However, the ripening process of cheese can take up to 12 months, and therefore, even a slow rate of chemical degradation of phenylpyruvic acid can be expected to contribute to benzaldehyde formation in cheese.

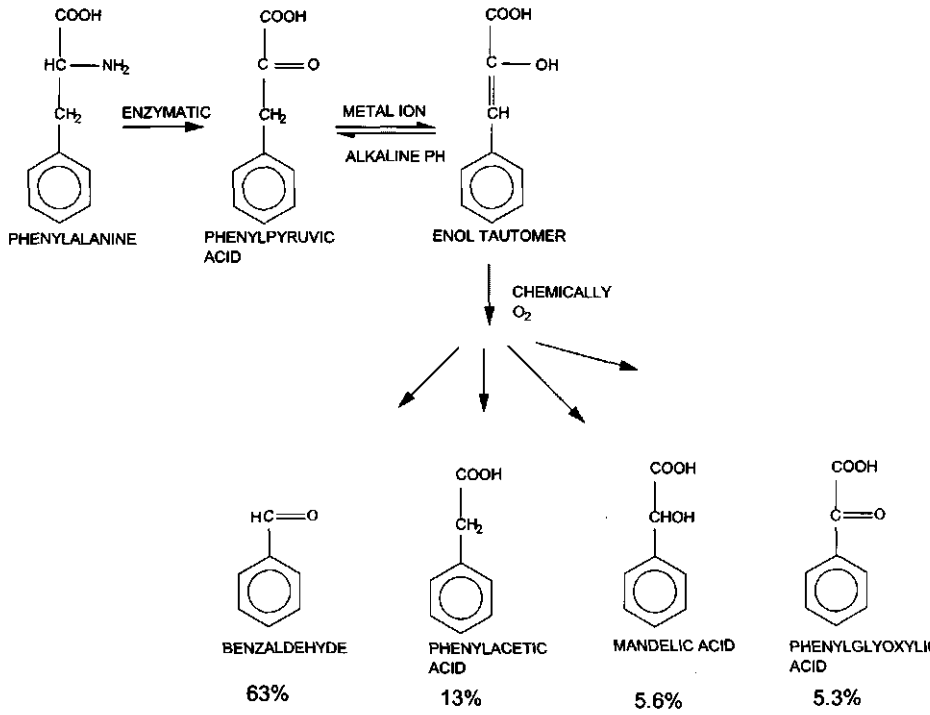

Figure 1: Proposed pathway for the conversion of phenylalanine to benzaldehyde in *L. plantarum*. Percentages indicated refer to molar yields obtained after 6 h incubation of phenylpyruvic acid (PPA) at 37° C in the presence of 350 µM of $CuSO_4$. The chemical oxidation of PPA to benzaldehyde by Mn^{2+} ions has been reported previously (Villablanca and Cilento (1987).

This is also supported by recently published results that confirm that chemical oxidation of phenylpyruvic acid to benzaldehyde occurs under cheese ripening conditions (Gummalla and Broadbent, 2001). Moreover, it has been demonstrated that aromatic aminotransferases from LAB are still active in a cheese environment, and thus can generate the corresponding α-keto acids in a cheese matrix (Gao *et al.*, 1997; Yvon *et al.*, 1997). Based on these findings, and on the results described in Chapter 3, it can be concluded that LAB contribute to the formation of phenylalanine-derived flavor compounds in cheese. The impact of chemical steps on flavor formation is now receiving more attention. The combination of

enzymatic- and chemical steps in the conversion of methionine and tryptophan, leading to methanethiol and indole aldehyde, respectively, has been reported (Goa *et al*, 1998; Gummala and Broadbent, 1999).

Genes for phenylalanine conversion

Analysis of the genome sequence of *L. plantarum* provides additional proof for the proposed pathway for benzaldehyde production (Figure 1). The initial step requires the presence of an amino transferase to convert phenylalanine to phenylpyruvic acid. Analysis of the *L. plantarum* genome sequence reveals the presence of two genes encoding proteins, that share 57% and 48% identity over the complete sequence, with the aromatic aminotransferase (*AraT*) of *L. lactis* (Rijnen *et al.*, 1999). AraT is the major aminotransferase in the conversion of aromatic amino acids in *L. lactis*. Presumably, one of the two or both genes, in *L. plantarum* are responsible for the pyridoxal 5′-phosphate-dependent aminotransferase activity reported in Chapter 3. In the genome sequences of *L. plantarum* and *L. lactis*, no genes are present that could encode enzymes involved in conversion of phenylpyruvic acid to subsequently phenylacetaldehyde (EC 4.1.1.43) and phenylacetate (EC 1.2.1.39 or EC 1.2.1.5). Moreover, both LAB do not seem to harbor genes encoding the mandelate dehydrogenase or benzoylformate decarboxylase activity which was reported for *P. putida* (Tsou *et al.*, 1989, 1990). Mandelic acid dehydrogenase activity has been reported for a *Lactobacillus* species (*L. curvatus*), however, this enzyme activity is only present in a limited number of lactobacilli (Hummel *et al.*, 1998). The protein sequence of benzoylformate decarboxylase shows similarity to that of pyruvate decarboxylase (PDC). It can therefore not be excluded that PDC can have activity towards phenylpyruvic acid. However, in *L. plantarum* PDC has no effect on benzaldehyde formation because the rate of conversion of the keto acid was not altered in a boiled cell extract (Chapter 3).

