Regulation of the genes involved in D-xylose catabolism of *Lactobacillus pentosus*

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Contents

CHAPTER 1

General introduction

Lactobacillus. Lactobacilli are members of the lactic acid bacteria, a group of microorganisms that is widely used in various food and feed fermentations. These non-pathogenic, Gram-positive, bacteria appear as small chains of rod-shaped cells with variable length, depending on the strain and physiological conditions (Rose, 1982, Kandler, 1984, Kandler and Weiss, 1986, Chassy, 1985, Chassy, 1987, McKay and Baldwin, 1990). Lactobacilli are naturally present on plants (Daeschel *et al.*, 1987). Furthermore, the mouth and the gastrointestinal tract of mammals, and the female urogenital tract are colonized by different lactobacilli (Tannock et al., 1982). Based on sugar fermentation three groups of lactobacilli can be identified. The first group consists of obligatory homofermentative lactobacilli, such as *L. acidophilus, L. delbrueckii* and *L. helveticus*. In the second group the facultatively heterofermentative strains are represented, like *L. casei, L. plantarum* and *L. sake*. And the third group is formed by obligatory heterofermentative lactobacilli, such as *L. buchneri, L. brevis, L. fermentum* and *L. hilgardii*. The homofermentative and facultative heterofermentative lactobacilli ferment hexoses to lactic acid. In addition, the latter are also able to convert pentoses into lactic acid and acetic acid. The obligatory heterofermentative lactobacilli ferment hexoses to lactic acid, acetic acid or ethanol and CO_2 (Buyze *et al.*, 1957, Heath *et al.*, 1958). Although bacteria of the genus *Lactobacillus* have been extensively studied, their taxonomical classification on the basis of biochemical characteristics is considered unsatisfactory. The introduction of improved techniques for bacterial classification resulted in a taxonomy that does not correlate any more with the subgrouping based on metabolic traits of homo- and heterofermentative pathways (Collins *et al.*, 1991, Schleifer, 1987, Schleifler *et al.*, 1992, Stackebrandt *et al.*, 1983, Stackebrandt and Teuber, 1988). Comparative analysis of rRNA sequence data revealed the presence of three phylogenetically distinct clusters comprising the majority of the *Lactobacillus* species. These groups are designated the *L. delbrueckii* group (cluster 1), the *L. casei/Pediococcus group* (cluster 2) and the *Leuconostoc paramesenteroides* group (cluster 3). In Fig.1 a phylogenetic tree of lactic acid bacteria and related bacteria is depicted.

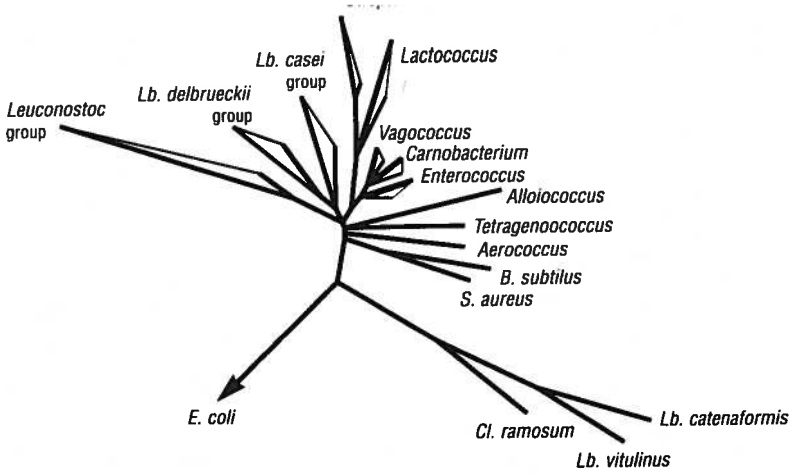

Fig.1. Phylogenetic tree of lactic acid bacteria and related bacteria (Schleifer and Ludwig, 1995)

***Lactobacillus* in food industry.** Historically, fermented foods must have originated from incidental or natural contamination of foods by microbes. Since this spontaneous fermentation process is uncontrolled, the quality of the fermented food and the reproducibility of the process were not always optimal. Throughout the years, the use of spontaneous fermentation was mostly replaced by a better controlled fermentation process, in particular in industrialized countries. For specific products, like fermented dairy products, commercially prepared single and multiple strain startercultures have been developed (Gilliland, 1985, Kandler, 1984, Kilara *et al.*, 1984, Rose, 1981). A well known example of the application of startercultures is the production of butter, yoghurt and cheese from milk by different lactic acid bacterial strains. Lactobacilli and other lactic acid bacteria contribute to food fermentation by producing lactic acid which suppresses the outgrowth of spoilage organisms and pathogens. Beside causing preservation, fermentation also contributes to the taste, aroma, and texture of the fermented food. Furthermore, some lactobacilli produce bio-antagonists such as bacteriocins which also control the growth of spoilage organisms (Kandler, 1984,Rose, 1981, Chassy, 1985, Chassy, 1986). Nowadays, lactobacilli find commercial application in the dairy industry, in baking and alcoholic beverage production, in production and preservation of sausages and meats, in pickling vegetables and in preparing silage.

***Lactobacillus* and health.** A number of *Lactobacillus* strains have the capacity to efficiently colonize external cavities of the body, such as the mouth, throat, stomach,

gut, and urogenital tract (Tannock *et al.*, 1982). The adherence of lactobacilli to the epithelial cells of these surfaces has been demonstrated to be strain and tissue-specific (Redondo-Lopez, 1990, Reid *et al.*, 1990). Presence of these microorganisms is thought to have a beneficial effect on health of the host organism. It has been reported that lactobacilli may play a role in detoxifying carcinogens, enhancing the immune response and reducing serum cholesterol levels (Fernandes *et al.*, 1987, Gilliland, 1990). In 1908 Metchnikoff already claimed a role for lactobacilli in the prolongation of life (Metchnikoff, 1908). Although their potential value in human health is still discussed, it seems clear that they have at least some capacity to enhance the nutritive value of food, discourage contamination by pathogens, and protect the host from toxic compounds.

Genetics of lactobacilli. Due to its wide use in the food and feed fermentation, and its potential value in human health, lactobacilli are of significant economic importance. As a consequence, there is considerable interest in the improvement of the properties of *Lactobacillus* strains. Since plasmid transfer by electroporation was first demonstrated, considerable progress has been made in developing the methodology for genetic manipulation of lactobacilli by recombinant DNA techniques (Chassy and Flickinger, 1987, Luchansky *et al.*, 1988, Posno *et al.*, 1988). Suitable cloning vectors for lactobacilli have been constructed, allowing the molecular cloning and characterization of *Lactobacillus* genes (Chassy, 1987, Posno *et al.*, 1991, Leer *et al.*, 1992). Although, more than 150 *Lactobacillus* genes from different strains have been cloned and sequenced, specific knowledge about gene expression and control of gene expression in *Lactobacillus* is still scarce. The genetic analysis of lactic acid bacteria has mostly been focused on lactococci strains used in the dairy industry. In this context, lactose metabolism has been studied in greatest detail (for a review see, de Vos and Vaughan, 1994).

In the past few years progress has been made in the identification and characterization of elements that are involved in the control of gene expression in lactobacilli. Promoter sites of lactobacilli resemble the consensus sequences of *Escherichia coli* and *Bacillus subtilis*. The putative *Lactobacillus* ribosome binding site (RBS) sequence, AGGAGG, is strongly conserved and closely resembles that of *E. coli* and *Bacillus* spp.. The region encompassing the translation start codon, AUG, also shows extensive homology to that of *E. coli* and *B. subtilis*. However, rare start codons like GUG and UUG are also used. Statistical analysis reveals a biased codon usage by lactobacilli (Pouwels and Leunissen, 1994). Knowledge about protein secretion in lactobacilli is also increasing. Several species are known to produce extra-

cellular enzymes like α-amylase, inulinase, proteinase, and surface-layer proteins. Cloning and sequence analysis of the respective genes revealed signal sequences that are typical for secreted proteins (Jore *et al.*, in preparation, Szilágyi *et al.*, personal communication, Boot *et al.*, 1993). Expression of heterologous proteins under the transcriptional control of *Lactobacillus* promoters was demonstrated for a number of enzymes and fusion proteins (for a review about genetics of lactobacili; see Pouwels and Leer, 1993).

Nowadays the obtained knowledge is more and more used in research that is focused on the proposed beneficial effects of lactobacilli to human health, like the reduction of serum cholesterol levels by lactobacilli. Several studies have indicated that serum cholesterol levels can be influenced by the intestinal microflora. A decrease of cholesterol levels might be attributed to the ability of the intestinal microflora to hydrolyse conjugated bile acids. The gene coding for conjugated bile acid hydrolase (Cbh) was identified in *L. plantarum*. The availability of Cbh-deficient (Leer *et al.*, 1993) and Cbh-overproducing (Christaens *et al.*, 1992) *L. plantarum* strains provides the opportunity to scientifically evaluate the role of Cbh in explaining the cholesterol lowering effect of lactic acid bacteria present in food.

Furthermore, since lactobacilli are thought to have a general beneficial influence on health, display adjuvant properties and are already present in the gastro-intestinal tract of humans and animals, they are attractive vehicles for oral immunisation. Oral administration of a vaccine is more convenient than the parenteral route, especially in large-scale vaccination programmes. Therefore, the capabilities of *Lactobacillus* species as carriers and vehicles for antigens from pathogens (such as rota virus, which causes diarrhoea in children, and influenza virus, the causative agent of influenza) are investigated (Claassen *et al.*, 1993a, Claassen *et al.*, 1993b, Pouwels *et al.*, 1993). For this purpose *Lactobacillus* expression vectors have been constructed to produce foreign antigens, either intracellularly or extracellularly, or anchored to the cellwall (Pouwels *et al.*, 1996).

With the genetic tools at hand and the present knowledge, strain improvement programmes are becoming within reach. However, for the first step in the process of making a genetically modified *Lactobacillus* strain accepted by regulatory authorities, substitution of antibiotic resistance markers by food-grade markers seems obligatory. Since only a few *Lactobacillus* species can utilize D-xylose as an energy source (Kandler and Weiss, 1986), the potential of D-xylose fermentation might be exploited as a food-grade selection marker for *Lactobacillus* spp. (Posno *et al.*, 1991). Until now, no other food-grade selection system for *Lactobacillus* spp. has been described (Pouwels and Leer, 1996). The study of xylose catabolism of *Lactobacillus pentosus*

which could serve as a model for regulation of gene expression in *Lactobacillus*, is the subject of this thesis.