Although various authors have proposed that phenylacetic acid is hydroxylated to mandelic acid, untill now, there is no evidence for the existence of an enzyme that can perform this reaction. It can therefore be questioned if such an enzyme exists, and whether the enzymatic conversion of phenylacetic acid (Figure 1 in Chapter 1) to mandelic acid proposed by several authors for white-rot fungi actually occurs. It is possible that in these fungi, mandelic acid is formed through chemical degradation of phenylpyruvic acid rather than by an enzyme-catalyzed conversion step. Considering the time course of the experiments involving

fungi (up to 14 days), even at the low pH (pH 4.5-5.5) used to culture fungi, chemical degradation is to be expected.

In *L. plantarum*, Mn^{2+} ions that are accumulated intracellularly are involved in the chemical conversion step of phenylpyruvic acid leading to benzaldehyde (Chapter 3). The formation of benzaldehyde via phenylalanine conversion is not limited to *L. plantarum,* but occurs in various strains belonging to the genera *Lactobacillus* and *Leuconostoc* that contain high levels of Mn^{2+} (see Chapter 4). *L. lactis,* a bacterium containing low Mn^{2+} levels, therefore produces high amounts of phenylpyruvic acid while only minor amounts of benzaldehyde are formed.

Manganese uptake systems

The extremely high Mn^{2+} level in *L. plantarum* is an intriguing characteristic of this species. The millimolar levels intracellularly exceed by far the micromolar level of Mn^{2+}, required as cofactor for enzymes. Although it has for long been known that *L. plantarum* lacks superoxide dismutase (SOD), and therefore depends on Mn^{2+} as a non-enzymatic mechanism to scavenge toxic oxygen species, our knowledge on the transport systems that can accomplish the Mn^{2+} uptake in this species is very limited. Considering the importance for *L. plantarum* to maintain millimolar levels of Mn^{2+} intracellularly, a highly efficient system for Mn^{2+} homeostasis can be expected. The only transporter with affinity for Mn^{2+} that has been reported to date in *L. plantarum,* is a P-type ATPase transporter (MntA, Hao *et al.*, 1999b). In Chapter 5, we demonstrate that *L. plantarum* harbors three more genes encoding transport systems that are induced upon Mn^{2+} starvation. These transport proteins belong either to the ATP-binding cassette (ABC) transporter family or the natural resistance-associated macrophage protein (Nramp) family.

ABC-transporter

To investigate the role of the ABC transporter in Mn^{2+} homeostasis in more detail, the *mtsCBA* genes encoding ABC-transport systems in *L. plantarum* and *L. casei* have been identified and characterized. The *Lactobacillus mts* gene products are homologous to ABC transporters involved in Mn^{2+} and Fe^{2+} uptake in other gram-positive and -negative bacteria (Chapter 5). The genes, encoding a similar system are present in the *L. lactis* genome, suggesting that this type of metal transporter represents a common system in LAB (Bolotin *et al.*, 2001). The substrate-binding protein (MtsA) of *L. plantarum* is 47% and 50% identical to ScaA (*S. gordonii*,

Kolenbrander *et al.*, 1998) and PsaA (*S. pneumoniae*, Dintilhac *et al.*, 1997), respectively. ScaA and PsaA encode the binding components of the ABC-type uptake system for Mn^{2+}, and inactivation of the corresponding genes affects Mn^{2+} uptake and results in increased Mn^{2+} requirements in these species. The organization of the operon encoding this type of transporter in the two *Lactobacillus* species is similar to equivalents in Gram-positives; the ATP-binding gene at the 5′-end and the lipoprotein gene at the 3′-end. Downstream of this operon, a gene encoding a putative thiolperoxidase is located in *S. pneumoniae* (Novak *et al.*, 1998) and *S. gordonii* (Kolenbrander *et al.*, 1998), or a gene encoding an iron-dependent repressor homolog (DtxR) in *S. mutans* (Kitten *et al.*, 2000) and *T. pallidum* (Posey *et al.*, 1999). Although homologs to both genes are present in the *L. plantarum* WCFS1 genome, these genes are not clustered with the *mtsCBA* genes. In agreement with findings for similar gene clusters in *S. pneumoniae*, *S. gordonii*, and *S. mutans*, the *mtsCBA* genes are transcribed as a monocistronic transcript (Chapter 5). Transcription is initiated from a promoter upstream of *mtsC* that is induced upon Mn^{2+} starvation. The *mtsCBA* transcript level was 6-fold increased in cells grown in 1.5 μM Mn^{2+} compared to growth on 300 μM Mn^{2+}, supporting a role of these genes in Mn^{2+} uptake similar to *S. gordonii* (Kolenbrander *et al.*, 1998) and *S. pneumoniae* (Dintilhac *et al.*, 1997).

Nramp type transporters

Recently, homologues of the mammalian Nramp transporters, that are high-affinity uptake systems for Mn^{2+}, were identified in *Bacillus subtilis* (Que and Helmann, 2000), *Salmonella typhimurium* (Kehres *et al.*, 2000) and *Escherichia coli* (Makui *et al.*, 2000). In *L. plantarum*, the genes *mntH1*, *mntH2* and *mntH3* are present, encoding proteins with homology to these transporters (Chapter 5). The genes *mntH1* and *mntH2* were found to be expressed upon Mn^{2+} starvation in *L. plantarum*, but expression of *mntH3* was not detected. The presence of a gene predicted to encode a ferrochelatase upstream of *mntH3*, suggests a role for MntH3 in iron uptake rather than Mn^{2+} transport. MntH1 and MntH2 are typical Nramp proteins, being highly hydrophobic (11 and 12 putative transmembrane domains, respectively) and the presence of a conserved transport motif in the fourth intracellular loop. Expression of both *mntH1* and *mntH2* was induced when the Mn^{2+} level in the medium was reduced from 300 to 1.5 μM, resulting in a 6- and 13-fold increase in transcript

level, respectively. The transcription initiation site of *mntH2* was determined to be 39 nucleotides upstream of the TTG translational start codon. The 2.1 kb transcript length detected with a *mntH2*-specific probe indicates that this gene is expressed as a bicistronic transcript with a downstream located gene of unknown function.

Manganese homeostasis in *L. plantarum*

L. plantarum is different from other bacteria for which Mn^{2+} uptake systems have been reported because of its extreme high level of intracellular Mn^{2+}. Remarkably, the *mtsA*, *mntH2* and *mtsCBA-mntH2* double mutants constructed in Chapter 5 were not affected in growth in Mn^{2+} limited medium compared to the parental strain, suggesting that *L. plantarum* is highly adaptive to changes affecting Mn^{2+} homeostasis. We demonstrated that this apparent lack of phenotype can be explained by upregulation of additional transporters in the various mutant strains. Inactivation of *mtsA* resulted in increased transcription of *mntH2*. Vice versa, in the *mntH2* mutant strain, the *mtsCBA* transcription was found to be upregulated. Upregulation of *mntH* upon inactivation of the *mntA* gene, encoding the ATP-binding protein of the ABC transporter, was also reported for *B. subtilis* (Que and Helmann, 2000). The *mntH1* gene was expected to be upregulated in the *mtsA-mntH2* double mutant in *L. plantarum,* however Northern analysis revealed no increase in transcription of the this gene. Another option is that the *mntA* gene, encoding the P-type ATPase, is upregulated. Expression of this transporter in *L. plantarum* has been demonstrated by immunoblotting (Hao *et al.*, 1999b), but we could not detect *mntA* transcription by Northern analysis. However, the presence of additional transporters with affinity for Mn^{2+} is not unlikely in *L. plantarum*, considering the importance of this metal ion for this bacterium. Findings that a *mntH-mntABCD* double-mutant of *B. subtilis* is still able to grow in the presence of micromolar levels of Mn^{2+} also suggest the presence of at least one more pathway for Mn^{2+} uptake in this bacterium (Que and Helmann, 2000). The identity of these transporters remains to be elucidated. Findings that the transporters MgtB and CorA involved in uptake of Mg^{2+} in *Salmonella typhimurium* (Snavely *et al.*, 1989) may also transport Mn^{2+}, suggests that transporters primarily involved in the transport of metal ions other than Mn^{2+}, can not be excluded. Homologues of the metal-citrate transporter (CitM) in *B. subtilis* (Krom *et al.*, 2000) and the phosphate-inorganic transport system (Pit) in *E. coli* and *A. johnsonii* (Van Veen *et al.*, 1994a;1994b), that can transport a Mn^{2+}-citrate or Mn^{2+}-