Xylose catabolism. Catabolism of D-xylose involves the transport of xylose into the cell, the isomerization of xylose to D-xylulose, and the phosphorylation of xylulose to D-xylulose-5-phosphate (Jeffries, 1983). At least four genes are involved in xylose utilization. They are coding for a D-xylose transport protein, for D-xylose isomerase, D-xylulose kinase, and for a regulatory protein. In all bacteria studied sofar, the xylose genes are organized in a cluster on the chromosome. Expression of the xylose genes is induced by xylose in the growth medium. Depending on the organism the expression of xylose genes is either positively regulated (*E. coli* and *Salmonella typhimurium*) (Malezka et al., 1982, Shamanna and Sanderson, 1979b) or negatively regulated (*Bacillus* spp. and *Staphylococcus xylosus*) (Kreuzer *et al.*, 1984, Rygus *et al.*, 1991, Scheler *et al.*, 1991, Sizemore *et al.*, 1991). The genetic organization of *xyl* regulons in various prokaryotes is depicted in Fig.2 and will be discussed on the next pages.

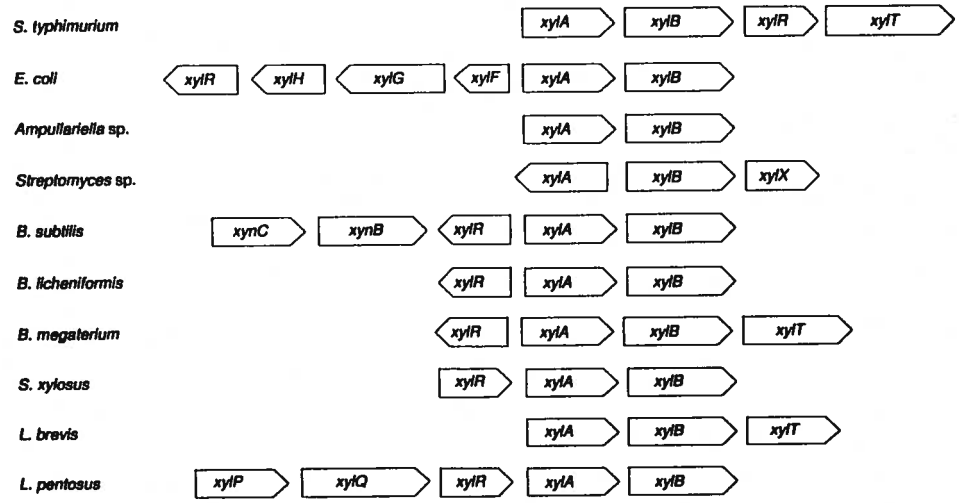

Fig.2. Organization of *xyl* operons in various prokaryotes. The transcription orientation is indicated by an arrow. *xylA*, D-xylose isomerase; *xylB*, D-xylulose kinase; *xylR* and *xylX*, regulatory proteins, *xylP*, putative xylose permease; *xylQ*, unknown function, *xylT*, D-xylose transport; *xylF*, D-xylose binding protein; *xylG*, ATP-binding protein; *xylH*, membrane

In *S. typhimurium* the xylose genes are clustered together at 78 min on the linkage map in the order *xylT* (transport), *xylR* (positive regulator), *xylB* (D-xylulose kinase), *xylA* (D-xylose isomerase) (Shamanna and Sanderson, 1979b). For a long time, the actual gene order of the xylose operon in *E. coli* was unclear. It was suggested that in *E. coli* the genetic organization was similar to that of *Salmonella* (Lawliss et al., 1984, Briggs *et al.*, 1984; Batt *et al.*, 1985). However, Rosenfeld *et al* (1984) proposed the order to be *xylB, xylA, xylR, xylT*. Finally, during sequence analysis of the *E. coli* genome two divergent gene clusters were identified, one for metabolism and one for transport of xylose in the gene order *xylB, xylA, xylT(FG), xylH, xylR* (Sofia *et al.*, 1994). The transport gene *xylT* actually consists of two genes, designated *xylF* and *xylG*. The *xylF* gene is coding for a periplasmic D-xylose binding protein that is involved in the high affinity transport system of D-xylose (Shamanna and Sanderson, 1979a; Ahlem *et al.*, 1982). Analysis of the 330 amino acid sequence of the XylF receptor protein revealed significant homologies with other sugar receptor proteins for ribose (RbsB), galactose (MglB) and arabinose (AraF) (Sumiya *et al.*, 1995). The *xylG* gene is coding for an ATP-binding protein and *xylH* for the membrane component of the transporter (Sofia *et al.* 1994). In *E. coli* xylose transport across the cytoplasmic membrane can also take place by means of a low affinity system that utilizes the proton-motive force and is encoded by the *xylE* gene (Lam *et al.*, 1980, Davis *et al.*, 1984, Davis and Henderson, 1987). This *xylE* gene was not found within the cluster of *xyl* genes but is present on a separate locus on the *E. coli* chromosome. The *xylR* gene codes for a regulatory protein involved in the activation of expression of the xylose genes in the presence of D-xylose. Beside this positive regulation, the *xyl* operons of *E. coli* and *S. typhymurium* are negatively regulated by catabolite repression, a phenomenon which will be discussed on page 16.

Xylose operons of several other organisms, like *Bacillus* spp., *S. xylosus, Lactobacillus brevis, Ampullariella* sp., and *Streptomyces* spp., have been characterized as well (Wilhelm and Hollenberg, 1984, Wilhelm and Hollenberg, 1985, Rygus *et al.*, 1991, Scheler *et al.*, 1991, Sizemore *et al.*, 1991, Bor *et al.*, 1992, Saari *et al.*, 1987, Drocourt *et al.*, 1988, Kikuchi *et al.*, 1990, Wong *et al.*, 1991). The deduced amino acid sequences of the metabolizing genes *xylA* and *xylB*, which are also clustered on the chromosome, are very homologous to those of *E. coli* and *Salmonella*. The regulation of the *xyl* operons of *Bacillus* spp. and *S. xylosus* has been studied extensively and differs from that of *E. coli* and *Salmonella*. In *B. subtilis*, *Bacillus megaterium*, *Bacillus licheniformis*, and *S. xylosus* the *xyl* operons are negatively regulated on the transcriptional level by binding of the Xyl repressor (XylR) to *xyl* operators in the absence of the inducer, xylose (Kreuzer *et al.*, 1984, Rygus *et*

al., 1991, Scheler *et al.*, 1991, Sizemore *et al.*, 1991). In these organisms, inactivation of *xylR* led to constitutive expression of *xylAB* (Rygus *et al.*, 1991, Scheler *et al.*, 1991, Sizemore *et al.*, 1992, Gärtner *et al.*, 1992). The *xyl* operator has been identified just downstream of the *xylAB* promoter site and its complex formation with XylR was demonstrated in *B. subtilis, B. licheniformis*, and *S. xylosus* by gel mobility experiments and DNA-footprinting studies (Gärtner *et al.*, 1992, Scheler and Hillen, 1994, Sizemore *et al.*, 1992). The *B. subtilis xyl* operator sequence consists of two XylR binding sites, O_L and O_R, which are spaced by 4 bp and both of which contribute to efficient regulation *in vivo* (Dahl *et al.*, 1994). Scheler and Hillen (1993) demonstrated that in *B. licheniformis* glucose is a non-inducing competitor of xylose for binding to the Xyl repressor. Recent studies also showed that glucose-6-phosphate is an anti-inducer of *xyl*-operon transcription. Apparently, glucose-6-phosphate is the active form of glucose which competes with xylose in the interaction (*in vitro*) with XylR from *B. subtilis, B. megaterium,* and *B. licheniformis* (Dahl *et al.*, 1995). Moreover, in *B. subtilis* the XylR-mediated effect is specific for the presence of glucose and does not occur with fructose or glycerol (Kraus *et al.*, 1994, Dahl and Hillen, 1995). No XylR-like protein could be detected in *L. brevis*, suggesting a different mechanism of regulation of the *xyl* operon. However, the existence of a functional XylR is supported by the presence of a *xyl* operator sequence upstream of *xylA* and the inducibility of D-xylose isomerase activity by D-xylose. D-xylose-isomerase activity was similar when *L. brevis* was cultivated in a mixture of xylose and glucose or in xylose only, indicating that the expression of the *xyl* operon is not catabolite repressed (C. Batt, personal communication). Like in *E. coli* and *S. typhimurium* transcription of the *xyl* operon in the other Gram-positive organisms discussed sofar is catabolite repressed. Mechanisms involved in catabolite repression will be discussed on page 16.

While most research is focused on the regulation of the metabolizing genes in these organisms, not much is known about the transport of xylose into the cell. Hastrup (1988) showed that beside the *xylR, xylA* and *xylB* genes, two additional genes belonged to the *B. subtilis* xylose regulon, the *xynB* and *xynC* genes. The *xynB* gene is coding for an intracellular ß-xylosidase (Roncerco, 1983). The *xynC* gene codes for a protein of 463 amino acids that has long stretches of hydrophobic residues suggesting location in the membrane and a possible function as a permease for xylose oligomers. Both, *xylAB* and *xynCB* are controlled by the same repressor (XylR). Experimental evidence for the putative transport function of *xynC* is still lacking. Recently, downstream of *xylAB* of *B. megaterium* an ORF (*xylT*) has been identified that is thought to code for the xylose permease. The deduced amino acid sequence showed 54% identity to *xylE* from *E. coli* (Schmiedel, personal communication).

Catabolite Repression. Enzymes involved in the metabolism of various carbon and energy sources are unnecessary under conditions of abundant, readily metabolizable alternatives such as glucose. The repression of these enzymes by glucose and other rapidly metabolizable C-sources is termed catabolite repression (CR) and is an important global regulatory system found in both prokaryotic and eukaryotic microorganisms (Saier, 1991, Chambliss, 1993).

In Gram-negative bacteria, in particular *E. coli*, catabolite repression is mediated by the cAMP receptor protein (CRP or CAP, catabolite gene activator protein), which in the presence of cAMP binds at specific DNA sites near promoters, and activates transcription (Magasanik 1961, Magasanik, 1970, Magasanik and Neihardt, 1987, Ullman and Danchin, 1983). Enzyme IIA^{Glc} of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is the central regulatory protein controlling the intracellular cAMP level and several non-PTS permeases in this organism (for a review see Postma *et al.*, 1993).