phosphate complex, are not present in *L. plantarum*. Suprisingly, several attemps to inactivate the *mntH1* gene failed, suggesting that inactivation of this transporter could be lethal to *L. plantarum*. Inactivation of *mtsA* and *mntH2* seperately, or together, did not significantly reduce the intracellular Mn^{2+} level. These findings suggest that Mn^{2+} homeostasis in *L. plantarum* is very efficiently and probably tightly controlled. A *L. plantarum* mutant was constructed via chemical mutagenesis (Hao *et al.*, 1999a), that was severely affected in growth and required a 5,000-fold increased Mn^{2+} level in the culture medium. Expression of the MntA protein could not be detected in this mutant strain (Hao *et al.*, 1999b). However, the results described in Chapter 6 demonstrate that the phenotype of the mutant strain can not result from a single inactivation of the *mntA* gene. Chapter 6 describes the targeted mutation in the *mntA* gene of *L. plantarum* WCFS1. The *mntA* mutant strain showed no significant decrease in intracellular Mn^{2+} concentration, but was severely affected in growth and the cells formed aggregates. However, complementation of the mutant with a plasmid-encoded copy of *mntA* strongly suggested that this phenotype results from a polar effect on the downstream genes and not from inactivation of *mntA*. In conclusion, the mutant strain constructed by Hao *et al.* (1999a) is likely to be mutated in other gene(s) than *mntA*.

Polyphosphate

The millimolar levels of Mn^{2+} present intracellular in *L. plantarum* raises questions about the form taken by this metal ion in the cell. There is no doubt that this amount can not be freely dissolved in the cytoplasm. Electron micrographs revealed the presence of large polyphosphate granules in *L. plantarum* grown in high Mn^{2+} medium that could not be identified in cells grown in Mn^{2+}-limited medium (Archibald and Duong, 1984). Polyphosphate accumulation is a phenomenon known to occur in a wide variety of micro-organisms (Kortstee and van Veen, 1999). Indeed, *L. plantarum* harbors a gene encoding a polyphosphoshate kinase that can couple phosphate to form the energy-rich polyphosphate. *L. lactis* lacks this enzyme and it is tempting to speculate about a link with the inability of this bacterium to accumulate Mn^{2+}. In the polyphosphate-accumulating bacteria *E. coli* and *Acinetobacter johnsonii*, phosphate uptake is mediated as a complex with divalent cations via the phosphate inorganic transport (Pit) system (van Veen *et al.*, 1994; van Veen *et al.*, 1993). In *A. johnsonii*, transport of the metal-phosphate complex is closely related to the metabolism of polyphosphate (van Veen *et al.*, 1994) and results in

accumulation of the metal-polyphosphate chelate. Although *L. plantarum* presumably accumulates Mn^{2+} in a similar form, the phosphate required for this complex must enter the cell via an alternative system because *L. plantarum* has no Pit system. However, the form of Mn^{2+} that is transported by the ABC-type transporter has not been determined. The possibility that Mn^{2+} is bound and transported as a chelated complex can not be ruled out.

Regulation of manganese uptake

Based on the presence of multiple systems for Mn^{2+} uptake in *L. plantarum,* it can be assumed that uptake via these systems must be coordinated by a regulatory system. This is also suggested by the fact that the three transport systems described in Chapter 5 are induced upon Mn^{2+} starvation. Recently, homologs of the diphteria toxin repressor (DtxR) family of iron repressors have been reported that are, unlike DtxR, active as a Mn^{2+} complex. Binding of this homolog to its target region was demonstrated to repress *mntH* expression in *E. coli* (Patzer and Hantke, 2001) and the ABC transporter genes in *S. gordonii* (*sca* operon, Jakubovics *et al.*, 2000) and in *T. pallidum* (*tro* operon, Posey *et al.*, 1999). A similar protein in *B. subtilis* is bifunctional and represses *mntH* expression under Mn^{2+}-replete conditions while it activates the expression of the *mntABCD* operon under low Mn^{2+} conditions. In *L. plantarum*, a gene predicted to encode a DtxR-homolog, is present with 41% and 38% identity to SloR (*S. mutants*, Kitten *et al.*, 2000) and ScaR (Jakubovics *et al.*, 2000) and other homologs in streptococci (Figure 2). The similarity to MntR (*B. subtilis*, Que and Helmann, 2000), TroR (*T. pallidum*, Posey *et al.*, 1999) and MntR (*E. coli*, Patzer and Hantke, 2001) was low being 23, 27, and 20% identical, respectively. These DtxR homologs lack the C-terminal (SH3-like) domain present in the streptococcal and the *L. plantarum* homologs. However, the residues involved in metal binding and most residues in the DNA binding helix are conserved. Northern analysis revealed that this MntR encoding gene was expressed in *L. plantarum*, and that expression was not affected by the Mn^{2+} concentration in the growth medium (unpublished results). Several different aproaches to inactivate the MntR-encoding gene in *L. plantarum* failed. Considering the likely role of this gene in regulation of the Mn^{2+} transport systems described in Chapter 5, this is not surprising. Inactivation of this gene may be lethal to *L. plantarum* in contrast to other bacteria that do contain SOD

such as *S. gordonii* (Kolenbrander *et al.*, 1998), *B. subtilis* (Que and Helmann, 2000), and *E. coli* (Patzer and Hantke, 2001).

```
lpmntR          MTPMKEDYLKIIFELGGTKKKVSNKQIALSLDIAAGSVTEMVGKLVQEGLAKHTPYAGIS 60
sgoscaR         MTPNKEDYLKCLYELGTRHNKITNKEIAQLMQVSPPAVTEMMKKLLAEELLIKDKKAGYL 60
spnpsaR         MTPNKEDYLKCIYEIGIDLHKITNKEIAARMQVSPPAVTEMIKRMKSENLILKDKECGYL 60
spymntR         MTPNKEDYLKCIYEIGEQEPKITNKMVAEKMHVSAPAVSEMIKKMISQGWIVKDKAKGYL 60
smusloR         MTPNKEDYLKIIYELSERDEKISNKQIAEKMSVSAPAVSEMVKKLLLEDLVLKDKQAGYL 60
                *** ****** ::*:.    *::** :*  : ::. :*:**: ::  :    :    *
                    #
                                                    DNA binding

lpmntR          LTKKGIRYAETLVRKHRIWEDFLVDKLDYDLPDVHTEAEVLEHVTSERLVDSLEAFLGNP 120
sgoscaR         LTDLGLKLVSDLYRKHRLIEVFLVHHLGYTTEEIHEEAEVLEHTVSDHFVERLDQLLDYP 120
spnpsaR         LTDLGLKLVSELYRKHRLIEVFLVHHLDYTSDQIHEEAEVLEHTVSDLFVERLDKLLGFP 120
spymntR         LKDKGYALVANLYRKLRLIEVFLIHQLGYNTQEVHQEAEVLEHTVSDSFIDRLDKILDFP 120
smusloR         LTKKGQILASSLYRKHRLIEVFLMNHLNYTADEIHEEAEVLEHTVSDVFVERLDKFLNYP 120
                *.. *   .  * ** *: * **:.:*.*   ::* *******..*: ::: *: :*. *
                              ^   ^               ^   #  ##

lpmntR          THCPHGGAIPDKDGHYQEDSHVSLADTQDGTTVTIERFIDNHDLLVYLHDTPLKIGQQVT 180
sgoscaR         KACPHGGTIPAKGELLVEKHKLTLEEAKEKGDYILARVHDNFDLLTYLERNGLQVGKTIR 180
spnpsaR         KTCPHGGTIPAKGELLVEINNLPLADIKEAGAYRLTRVHDSFDILHYLDKHSLHIGDQLQ 180
spymntR         DFCPHGGTIPRYGQPLVEMNTTTLNTITELGRFRLSRIHDHFDLIQYLEAHHLNINTELT 180
smusloR         KVCPHGGTIPQHGQPLVERYRTTLKGVTEMGVYLLKRVQDNFQLLKYMEQHHLKIGDELR 180
                  *****:**  .    *    .*    :     : *. * .::: *:.   *::.  :

lpmntR          VLKHDPFEGPVTVSIQKTGEEIPVSYKAAHNVFVK---- 215
sgoscaR         FLGYDDFSH--LYSLEVDGQEIQLAQPIAQQIYVEKI-- 215
spnpsaR         VKQFDGFSN--TFTILSNDEDLQVNMDIAKQLYVEKIN- 216
spymntR         LTQIDTFAK--TYTICYGDKELVIPENIAKQLYVTAL-- 215
smusloR         LLEYDAFAG--AYTIEKDGEQLQVTSAVASQIYIEKKAY 217
                .   * *      ::   .::: :    * ::::
```

Figure 2. Multiple sequence alignment of the the MntR protein in *L. plantarum* WCFS1 with DtxR-like proteins in *S. gordonii* (ScaR), *S. pneumoniae* (PsaR), *S. pyogenes* (MntR), and *S. mutans* (SloR). Alignment of the sequences was performed using the ClustalW program. The residues that form the metal-binding site in DtxR-like repressors (Qiu *et al.*, 1995) are perfectly conserved (indicated by #).

Target genes for MntR

The transcriptional analysis of *mtsCBA*, *mntH1* and *mntH2* described in Chapter 5 reveals that Mn^{2+} controls the expression of these genes. Moreover, the increased expression of *mtsCBA* and *mntH2* in the *mntH2*- and *mtsA*-mutant, respectively, suggest that the maintenance of Mn^{2+} homeostasis is regulated by an efficient regulatory system. Mn^{2+} homeostasis in *L. plantarum* is possibly regulated by a similar DtxR-like metalloregulatory protein as demonstrated for *S. gordonii* (ScaR; Jakubovics *et al.*, 2000), *E. coli* (MntR; Patzer and Hantke, 2001), *T. pallidum* (TroR; Posey *et al.*, 1999), and *B. subtilis* (MntR; Que and Helmann, 2000). A gene (*mntR*) encoding a homolog to these regulatory proteins is present in the *L. plantarum* genome. In Chapter 5, potential target regions for MntR were identified in the promoter regions of *mtsC*, *mntH1* and *mntH2*. Sequences similar to the ones identified in *B. subtilis* (Que and Helmann, 2000), *E. coli* (Patzer and Hantke, 2001), and *T.*