The mechanism of CR in Gram-positive bacteria like *Bacillus* species is different from that of *E. coli*, since neither the cAMP receptor protein nor relevant concentrations of cAMP have been detected (Chambliss, 1993, Setlow, 1973). Instead, the negative transcription factor CcpA, which is a member of the GalR-LacI family of transcriptional regulators (Weickert and Adhya, 1992), is mediating catabolite repression by interacting with a *cis*-acting catabolite responsive element (CRE) in carbon catabolic operons of these organisms (Hueck *et al.*, 1994, Hueck and Hillen, 1995, Kim *et al.*, 1995). As a result of this complex formation efficient transcription of the respective operons is prevented. The *cis*-acting sequence was also detected in the *xyl* operon of *B. subtilis, B. megaterium*, and *S. xylosus* (Jacob *et al.*, 1991, Rygus and Hillen, 1992, Sizemore *et al.*, 1991). Furthermore the presence of a *ccpA* gene in these organisms was established (Henkin *et al.*, 1991, Hueck *et al.*, 1994, Hueck *et al.*, 1995, R. Brückner and F. Götz, personal communication). Mutations in either *ccpA* or CRE demonstrated the involvement of these factors in CR of the *xyl* operon. Another factor involved in CR is the heat stable protein, HPr, a component in the phosphate-transfer chain of the PEP-PTS. HPr can be phosphorylated at two different sites: (I) in a PEP-dependent reaction catalysed by enzyme I of the PTS, at His-15 (the phospho donor for the PTS-catalysed carbohydrate uptake (Postma *et al.*, 1993)); and (II) at Ser-46 in an ATP-dependent reaction catalysed by a fructose-1,6-diphosphate activated protein kinase (Reizer *et al.*, 1993). CcpA interacts with HPr, but only when it is phosphorylated at Ser-46 (Deutscher *et al.* 1994, Deutscher *et al.*, 1995, Hueck and Hillen, 1995). Binding of this protein complex to the *B. subtilis gnt* (gluconate) and *B. megaterium xyl* catabolite-responsive element has been demonstrated (Fujita *et al.*,

1995, Küster and Deutscher, personal communication). In contrast, however, Ramseier *et al.* (1995) showed that binding of *B. megaterium* CcpA to several CREs was inhibited by the presence of HPr(Ser-P). Moreover, Kim *et al.* (1995) noted that the purified *B. subtilis* CcpA protein binds specifically and with high affinity to the CRE in the *amyO* control region in the absence of HPr(Ser-P). Whether the differences reported represent differences in experimental conditions of physiologically relevant differences due to the different systems studied remains to be established. Although future experiments have to clarify how the HPr(Ser-P)/CcpA complex affects catabolite repression, it seems clear that carbon catabolite repression in Gram-positive bacteria is a protein kinase-triggered mechanism. A link between glycolytic activity and carbon catabolite repression is suggested since the glycolytic intermediate fructose-1,6-diphosphate, stimulates the corresponding protein kinase and HPr(Ser-P)/CcpA complex formation. The sensitivity of this complex formation to phosphorylation of HPr at His-15 also suggests a link between carbon catabolite repression and PTS transport activity (Deutscher *et al.*, 1995). A proposed mechanism for the regulation of the transcription of catabolite-sensitive operon in *B. subtilis* is depicted in Fig. 3.

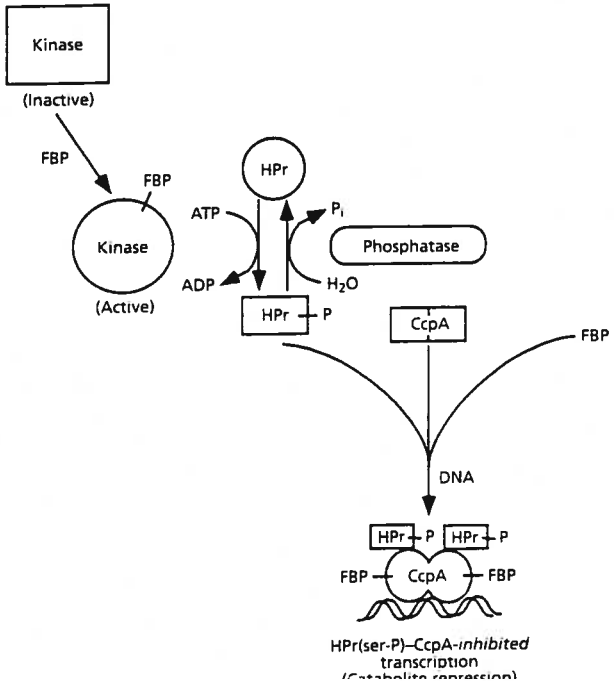

Fig.3. Proposed mechanism for the regulation of the transcription of catabolite sensitive operons in *B. subtilis*. The metabolite-activated HPr(ser) kinase phosphorylates Ser-46 in HPr, converting it to a form that can bind to the transcription factor, CcpA. These two proteins, possibly together with a metabolite such as fructose 1,6-biphosphate (FBP), form a complex which binds to CRE's in or near the promoter regions of catabolite-sensitive target operons to promote catabolite repression (Saier *et al.*, 1996).

Saier *et al.* (1996), report on the presence of a second catabolite control protein, CcpB. This protein exhibits 30% sequence identity with CcpA. CcpA and CcpB are suggested to function in parallel in response to HPr(Ser-P) concentrations, to allow the catabolite repression phenomenon to be sensitive to environmental conditions.

In *Streptomyces coelicolor*, another Gram-positive organism, HPr(Ser-P)-dependent or cAMP-dependent CR has not been demonstrated so far (Titgemeyer *et al.*, 1995). In this organism a gene encoding a glucose kinase is involved in CR of agarase and glycerol kinase (Angell *et al.*, 1992, Angell *et al.*, 1994, Kwakman and Postma, 1994). Recent studies showed that also in *S. xylosus*, beside CcpA and HPr the enzyme glucose kinase participates in catabolite repression as well (Wagner *et al.* 1995).

Outline of this thesis

The economic importance of lactobacilli is beyond doubt. Their properties and potential for food and feed industry, and for human and animal health might be considerably improved by elucidating at a molecular level the mechanisms involved in regulation of gene expression. As a model system for gene expression in *Lactobacillus*, the xylose catabolizing genes of *Lactobacillus pentosus* were studied. The *xyl* genes in *Lactobacillus* are negatively controlled by various carbon sources, e.g. glucose. In this thesis, mechanisms involved in repression of the *xyl* operon are investigated.

In **Chapter 2** the cloning and sequence analysis of three genes (*xylR, xylA, xylB*) involved in D-xylose catabolism in *L. pentosus* is described. A functional analysis by NMR studies of *L. casei* cells transformed with the xylose genes is reported.

In **Chapter 3** the promoter analysis and transcriptional regulation of the *xyl* genes of *L. pentosus* is described. Transcription start sites for *xylA* and *xylR* are mapped and the expression of the chloramphenicol acetyltransferase gene under control of the *xylA* and *xylR* promoter is determined. The presence of two operator-like elements involved in negative regulation of the *xyl* operon is suggested. Furthermore, the involvement of two additional genes (*xylP* and *xylQ*) in xylose catabolism is reported.

In **Chapter 4** the identification of the *ccpA* gene is described. Sequence and transcriptional analysis of this gene is reported. Furthermore, its role in catabolite repression of the *xyl* operon and of the α-amylase gene of *L. amylovorus* is established by the analysis of a *ccpA* disruption mutant.

Chapter 5 describes the role of XylR in the regulation of expression of the *xyl* genes. Expression of the *xyl* genes in a *xylR* disruption mutant, under inducing and non-inducing conditions, is analyzed. The results suggest a repressor function for XylR. However, growth analysis of the disruption mutant in xylose suggests an activator role as well. The function of XylR is also determined in the xylose non-fementing bacterium, *L. casei*, by introducing the *xyl* genes on a multi-copy plasmid.

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CHAPTER 2

Organization and characterization of three genes involved in D-xylose catabolism in *Lactobacillus pentosus*

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SUMMARY

A cluster of three genes involved in D-xylose catabolism (viz. xylose genes) in *Lactobacillus pentosus* has been cloned in *Escherichia coli* and characterized by nucleotide sequence analysis. The deduced gene products show considerable sequence similarity to a repressor protein involved in the regulation of expression of xylose genes in *Bacillus subtilis* (58%), to *E. coli* and *B. subtilis* D-xylose isomerase (68% and 77%, respectively), and to *E. coli* D-xylulose kinase (58%). The cloned xylose genes represent functional genes on the basis of the following criterium. The inability of a *L. casei* strain to ferment D-xylose could be complemented by introduction of *L. pentosus* xylose genes. NMR analysis revealed that ^{13}C-xylose was converted into ^{13}C-acetate in *L. casei* transformed with *L. pentosus* xylose genes but not in untransformed *L. casei* cells. Comparison with the aligned amino acid (AA) sequences of D-xylose isomerases of different bacteria suggests that *L. pentosus* D-xylose isomerase belongs to the same similarity group as *B. subtilis* and *E. coli* D-xylose isomerase and not to a second similarity group comprising D-xylose isomerases of *Streptomyces violaceoniger*, *Ampullariella* sp. and *Actinoplanes*. The organization of the *L. pentosus* xylose genes, 5'- *xylR* (1167 bp, repressor) - *xylA* (1350 bp, D-xylose isomerase) - *xylB* (1506 bp, D-xylulose kinase) -3' is similar to that in *B. subtilis*. In contrast to *B. subtilis xylR*, *L. pentosus xylR* is transcribed in the same direction as *xylA* and *xylB*.

INTRODUCTION

In many bacteria utilizing D-xylose as an energy source, catabolism of D-xylose involves the coordinated expression of at least four genes (viz. xylose genes) coding for one or more D-xylose transport proteins, for D-xylose isomerase and D-xylulose kinase and for a regulatory protein. These proteins mediate the uptake and the conversion of D-xylose via D-xylulose into D-xylulose-5-phosphate (D-xylulose-5-P) (Jeffries, 1983). This intermediate, which plays a key-role in the 6-phospho-gluconate pathway and in the fermentation of pentoses and pentitols (Kandler, 1983), is subsequently metabolized along the Emden-Meyerhoff and/or pentose-phosphate pathways. In *Escherichia coli* and *Salmonella typhimurium*, the xylose genes are organized in a cluster on the chromosome, in the order 5'- *xylA* (D-xylose isomerase) - *xylB* (D-xylulose kinase) - *xylR* (positive regulator) - *xylT* (permease) -3'. Expression of the xylose genes in these bacteria is regulated by a positive control mechanism (Maleszka *et al.*, 1982; Shamanna and Sanderson, 1979). In *Bacillus subtilis*, three genes involved in D-xylose catabolism, coding for D-xylose isomerase, D-xylulose kinase and a regulatory protein, respectively, have been cloned and characterized (Hastrup, 1988; Wilhelm and Hollenberg, 1984). In this bacterium, expression of the D-xylose isomerase and D-xylulose kinase genes is negatively controlled by an operator-repressor mechanism (Hastrup, 1988; Kreuzer *et al.*, 1989).

We have recently started a molecular genetic investigation on D-xylose catabolism in another Gram-positive bacterium, viz. *Lactobacillus*. It has been demonstrated that only very few *Lactobacillus* species, notably *L. pentosus*, are able to use D-xylose as an energy source (Kandler, 1983; Kandler and Weiss, 1986). The synthesis of D-xylose isomerase in *L. pentosus* is induced by D-xylose in the growth medium (Mitsuhashi and Lampen, 1953). All heterofermentative and homofermentative lactobacilli that can utilize pentoses for growth, however, have the capacity to convert D-xylulose-5-P into lactate and acetate or ethanol (Kandler, 1983). Therefore, *Lactobacillus* strains, which are incapable to utilize D-xylose, are likely to be functionally deficient in (or lack) one or more of the xylose genes.

In this article we describe the structural and functional characterization of three genes involved in D-xylose catabolism in *L. pentosus*. Based on the sequence similarities to the corresponding xylose genes of *B. subtilis*, these genes were designated *xylR*, *xylA* and *xylB*.

MATERIALS AND METHODS

Materials. Enzymes for molecular cloning were purchased from Boehringer or Bethesda Research Laboratories and were used according to the specifications of the manufacturer. Lysozyme was from Boehringer, proteinase K from Merck, [α-^{35}S]dATP and [γ-^{32}P]ATP from Amersham, D-xylose-1-^{13}C (99% enriched) from Cambridge Isotope Laboratories, agar from Difco and D_2O from Aldrich.

Bacterial strains, plasmids and media. *L. pentosus* MD353 (kindly provided by Dr. M. Daeschel), isolated from a natural cucumber fermentation, was the source of xylose genes. *E. coli* JM109 was used for all cloning experiments and as a host strain for M13 infection. *L. casei* ATCC 393 (Chassy and Flickinger, 1987) was used as a D-xylose non-fermenting *Lactobacillus* strain in complementation experiments (see below). Plasmid pUC19 was used as a vector for gene cloning, bacteriophages M13mp18 or M13mp19 for subcloning and sequence analysis. *Lactobacillus* strains were routinely cultivated at 37 °C in MRS medium (Difco) (deMan *et al.*, 1960) or in MRSX medium in which glucose was replaced by 1 % (w/v) D-xylose. For NMR experiments (see below) NMRX medium was used (modified LCM medium (Efthymiou and Hansen, 1962) containing per litre 10 g proteose peptone, 4 g yeast extract, 1 g tween-80, 4 g dipotassium phosphate, 4 g monopotassium phosphate, 1 g sodium acetate, 200 mg ammonium citrate, 200 mg magnesium sulphate and 5 mg manganese sulphate. The pH was adjusted to 6.8. After autoclaving D-xylose was added to a final concentration of 0.6% (w/v)). For plating, MRS was solidified with 1.5 % agar. Erythromycin was used at 5 μg/ml

DNA isolation. *L. pentosus* MD353 DNA was isolated from a 500 ml culture of logarithmically growing *L. pentosus* MD353 cells. Cells were harvested by centrifugation and washed once with 20 mM Na-maleate pH 6.2. Protoplasts were formed by incubating the cells in 40 ml protoplast buffer (20 mM Na-maleate pH 6.2, 0.6 M lactose, 20 mM $MgCl_2$ and 80 mg lysozyme) for 1 hour at 37 °C. After harvesting by centrifugation, protoplasts were resuspended in 20 ml 20 mM Tris-HCl pH 8.2; 0.1 M Na_2EDTA and subsequently lysed at room temperature by addition of sarkosyl up to 1 % (w/v). The lysate was extracted once with phenol and once with chloroform. High-molecular weight DNA was precipitated at room temperature by the addition of 1 volume 96 % ethanol, spooled out with a glass rod, washed once with 70 % ethanol and dissolved in 0.1 x SSC (15 mM NaCl/1.5 mM Na_3-citrate pH 7.0). The solution was treated with RNAse (0.8 mg/ml, 1 hour at 37 °C) and with proteinase K (3.2 mg/ml, 1

hour at 55 °C) according to standard procedures and extracted once again with phenol, phenol/chloroform (1:1) and chloroform. High-molecular weight DNA was spooled out with a glass rod, washed once with 70 % ethanol and finally dissolved in 0.1 x SSC.

Complementation of a D-xylose non-fermenting Lactobacillus strain. The 2.4 kb *Pst*I-*Hin*dIII fragment of pXH50A (encompassing *xylR* and the 5' part of *xylA*; Fig. 1) was cloned in *E. coli* between the *Pst*I and *Hin*dIII sites of the *E. coli-Lactobacillus* shuttle vector pLP3537 (Posno *et al.*, 1991.) The resulting hybrid vector was linearized with *Hin*dIII and ligated with the 3.7 kb *Hin*dIII fragment of pXH37A (containing the remaning part of *xylA* and *xylB*; Fig. 1.) The ligation mixture was introduced into *L. casei* ATCC 393 by electroporation (Posno *et al.*, 1991). Transformants were analyzed on MRSX agar plates with erythromycin and bromocresol purple as an indicator for acid production.

NMR experiments. For NMR experiments, *Lactobacillus* cells were cultivated overnight in MRS medium (with 10 μg/ml erythromycin in the case of *L. casei* transformed with xylose genes). The cells were harvested by centrifugation, washed twice in 0.9 % (w/v) NaCl and resuspended in NMRX medium. After incubating the cell suspension (approximately 10^9 CFU/ml) for 1 hour at 37 °C, the cells were harvested by centrifugation at 4 °C, washed once in 0.9% NaCl and twice in NMRX medium (4 °C) and finally resuspended in NMR medium (NMRX medium without D-xylose) (approximately 10^{11} CFU/ml). Following addition of D-xylose-1-^{13}C (final concentration 4 mM) and D_2O (10 % v/v), the cell suspension was kept on ice until the start of the experiment (for further experimental details see legend Fig. 4). For NMR measurements 5 mm sample tubes were used containing 0.5 ml of the cell suspension. ^{13}C spectra were obtained at 100.577 MHz on a Varian VXR 400 spectrometer operating in the Fourier transform mode. Field stabilization was achieved by locking on D_2O. Broad-band proton decoupled spectra were recorded at 37 °C using 40° pulses with a repetition time of 0.65 s. The spectra were obtained from blocks of 7200, 14400 or 28800 accumulated transients. Chemical shifts are expressed relative to external tetramethylsilane.

Other techniques. Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, end labelling of oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase, DNA transfer to nitrocellulose filters and filter hybridizations were performed according to Sambrook *et al.*, (1989). Nucleotide sequencing was performed by the dideoxy chain-

termination method (Sanger *et al.*, 1977). The M13 universal primer (Boehringer) and sequence specific primers were used for sequencing reactions. The nucleotide sequence of both strands was determined and assembled from a set of overlapping fragments.

RESULTS

Cloning of genes involved in D-xylose catabolism. Initially we attempted to clone *L. pentosus* MD353 *xylA* by complementation of D-xylose isomerase defective *E. coli* mutants. This approach, though successful for the cloning of D-xylose isomerases of a number of different bacteria (Maleszka *et al.*, 1982; Saari *et al.*, 1987; Wilhelm and Hollenberg, 1985), however, proved to be unsuccessful for the cloning of *L. pentosus* MD353 *xylA*. Therefore, as an alternative approach, two mixed oligonucleotide probes (a 17-mer and a 23-mer, respectively) were synthesized, both of which were based on highly conserved regions in the AA sequences of D-xylose isomerase of *E. coli* (Lawliss *et al.*, 1984), *B. subtilis* (Wilhelm and Hollenberg, 1985), *Streptomyces violaceoniger* (Drocourt *et al.*, 1988) and *Ampullariella* sp. (Saari *et al.*, 1987). From a (partial) *Eco*RI library of *L. pentosus* MD353 chromosomal DNA, a plasmid with a 600 bp insert (pXE600) was isolated, which hybridized to both the 17-mer and 23-mer probe. The AA sequence, deduced from the DNA sequence of the insert, showed strong sequence similarity with a part of the D-xylose isomerases of *E. coli* and *B. subtilis*. Two DNA fragments comprising the complete *L. pentosus* MD353 D-xylose isomerase gene and flanking regions were isolated from a (partial) *Hin*dIII library of *L. pentosus* MD353 chromosomal DNA in pUC19, using the 600 bp *Eco*RI insert of pXE600 as a probe. A resulting clone containing the 5' part of *xylA* on a 5.0 kb *Hin*dIII fragment was designated pXH50A and a clone containing the remaining part of *xylA* on a 3.7 kb *Hin*dIII fragment was designated pXH37A. The cloned DNA fragments do originate from *L. pentosus* MD353 as they specifically hybridize to fragments of the same size in a Southern blot of *Hin*dIII digested *L. pentosus* MD353 chromosomal DNA (not shown). The restriction map of the *Hin*dIII insert of both clones is shown in Fig. 1.

Identification of three genes involved in D-xylose catabolism. To confirm the presence of the entire *xylA* gene within pXH50A and pXH37A (Fig. 1) and to examine whether genes involved in D-xylose catabolism are also clustered in *L. pentosus* MD353, the nucleotide sequence of a contiguous stretch of about 4.6 kb, starting from

the *Pst*I site at position 1 in pXH50A to just beyond the *Eco*RI site at position 4522 in pXH37A (Fig. 2),was determined.