pallidum (Posey *et al.*, 1999) were present in the *mntH1*, *mntH2* and *mtsC* –35 promoter regions in *L. plantarum*. These findings suggest, that regulation of Mn^{2+} uptake in *L. plantarum* may be accomplished by the identified DtxR homolog. It would be highly speculative to draw conclusions on the mode of action (activation or repression) of MntR in *L. plantarum* since similar sequences were found to result in repression (*mntH*) or activation (*mntABCD*) *in B. subtilis* (Que and Helmann, 2000). An additional sequence can be identified in the *mntH2* promoter region that is similar to the conserved palindromic sequence present in *S. gordonii* and other streptococci (Jakubovics *et al.*, 2000). minvenw.nl

Considering the role of Mn^{2+} in *L. plantarum* in oxygen stress tolerance, additional binding boxes may be present in control regions of other genes, that are involved in protection against oxygen stress. Analysis of the promoter regions of the genes encoding glutathione peroxidase, catalase, thiolperoxidase, NADH oxidase (7 genes are present in *L. plantarum*) suggest the presence of similar sequences for several genes. Sequences with similarity to the conserved box in the *mtsC* and *mntH2* promoter region can be found in the promoter region of a gene, encoding a putative gamma glutamate cysteine ligase (gamma-ECS, Figure 3).

γ-ecs	**AATTATTTG**AAT**A**CA**C**AAG**AA**GA-226-ATG
mtsC	**AATTATTTG**C**AT**TAG**C**GTT**AA**TT- 59-ATG
nox_{1925}	TG**TT**T**TTTG**GTT**C**A**T**AA**TT**G**AT**C-175-ATG
mtsC	AA**TT**A**TTTG**CA**TT**A**G**CG**TT**AA**TT**- 59-ATG
nox_{1069}	**A**CTG**ATTTG**AA**TT**TCATGAT**AT**C-275-ATG
mtsC	**AATTATTTG**C**ATT**AGCGTTA**AT**T- 59-ATG

Figure 3. Alignment of the potential binding site for MntR in the *mtsC* promoter region with that of genes predicted to encode γ-glutamate cysteine ligase (γ-ecs), and two representatives of potential NADH oxidase-encoding genes in *L. plantarum* WCFS1.

This gene is located upstream of the thiolperoxidase encoding gene. Gamma-ECS is the first enzyme involved in glutathione biosynthesis. There is no strong transcriptional terminator between the two genes, thus a bicistronic transcript is not unlikely. Similar sequences are also present in the region upstream of 4 out of the 7 genes encoding NADH oxidase. It

remains to be determined whether this consensus sequence has significance and has a function in *L. plantarum*.

Perspectives

In order to increase benzaldehyde formation in lactic acid bacteria, it may be desirable to combine the level of phenylpyruvic acid formed by the aminotransferase activity of *Lactococcus lactis*, with the high intracellular Mn^{2+} levels present in *L. plantarum*. We tried to achieve this, by expressing the *mntA* gene from *L. plantarum* or the *mtsCBA* genes from *L. casei* in *L. lactis* using the nisin-inducible expression system. The overexpression of the *mtsCBA* genes in *L. lactis* was confirmed by SDS-PAGE. The raised level of the ABC transporter resulted in only a small increase in the intracellular Mn^{2+} concentration. It may be possible that *L. lactis* can not store high amounts of Mn^{2+} and subsequently, excess of this metal ion is exported. Considering the fact that Mn^{2+} is accumulated as a polyphosphate complex in *L. plantarum* as suggested by Archibald and Duong (1984), it is possible that *L. lactis* can not realise this since a gene potentially encoding polyphosphate kinase is not present in the *L. lactis* genome. A combined overexpression of the *mtsCBA* genes and the polyphosphate kinase encoding gene in *L. lactis* could reveal if this is indeed true. The results described in this thesis show that the high intracellular level of manganese is probably not accomplished by a single transporter in *L. plantarum*, but is likely the overall effect of the concerted activity of several transporters that are presumably subjected to a strict regulation mechanism. Therefore, it remains to be established whether the formation of benzaldehyde in *L. lactis* can be increased by the overexpression of one or more Mn^{2+} transporters. An alternative would be to increase the phenylpyruvic acid generation in *L. plantarum*. This could be realised via overexpression of the aminotransferase of *L. lactis*. However, the conversion of amino acids to flavor compounds in cheese curd can be enhanced by the addition of α-ketoglutaric acid (Yvon *et al.*, 1999). This demonstrates that for most starter cultures, the availability of the aminogroup acceptor, rather than the aminotransferase activity, is limiting. Indeed, heterologous expression of glutamate dehydrogenase, in *L. lactis* increased the conversion of amino acids to flavor compounds (Rijnen *et al.*, 2000). A potentially succesful approach may therefore be, to improve the regeneration of transaminated α-ketoglutaric acid in *L.*

plantarum by overexpression of the glutamate dehydrogenase encoding gene.