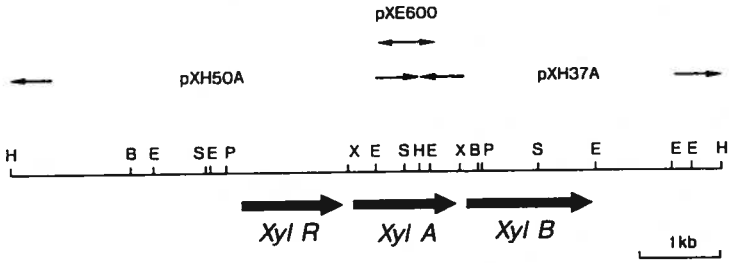
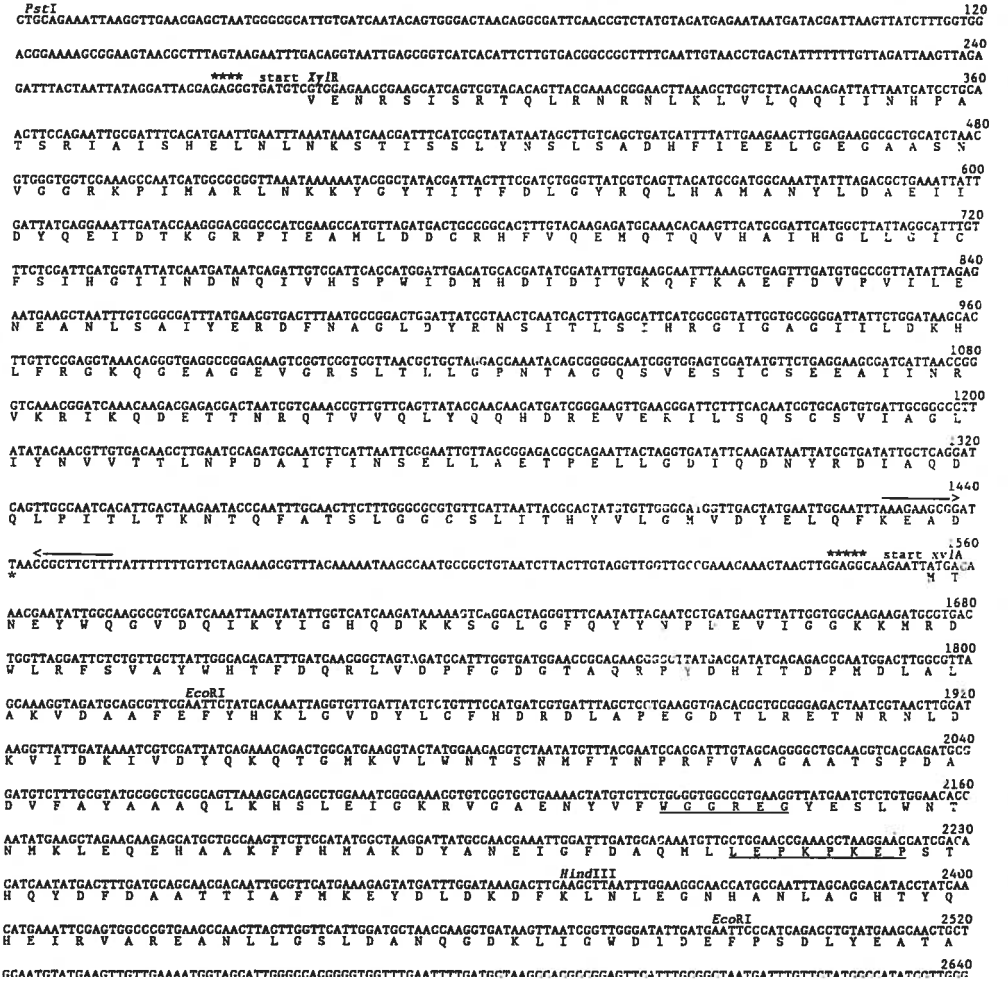


Fig. 1. Restriction map and genetic organization of the *L. pentosus* MD353 chromosomal DNA region containing three genes involved in D-xylose catabolism. The direction of transcription and the positions of the ORFs *xylR*, *xylA* and *xylB* are presented by thick arrows. The sites of the following restriction enzymes are shown: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I; X, *Xba*I. The clones pXE600, pXH50A, and pXH37A consist of pUC19 with a 600 bp *Eco*RI fragment, a 5.0 kb *Hin*dIII fragment or a 3.7 kb *Hin*dIII fragment of *L. pentosus* chromosomal DNA, respectively. The position of the inserts are indicated.

The sequence, shown in Fig. 2, contains three ORFs (i.e. open reading frames starting with an initiation codon) with the same transcription polarity. The first ORF begins at position 277 with GTG and terminates at position 1443 with the termination codon TAA. This ORF could code for a protein of 388 AA residues. As shown in Fig. 3A, the AA sequence of the protein is significantly similar (58% when conservative AA replacements are included) to that of the gene product of *B. subtilis xylR*, a repressor protein involved in regulation of D-xylose gene expression (Gärtner *et al.*, 1988; Kreuzer *et al.*, 1989). The calculated Mw of the *L. pentosus* MD353 protein (42.9 kD) is in good agreement with that of the *B. subtilis* repressor (42.3 kD) (Kreuzer *et al.*, 1989). The second ORF starts at position 1555 and terminates at position 2904 (including the initiation codon ATG and termination codon TAA) and could code for a protein of 449 AA residues. This protein exhibits sequence similarity with D-xylose isomerase (Fig. 3B) of, for instance, *E. coli* (68%), *B. subtilis* (77%), *Streptomyces violaceoniger* (51%) or *Ampullariella* sp. (50%). The Mw of the *L. pentosus* MD353 protein (50.7 kD) compares very well with that of the D-xylose isomerases of *E. coli* (49.7 kD) (Schellenberg *et al.*, 1984) and *B. subtilis* (49.7 kD) (Wilhelm and Hollenberg, 1985). The third ORF, finally, starts at position 2971 and terminates at position 4476

(including the initiation codon ATG and termination codon TAA) and could code for a protein of 501 AA residues. Comparison of the corresponding AA sequence with *E. coli* D-xylulose kinase demonstrates a sequence similarity of 57% (Fig. 3C). In addition, the N-terminus of the *L. pentosus* MD353 protein is very similar to the N-terminus of D-xylulose kinase from *Klebsiella aerogenes* (Neuberger *et al.*, 1981). The Mw of the *L. pentosus* MD353 protein (54.8 kD) is similar to that of the corresponding *E. coli* protein (54.0 kD) (Lawliss *et al.*, 1984). Based on these sequence similarities, the three *L. pentosus* MD353 ORFs were designated *xylR*, *xylA* and *xylB*, respectively (Fig. 1).



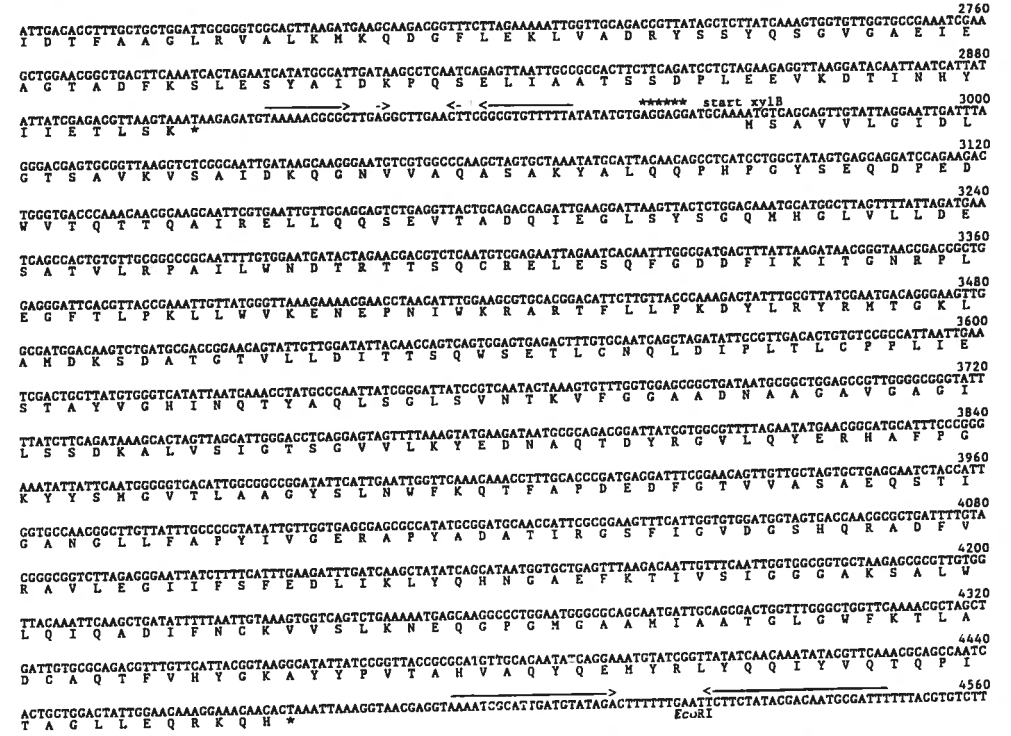


Fig. 2. Nucleotide sequence of *L. pentosus* MD353 *xylR*, *xylA* and *xylB* and their flanking regions. The RNA-like strand is shown, together with the deduced amino acid (AA) sequence (in single letter code) of the coding regions. The conserved regions of D-xylose isomerase, on which the 17-mer and 23-mer oligonucleotide probes were based, are underlined in the AA sequence. *Eco*RI, *Hin*dIII and *Pst*I restriction sites are indicated. Putative Shine and Dalgarno sequences are marked with asterisks, inverted repeats are indicated by arrows above the sequence. The nucleotide sequence will appear in the GenBank database under the accession number M57384.

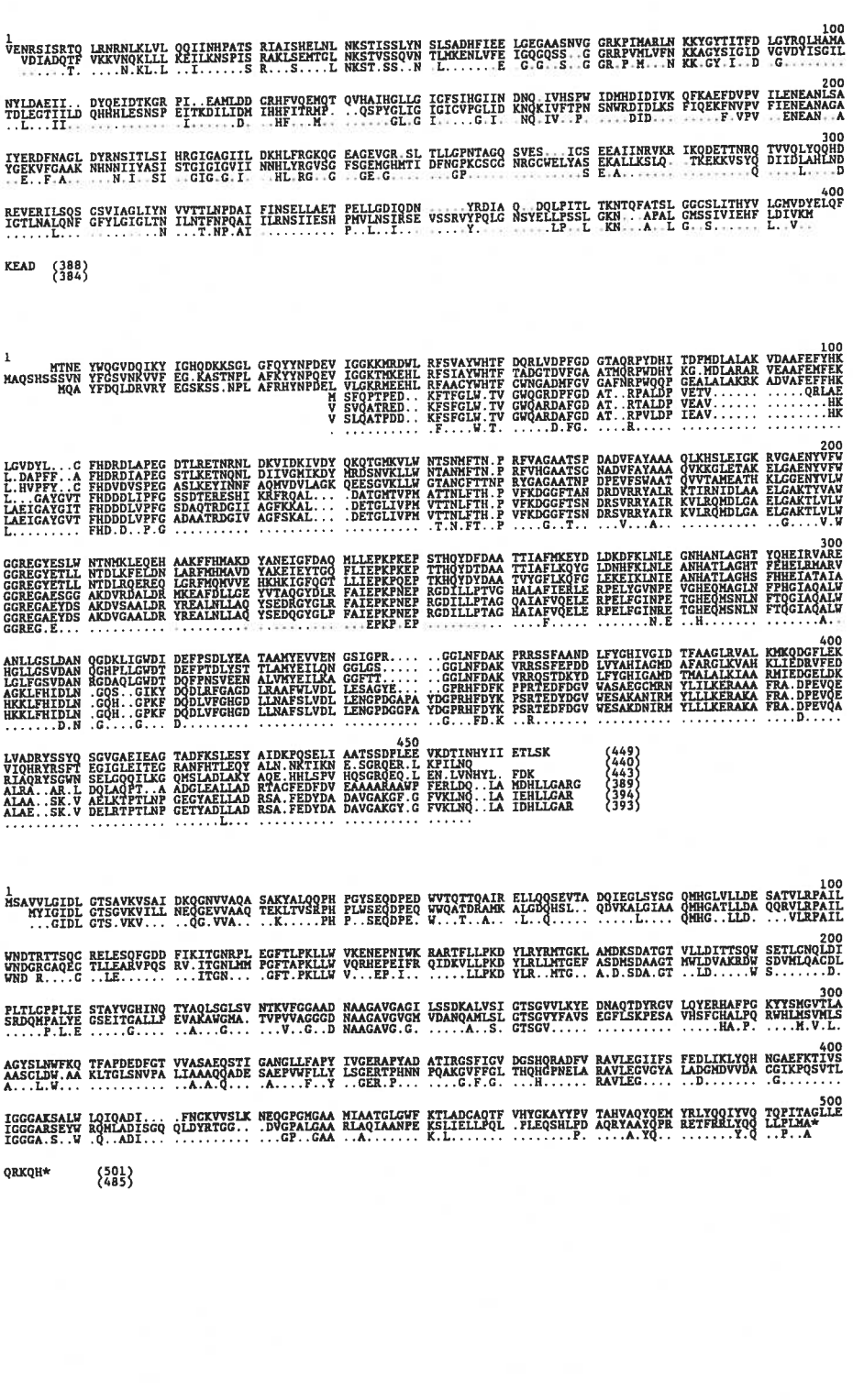
The cloned L. pentosus genes are functional xylose genes. To verify the hypothesis that the *L. pentosus* MD353 xylose genes had been cloned, we introduced the genes into a D-xylose non-fermenting *Lactobacillus* strain, *L. casei* ATCC 393. Heterologous hybridization with *L. pentosus* MD353 xylose gene probes revealed that *xylR*, *xylA* and *xylB* sequences are absent from this *L. casei* strain. *L. casei* transformants were obtained that could ferment D-xylose (manuscript submitted). Further to confirm the functionality of the cloned xylose genes, the capability of *L.*

pentosus MD353 and of untransformed and transformed *L. casei* cells to ferment ^{13}C-xylose was analyzed in vivo by NMR. As illustrated in Fig. 4, *L. pentosus* MD353 is able to convert a substantial fraction of D-xylose-1-^{13}C into ^{13}C-acetate, the end product of the fermentation; δ about 23 ppm) (Fig. 4A), whereas *L. casei* is not (Fig. 4B). Transformed *L. casei* cells, however, efficiently convert ^{13}C-xylose into ^{13}C-acetate (Fig. 4C). Moreover, it appeared that formation of ^{13}C-acetate in *L. pentosus* MD353 and transformed *L. casei* cells was preceded by the (temporary) accumulation of the intermediate ^{13}C-xylulose (result not shown). From these results it can be deduced that: (1) complementation of the D-xylose negative phenotype of *L. casei* is directly correlated with introduction of *L. pentosus* MD353 xylose genes; (2) *L. pentosus* MD353 *xylA* codes for a functional D-xylose isomerase (i.e. formation of ^{13}C-xylulose in transformed *L. casei* cells); and (3) *L. pentosus* MD353 *xylB* most likely codes for a functional D-xylulose kinase (i.e. no hybridization of *L. casei* chromosomal DNA with *L. pentosus xylB* under heterologous conditions; formation of ^{13}C-acetate in transformed *L. casei* cells.

Characteristics of the flanking regions. In the upstream region of *xylA*, *xylB* and *xylR*, at a distance of 7 to 8 bp from the presumed initiation codon, a sequence 5'-(AG)GAGG-3' (indicated in Fig. 2) is found, which may serve as an analogon of the Shine and Dalgarno sequence preceding the start codon of *B. subtilis* (Moran *et al.*, 1982) or *E. coli* genes (Gold *et al.*, 1981).

Promoter motifs which exactly match the consensus promoter sequence of genes in Gram-positive bacteria (Graves and Rabinowitz, 1986) or *E. coli* (Harley and Reynolds, 1987) are not present upstream from either *L. pentosus xylR, xylA* or *xylB*.

Inverted repeats are found within the 3' flanking regions of *xylR* and *xylB* (indicated by arrows in Fig. 2), which can be folded into possible stem-loop structures followed by a thymidine-rich region (free energy of formation ΔG= -19.4 and -27.0 kcal, respectively; calculated according to Tinoco *et al.*, (1973)) . Although the functional role of such structures remains to be established, it seems likely that these sequences serve as rho-independent transcription termination signals (Friedman *et al.*, 1987; Platt, 1986). A stem-loop structure can also be folded in the region directly downstream from *xylA* (indicated in Fig. 2; ΔG= -14 kcal). However, a functional role in transcription-termination remains speculative, since a thymidine-stretch is lacking. It has been reported that in *E. coli* inverted repeat regions may play a role in the stabilization of mRNA (protection from 3' -> 5' exonuclease degradation) (Newbury *et al.*, 1987). Whether the palindromic sequence between *L. pentosus* MD353 *xylA* and *xylB* controls the level of expression of one or both genes is, at the moment, unknown.



A

B

C

Fig. 3. Alignment of AA sequences of: **A** *L. pentosus* MD353 (L.p) *xylR* gene product with *B. subtilis* (B.s) repressor protein (Kreuzer *et al.*, 1989); **B** *L. pentosus* MD353 D-xylose isomerase and D-xylose isomerases of *E. coli* (E.c) (Lawliss *et al.*, 1984; Schellenberg *et al.*, 1984), *B. subtilis* (Wilhelm and Hollenberg, 1984), *Ampullariella* sp. (Am) (Saari *et al.*, 1987), *Streptomyces violaceoniger* (S.v) (Drocourt *et al.*, 1988) and *Actinoplanes* (Ac) (Amore and Hollenberg, 1989); **C** *L. pentosus* MD353 D-xylulose kinase with *E. coli* D-xylulose kinase (Lawliss *et al.*, 1984). The consensus sequence (*con*) represents identical residues in the respective proteins. The total number of residues in the protein is given between brackets.

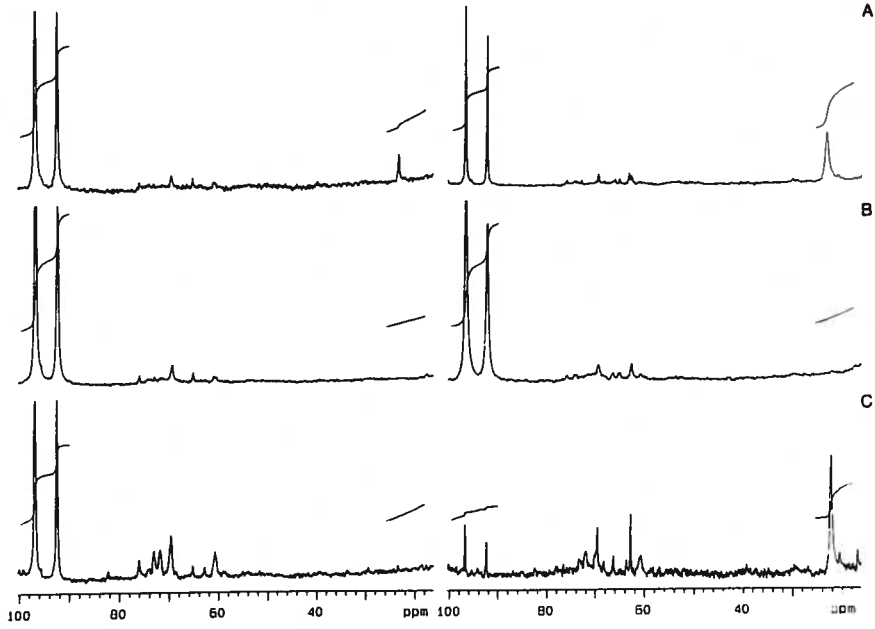


Fig. 4. NMR analysis of ^{13}C-fermentation products of ^{13}C-xylose in *L. pentosus* MD353 (**A**), *L. casei* ATCC 393 (**B**) and *L. casei* ATCC 393 transformed with the xylose genes of *L. pentosus* MD353 (**C**). The panels at the left represent NMR spectra shortly after addition of ^{13}C-xylose to the cell suspensions; the panels at the right represent NMR spectra of ^{13}C-fermentation products of ^{13}C-xylose after 42 (**A, B**) or 85 (**C**) hours of incubation at 37 °C. Traces of integrals are drawn at the resonance positions of ^{13}C-xylose (96.8 ppm and 92.4 ppm for the β-anomer and the α-anomer, respectively) and of ^{13}C-acetate (about 23 ppm). Peaks between 60 and 80 ppm partly orginate from a constant background and partly from transient fermentation intermediates.