The increasing number of papers on Mn^{2+} uptake may result from the awareness that Mn^{2+} is not only required as cofactor, but probably has a broad role in various important cellular processes such as host interaction and virulence. Further analysis of Mn^{2+} homeostasis in bacteria will provide valuable information that may be usefull in the development of antimicrobial drugs. The identification of the three transport systems that are induced upon Mn^{2+} starvation in *L. plantarum* provides more insight in the mechanisms available to import this essential metal from the environment. The *mtsA* and *mntH2* mutants of *L. plantarum* suggest, that Mn^{2+} accumulation in this bacterium is probably very efficiently, and possibly tightly controlled. An intriguing question is what other system can, in addition to MtsCBA, MntH1, MntH2, and MntA, transport Mn^{2+} in *L. plantarum*. Results described in Chapter 5 suggest that at least one more high-affinity transporter should be present in addition to the ABC transporter and the two Nramp-type transporters. Although we did not detect transcription of *mntA* in the wild type strain, the MntA transporter can not be excluded as candidate. DNA micro-array analysis would be a powerful tool to reveal additional genes encoding transporters or other cellular functions that are regulated by Mn^{2+}. Determining the relative significance of multiple systems involved in Mn^{2+} homeostasis in individual species is a challenge for future research. From the increased knowledge of Mn^{2+} homeostasis, valuable information can be gained to understand the observed link of Mn^{2+} homeostasis to host-microbe interactions and virulence.

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Samenvatting

Benzaldehyde is een geur- en smaakstof die van nature voorkomt in de pit van o.a. kersen, abrikozen en perzikken, en in amandelen. Deze stof wordt, samen met vanilline, op grote schaal toegepast in de geur- en smaakstoffen industrie (Hoofdstuk 1). Daarnaast is benzaldehyde ook een componenten die in verscheidene kaastypes gevormd wordt. In kaas kan deze geur- en smaakstof gevormd worden uit het aminozuur fenylalanine. De bijdrage van melkzuurbacteriën aan de omzetting van fenylalanine, maar ook andere aminozuren, naar geur- en smaakstoffen is nog niet uitvoerig bekend. Dit proefschrift beschrijft de resultaten van een onderzoek naar de vorming van benzaldehyde door melkzuurbacteriën. Een belangrijke factor in de vorming van deze component is de intracellulaire concentratie aan mangaan in melkzuurbacteriën. Dit mangaan komt de cel binnen via speciale transport systemen die tot een aantal jaren geleden nog onbekend waren. Recent is meer bekend geworden over de transport systemen voor dit metaal ion en over de regulatie van deze opname systemen. Een overzicht van de recente literatuur op dit gebied staat beschreven in Hoofdstuk 2.

In Hoofdstuk 3 wordt de omzetting van fenylalanine naar benzaldehyde door de melkzuurbacterie *Lactobacillus plantarum* beschreven. In deze bacterie wordt fenylalanine omgezet via een route, die zowel een enzymatische als een chemische stap omvat. Het fenylalanine wordt in een eerste stap omgezet naar fenylpyruvaat door een aminotransferase in *L. plantarum*. Fenylpyruvaat is een instabiele verbinding, die in aanwezigheid van metaal ionen, en bij alkalische pH, vrij snel omgezet wordt naar benzaldehyde. Deze chemische omzetting verloopt alleen in aanwezigheid van zuurstof en na toevoeging van Cu^{2+}, Fe^{2+}, Fe^{3+} of Mn^{2+} ionen.

In Hoofdstuk 4 is aangetoond dat Mn^{2+} het metaal ion is dat verantwoordelijk is voor de bovengenoemde omzetting van fenylpyruvaat naar benzaldehyde in melkzuurbacteriën. In een gedialyseerd celextract van *L. plantarum* wordt geen benzaldehyde gevormd en accumuleert het fenylpyruvaat dat gevormd is door een aminotransferase in *L. plantarum*. De toevoeging van Mn^{2+}, Fe^{2+}, Fe^{3+} of Cu^{2+} ionen aan het gedialyseerde celextract herstelt de omzetting van fenylpyruvaat naar benzaldehyde waarbij in aanwezigheid van mangaan de hoogste omzettingssnelheid gemeten werd. De omzetting van fenylalanine naar benzaldehyde is niet uniek voor *L. plantarum*, maar kan ook uitgevoerd worden door een aantal andere melkzuurbacteriën van de genera *Lactobacillus* en *Leuconostoc* (Hoofdstuk 4). Bacteriën die benzaldehyde vormen hebben allen een hoge

intracellulaire mangaan concentratie, hetgeen suggereert dat dit metaal betrokken is bij de chemische omzetting in de melkzuurbacteriën. *Lactococcus lactis* heeft een hoge omzetting van fenylalanine naar fenylpyruvaat maar kan dit vervolvens niet omzetten naar benzaldehyde als gevolg van de lage concentratie aan mangaan in de cel.