In *B. subtilis*, a *xyl*-operator was identified in the region upstream from *xylA* as a highly conserved palindromic sequence (Hastrup, 1988; Kreuzer *et al.*, 1989). Comparison of the DNA sequence upstream from *L. pentosus* MD353 *xylA* with the nucleotide sequence of the *B. subtilis xyl*-operator revealed that 26 to 17 bp 5' to the ATG initiation codon of *L. pentosus* MD353 *xylA* a sequence is present, which is identical to the right inverted repeat sequence of the *B. subtilis xyl*-operator (Fig. 5). However, the *L. pentosus* MD353 sequence 41 to 32 bp 5' to *xylA* shows only moderate similarity with the left inverted repeat of the *B. subtilis xyl*-operator (Fig. 5).

```
L.p    TAGGTTGGTTGCGGAAACAAACTAA  16 bp to ATG
       |  ||| |||     ||||||||||
B.s    TTAGTTTGTTTGGGCAACAAACTAA  69 bp to ATG
```

Fig. 5. Comparison of the nucleotide sequence of the xylose operator of *B. subtilis* W23 (Kreuzer *et al.*, 1989) (bottom) with the nucleotide sequence of a region 41 to 17 bp 5' to the ATG start codon of *L. pentosus* MD353 *xylA* (top). Identical nucleotides are indicated.

DISCUSSION

In this report we describe the cloning and characterization of three genes involved in D-xylose catbolism in *L. pentosus* MD353. The functionality of the genes was analyzed in a D-xylose non-fermenting *L. casei* strain. NMR analysis revealed that *L. casei* transformed with *L. pentosus* MD353 xylose genes is able to convert ^{13}C-xylose via the intermediate D-xylulose into ^{13}C-acetate, the end product of fermentation, whereas untransformed cells are not (Fig. 4). After addition of ^{13}C-xylose to cells of *L. pentosus* MD353 and the transformed *L. casei* strain, the intermediate ^{13}C-xylulose was formed first and then efficiently converted into ^{13}C-acetate (data not shown). On the basis of these results it can be deduced that the cloned *L. pentosus* MD353 *xylA* gene codes for a functional D-xylose isomerase and that it is highly likely that the cloned *xylB* gene codes for a functional D-xylulose kinase. It can be excluded that a specific D-xylose transport function is complemented in *L. casei*, since duch a function is not present in the DNA fragments used for complementation (see also below). As in other bacteria studied sofar, the genes encoding *L. pentosus* MD353 D-xylose isomerase D-xylulose kinase and a regulatory protein are tightly linked (Figs.

1 and 2). In particular, the organization of *xylA* and *xylB* seems to be highly conserved in all bacteria: these genes are always adjacent to each other. However, a closer inspection of the organization of the xylose genes in different bacteria reveals some marked differences. For instance, *L. pentosus* MD353 *xylR* is transcribed in the same direction as *xylA* and *xylB*, whereas *xylR* in *B. subtilis* has the opposite orientation with respect to *xylA* and *xylB* (Hastrup, 1988; Kreuzer *et al.*, 1989). Moreover, *L. pentosus* and B. subtilis *xylA* and *xylB* are transcribed from the same DNA strand (Hastrup, 1988; Wilhelm and Hollenberg, 1985), whereas in *Streptomyces violaceoniger xylA* and *xylB* are divergently transcribed (Tiraby *et al.*, 1989).

In *E. coli*, two open reading frames are found downstream from *xylB*, presumably encoding a permease (*xylT*) and a regulatory protein (*xylR*) (Lawliss et al. 1984), whereas an *xylF* gene, encoding a D-xylose binding protein, was also mapped in close proximity of the *xyl* operon (Sumiya and Henderson, 1989). DNA sequence analysis has indicated that such genes (either involved in regulation of D-xylose gene expression or D-xylose transport) are not present in the region downstream from *L. pentosus* MD353 *xylB* (not shown). However, (the 3' part of) a putative D-xylose transport gene could be identified in the region about 2.4 kb upstream from *L. pentosus* MD353 *xylR* (not shown). Currently we are cloning the *L. pentosus* MD353 chromosomal DNA adjacent to the 5.0 kb *Hin*dIII fragment to identify and characterize the remaining part of that gene.

It has been reported that on the basis of AA sequence similarity, two groups of D-xylose isomerases can be distinguished (Vangrysperre *et al.*, 1990). One group comprises the D-xylose isomerases of *E. coli* and *B. subtilis* (average similarity approximately 70%), in the other group the D-xylose isomerases of *Streptomyces violaceoniger*, *Ampullariella* sp. and *Actinoplanes* are found (intragroup sequence similarity 83 to 96%). The enzymes in the second group are considerably less similar to the enzymes in the first group (intergroup sequence similarity of 50%). As illustrated in Fig. 3B, *L. pentosus* MD353 D-xylose isomerase obviously belongs to the first group. Remarkably, all D-xylose isomerases of the second group miss the 30-40 AA residues, which are present in the N-terminus of D-xylose isomerase of *E. coli*, *B. subtilis* and *L. pentosus* MD353. Consequently, the Mw of these proteins is considerably smaller (e.g. 43.0 kD for *Streptomyces violaceoniger* D-xylose isomerase) (Drocourt *et al.*, 1988) than that of the proteins in the first group. When the primary structure of six D-xylose isomerases is compared, it appears that only a few regions are conserved in all proteins (consensus sequence in Fig. 3B). The two histidine residues, which are considered as essential components in the active site of D-xylose isomerase (Batt *et al.*, 1990), are conserved in *L. pentosus* MD353 (His-103

(Shamanna Sanderson, 1979) or negatively regulated (e.g. in *B. subtilis*) (Hastrup, 1988; Kreuzer *et al.*, 1989). The gene product of *L. pentosus* MD353 *xylR* is very similar to the *B. subtilis xylR* repressor (and distinct from the *xylR* gene product of Gram-negative microorganisms (Fig. 3A). It is interesting to note that in another Gram-positive bacterium, *Streptomyces violaceoniger*, a regulatory gene (*xylX*) is found directly downstream from *xylB*. This gene encodes a protein with significant sequence similarity to the *B. subtilis* repressor as well (Tiraby *et al.*, 1989; F. Martin, personal communication). In the *B. subtilis* repressor, a conserved N-terminal helix-turn-helix motif was identified, which has been reported to be involved in repressor-operator recognition in many prokaryotes (Aslanidis and Schmitt, 1990; Dodd and Egan, 1990; Sauer and Pabo, 1984). This motif is also conserved in the presumed *L. pentosus* MD353 repressor protein (AA 30-50; Fig.3A). On the basis of these observations, it is reasonable to assume that *L. pentosus* MD353 *xylR* codes for a protein with the same function as the *B. subtilis* repressor protein and that expression of the xylose genes in *L. pentosus* MD353 is under negative control. However, as shown in Fig. 5, the sequence in front of *L. pentosus* MD353 *xylA* shows (limited) similarity with the operator sequence for the *B. subtilis* repressor but is lacking the T-residue at the leftward end of the *xyl*-operator (second position), which is important for repressor-function in *B. subtilis* (Kreuzer *et al.*, 1989). Whether this region plays a functional role in binding of the *xylR* gene product in *L. pentosus* MD353 remains, therefore, to be established. Currently, experiments are in progress to analyze into detail transcription of *L. pentosus* MD353 xylose genes and to unravel the mechanism by which the expression of these genes is regulated.

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CHAPTER 3

Promoter analysis and transcriptional regulation of *Lactobacillus pentosus* genes involved in xylose catabolism

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SUMMARY

The *xyl* genes in *Lactobacillus pentosus* are induced by xylose and repressed by glucose, ribose, and arabinose. Northern blot analysis showed that regulation is mediated at the transcriptional level. Under inducing conditions two *xylA* transcripts were detected, a major transcript of 1500 b and a minor transcript of 3000 b. The 3000 b transcript also comprises sequences from *xylB*, suggesting that *xylA* and *xylB* are transcribed together. A 1200 b *xylR* transcript was found under, inducing and non-inducing conditions. In the presence of xylose, a second *xylR* transcript (> 7000 b) was detected, which comprises sequences from two upstream genes, *xylQ* and *xylP*. The transcription start sites for *xylA* and *xylR* were mapped by primer extension and S1 experiments at 42 and 83 b upstream of the translation start sites, respectively. Induction by xylose of the chloramphenicol acetyltransferase (CAT) gene under control of the *xylA* promoter, on a multicopy plasmid, was 60-80 fold, but only 3-10 fold in the presence of glucose and xylose. Expression of CAT under control of the *xylR* promoter was constitutive and was 10-fold less than expression under control of the *xylA* promoter. Sequence analysis suggests the presence of two operator-like elements, one overlapping with the promoter -35 region of *xylA* and controlling the expression of *xylA* by binding factors involved in catabolite repression, and a second operator downstream of the promoter -10 region of *xylA* which may bind the product of *xylR*, the repressor. Titration experiments with multiple copies of these elements showed that under

inducing conditions expression of *xylA* in wild-type *L. pentosus* is sub-optimal.

INTRODUCTION

Like many other microorganisms, but unlike most other lactobacilli, *Lactobacillus pentosus* MD353 is able to utilize D-xylose as energy source. Three genes involved in D-xylose catabolism in this organism coding for D-xylose isomerase (XylA), D-xylulose kinase (XylB), and a regulatory protein (XylR) have been cloned and sequenced (Lokman et al. 1991; Posno et al. 1991b). The product of the *L. pentosus* MD353 gene, *xylR* shows great similarity to that of the *B. subtilis* gene, *xylR* which functions as a repressor for the adjacent *xylAB* operon (Gärtner et al. 1992; Kreuzer et al. 1989). In the deduced amino acid sequences of both genes a putative N-terminal helix-turn-helix motif was identified, which reportedly is involved in repressor-operator recognition in many prokaryotes (Aslanidis and Schmitt 1990; Dodd and Egan 1990, Sauer and Pabo 1984). Based on the deduced amino acid sequence homology it was assumed that also *L. pentosus* MD353 *xylR* codes for a repressor and that expression of xylose genes in *L. pentosus* therefore is under negative control (Lokman et al. 1991). The organization of the xylose genes is different in the two organisms. *L. pentosus xylR* is transcribed in the same direction as *xylAB*, whereas *xylR* has the opposite orientation with respect to *xylAB* in *B. subtilis* (Hastrup 1988; Kreuzer et al. 1989). Other Gram-positive bacteria show either the same organization as *B.subtilis* (*B. licheniformis*, Scheler et al. 1991; *B. megaterium*, Rygus et al. 1991), or as *L. pentosus* (*Staphylococcus xylosus*, Sizemore et al. 1991). In *L. brevis* no *xylR*-like gene has been found in the vicinity of the *xylAB* operon (Batt, personal communication).