Naar aanleiding van de resultaten van Hoofdstuk 3 en 4 werden in Hoofdstuk 5 de mangaan transport systemen in *L. plantarum* onderzocht. Dit organisme staat bekend om zijn afhankelijkheid van mangaan om zich onder oxidatieve stress condities te handhaven. Hiermee onderscheidt deze bacterie zich van andere bacteriën, waaronder *L. lactis,* die hiervoor het enzym superoxide dismutase bezitten. Naast de reeds uit de literatuur bekende P-type ATPase in *L. plantarum*, zijn in Hoofdstuk 5 nog een drietal additionele systemen beschreven die geïnduceerd worden onder mangaan-gelimiteerde groeicondities. Dit is een ATP-binding cassette (ABC) transporter (MtsCBA) en twee Nramp (natural resistance-associated macrophage proteins) type transporters, genoemd MntH1 en MntH2. De genen coderend voor MtsA en MntH2 zijn afzonderlijk, en in combinatie, geïnactiveerd in *L. plantarum*. Deze mutanten waren echter niet vertraagd in groeisnelheid onder aerobe condities, noch was de intracellulaire mangaan concentratie significant verlaagd. Analyse van de transcriptie van de verschillende genen toonde aan, dat in de *mtsA* mutant het transcriptieniveau van *mntH2* verhoogd was, en vice versa, werden in de *mntH2* mutant hogere *mtsCBA* transcriptie niveaus gemeten. In de dubbel mutant (*mtsA-mntH2*) werd geen verhoogde transcriptie van het *mntH1* gen gemeten, hetgeen suggereert dat additionele transporters voor deze mutaties compenseren. De resultaten in Hoofdstuk 5 suggereren dat mangaan homeostase in *L. plantarum* erg efficient is, en bovendien strak gereguleerd wordt. Inderdaad is in het *L. plantarum* genoom een gen aanwezig dat kan coderen voor een eiwit dat homologie vertoont met recent geïdentificeerde regulatoren van Mn^{2+} opname systemen uit een aantal verschillende bacteriën.

In Hoofdstuk 6 wordt beschreven hoe het gen, coderend voor het hierboven genoemde P-type ATPase (MntA), uitgeschakeld kan worden in *L. plantarum*. De resulterende mutant vormt aggegraten in vloeibaar medium maar is niet significant vertraagd in groeisnelheid bij 1.5 µM mangaan. Ook was de intracellulaire mangaan concentratie in deze mutant niet verlaagd, hetgeen aantoont dat deze transporter niet cruciaal is voor handhaving van intracellulaire mangaan niveaus. Complementatie van deze mutant resulteerde niet in herstel van de aggegraat vorming en

dit suggereert dat dit fenotype veroorzaakt is door een polair effect op stroomafwaarts gelegen gen(en).

In Hoofdstuk 7 worden de resultaten uit de voorgaande hoofdstukken bediscussieerd en worden suggesties gedaan voor verder onderzoek.

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Met een glas rode wijn in de hand zit ik achter de computer om de allerlaatste regels van mijn proefschift te schrijven. Als ik op de afgelopen jaren terugkijk realiseer ik mij dat ik, afgezien van de bekende AIO dipjes, vooral een leuke, afwisselende tijd achter mij laat waar een groot aantal mensen aan bijgedragen hebben.

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Curriculum vitae

Masja Nathalie Nierop Groot werd geboren op 15 november 1971 te Alkmaar. In 1990 behaalde zij haar diploma VWO aan de Rijksscholengemeenschap Noord-Kennemerland te Alkmaar. In datzelfde jaar begon zij aan de studie Levensmiddelentechnologie, orientatie Biotechnologie, aan de toenmalige Landbouwuniversiteit Wageningen. In maart 1996 werd deze studie afgesloten met de afstudeervakken Zuivelkunde en Industriële Microbiologie en een stage bij het National Dairy Products Research Centre, Moorepark in Ierland. Aansluitend begon zij bij de leerstoelgroep Industriële Microbiologie aan het promotieonderzoek dat in dit proefschrift beschreven staat. Vanaf maart 2001 is zij voor 3 jaar aangesteld als post-doctoraal onderzoeker bij Wageningen Centre for Food Science en werkzaam bij NIZO food research te Ede.

The research described in this thesis was carried out at the Division of Industrial Microbiology, Department of Agrotechnology and Nutritional Sciences, Wageningen University and was part of the research carried out in the scientific programme 'Processing and Functionality' of the Wageningen Centre for Food Sciences.

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