Expression of the xylose genes in bacilli (Gärtner et al. 1988; Rygus et al. 1991; Scheler et al. 1991) and *S. xylosus* (Sizemore et al. 1992) is induced by xylose and repressed by glucose. Recently, it has been shown that glucose repression of the *xylAB* operon in *B. subtilis* involves a *cis*-acting element which is located within the *xylA* reading frame, near the 5' end (Jacob et al. 1991). This element shows considerable similarity to a sequence identified as the operator for catabolite repression in *B. subtilis* by Weickert and Chambliss (1990).

In this article we report on the transcriptional regulation of the *L. pentosus* MD353 *xyl* genes. Our data indicate that expression of the *xylAB* operon is induced by xylose and repressed by various other sugars. The expression is negatively

regulated at the level of transcription by a repressor, the product of *xylR*. Nucleotide sequence analysis suggests the presence of a *cis*-acting element in the promoter region of the *xylAB* operon of *L. pentosus* MD353 involved in glucose repression.

MATERIALS AND METHODS

Materials. Enzymes for molecular cloning were purchased from Boehringer or Bethesda Research Laboratories and were used according to the specifications of the manufacturer. Lysozyme was from Boehringer, and [α-^{35}S]dATP and [γ-^{32}P]ATP from Amersham.

Bacterial strains and media. *L. pentosus* MD353 isolated from a natural cucumber fermentation (Posno et al. 1991a), was the D-xylose fermenting strain used for studying D-xylose catabolism in *Lactobacillus*. *E. coli* strain JM109 was used for construction of recombinant DNA plasmids. *L. pentosus* MD353 was routinely cultivated at 37°C in MRS medium (Difco) (deMan et al. 1960). For RNA and protein isolations M-medium was prepared containing per litre 5 g yeast extract, 1 g proteose pepton, 1 g Tween 80, 5 g sodium acetate, 2 g dipotassium phosphate, 2 g ammoniumcitrate, 200 mg magnesium sulphate, 50 mg manganese sulphate. After autoclaving one of the following energy sources was added to a final concentration of 1% (w/v); glucose, D-xylose, ribose, arabinose or a combination of 1% xylose with 1% glucose, ribose or arabinose. For plating, all media used were solidified with 1.5 % agar (Difco). For selecting *L. pentosus* MD353 transformants, erythromycin was used at 5 μg/ml.

Plasmids and plasmid constructions. The plasmids used in this study are listed in Table 1. The shuttle vector pLP3537 was used for cloning of DNA fragments in *E. coli* and *Lactobacillus* (Posno et al. 1991a). The plasmids pXH50A and pXH37A that together comprise *xylR*, *xylA, xylB*, and flanking regions, were used as the source of *xyl* genes to be studied (Lokman et al. 1991).

Plasmid pRBE1 was constructed by cloning a *Pst*I-*BamH*I fragment, containing the erythromycin gene from plasmid pEI2 (Posno et al. 1991a), into the promoterscreening vector pRB394 (Brückner 1992) digested with *Pst*I and *Bgl*II. To determine promoter activity, different DNA fragments containing the putative promoter, ribosome bindingsite, startcodon, and 5'-end of the *xylR*, *xylA*, or *xylB*

gene, respectively, were cloned upstream of the promoterless chloramphenicol resistance gene (*cat*-86) of pRBE1. The resulting plasmids are designated pRBE2 (containing a 780 bp *Pst*I-*Eco*RV *xylR* promoter fragment), pRBE3 (containing a 105 bp *Bam*HI-*Sst*I *xylA* promoter fragment), pRBE4 (containing a 355 bp *Xba*I-*Eco*RI *xylA* promoter fragment), pRBE5 (containing a 750 bp *Bam*HI-*Sal*I *xylB* promoter fragment). Plasmid pLP3537-17 was made by cloning a 2.4 kb *Pst*I-*Hin*dIII fragment containing the *xylA* promoter preceded by the *xylR* gene which harboured a deletion. The deletion of 220 bp of the *xylR* gene was introduced by digestion with *Eco*RV and *Hpa*I followed by ligation. All cloned fragments described above were directly isolated from pXH50A or pXH37A, only the 105 bp *Bam*HI-*Sst*I fragment was made by the polymerase chain reaction (PCR), using pXH50A as a template.

Plasmids	Selection markers	Source of reference
pXH50A	Ap^r	Lokman *et al.*, 1991
pXH37A	Ap^r	Lokman *et al.*, 1991
pLP3537	Ap^r;Ery^r	Posno *et al.*, 1991a
pLP3537-17	Ap^r;Ery^r	Lokman *et al.*, 1994
pRB394	Ap^r;Cm^a	Brückner, 1992
pRBE1	Ap^r;Ery^r;Cm^a	this study
pRBE2	Ap^r;Ery^r;*xylR*-Cm^r	this study
pRBE3	Ap^r;Ery^r;*xylA*-Cm^r	this study
pRBE4	Ap^r;Ery^r;*xylA*-Cm^r	this study
pRBE5	Ap^r;Ery^r;*xylA*-Cm^a	this study

Table 1. Plasmids used in this study. [a] Promoterless *cat*-86 gene.

DNA and RNA isolation. *Lactobacillus* plasmid DNA isolation was performed according to Posno et al. (1991a). RNA isolation was performed as described by Pouwels et al. (1993).

Transformation of Lactobacillus strains. The electroporation protocol for *L. pentosus* MD 353 (Leer et al. 1992) was optimized. Briefly, an overnight culture was diluted (1:10) in MRS broth and incubated without shaking at 37 °C for 1 h. The cells were harvested by centrifugation, chilled on ice, and washed twice with cold

Millipore-treated water. The cells were resuspended in 1/100 of the original culture volume of ice-cold electroporation buffer (30% PEG-1450 in Millipore-treated water, pH 6.8). Plasmid DNA (0.5-1.0 μg) was mixed with 50 μl of the cell suspension in an ice-cold Gene Pulser cuvette (inter-electrode distance, 0.2 cm). A single pulse of 12,500 V/cm was delivered immediately (100 Ω parallel resistor and 25 μF capacitance settings; Gene Pulser and Pulsecontroller from Bio-Rad). Following the pulse, the cell suspension was directly diluted with 450 μl of MRS. An incubation period of 1-2 h at 37 °C was applied for expression of the erythromycin resistance gene. Erythromycin-resistant transformants were selected on agar plates at a sub-inhibitory concentration of the antibiotic (0.5 μg/ml), followed by replica plating at the selective concentration (5.0 μg/ml). After 24-48 h of anaerobic incubation at 37 °C, transformants became visible. The transformation efficiency for *L. pentosus* MD353 varies from 10^3 to 10^4 transformants per μg of plasmid DNA.

CAT assay. Cells were cultivated in 10 ml M-medium supplemented with 1% glucose, 1% xylose, or 1% glucose plus 1% xylose, respectively, until OD_{695} 0.3. After washing with 20mM Hepes pH 7.0 the cells were resuspended into 200 μl of 20mM Hepes pH 7.0. Bacterial extracts were prepared after addition of an equivalent of glass beads (BDH 0.4 mm), by vortexing the mixture for 60 seconds, followed by centrifugation. The supernatant was used to determine the chloramphenicol acetyltransferase (CAT) activity as described by Shaw (1975). The determination of protein concentrations was done by the Bio Rad protein assay (Bio Rad laboratories GmbH), using BSA as a standard.

Other techniques. Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, end-labelling of DNA-fragments and oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase, DNA and RNA transfer to Hybond N filters, and PCR was performed according to Sambrook et al.(1989). Primer extension analysis was performed by annealing 1 pmol of a ^{32}P-labelled oligonucleotide, complementary to *xyl* mRNA, to 20 μg of total RNA. Synthesis of cDNA with reverse transcriptase (M-MLV from BRL) was followed. Primer extended products were separated on a 6% polyacrylamide-8M urea sequencing gel together with the products of a single-stranded sequence reaction obtained with the same primer. Nucleotide sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977). For isolation of DNA fragments from agarose gels, the GeneClean kit from Bio101 (LaJolla) was used.

Induction of xylose genes. To analyze transcription of *L. pentosus* MD353 xylose genes, we prepared Northern blots of total RNA isolated from cells cultivated in medium containing different energy sources. As shown in Fig.1, *xylA* is not transcribed in the presence of glucose but transcription occurs in the presence of D-xylose. Two *xylA* transcripts were detected, a major transcript of about 1500 b in size and a minor transcript of about 3000 b in size. The same 3000 b transcript was found using a *xylB* DNA fragment as a probe. This suggests that *xylA* and *xylB* are transcribed together and are subsequently processed into separate transcripts. Since an expected 1500 b transcript of *xylB* could not be detected, the two products are probably each decaying at their specific rate: *xylB* RNA much faster than *xylA* RNA and *xylAB* RNA. A palindromic sequence is present in the intergenic region between *xylA* and *xylB* that can be folded into a stem-loop structure. A functional role in transcription-termination remains speculative, since a thymidine-stretch is lacking (Lokman et al. 1991). Therefore this sequence might function as a processing site and/or play a role in rendering *xylA* RNA more stable than *xylB* RNA. In *E. coli* and *S. thyphimurium* such inverted repeat structures called REP (repetitive extragenic palindromic) sequences stabilize upstream mRNA by protecting it from exonucleolytic attack (Stern et al. 1984; Newbury et al. 1987). However, the sequence present in the *xylAB* intergenic region does not show any homology with the consensus REP sequence. Melin et al. (1990) reported on the importance of the 5'-region in controlling the stability of *B. subtilis sdh* mRNA under different growth conditions. They found that the 5'-part of the mRNA decayed more rapidly than the 3'-part whereas from studies in *E. coli* it is known that specific 5'-terminal mRNA segments increase transcript stability (Belasco et al. 1986). Until now, not much is known about stability of mRNA in *Lactobacillus*. Whether the 5'-part of the *xylAB* mRNA or the stem-loop structure between *xylA* and *xylB* has a functional role in processing the 3000 b *xylAB* transcript and/or in stabilizing the 1500 b *xylA* transcript remains to be established.

Transcription of the *xylR* gene occurred in the presence of glucose or xylose. With both energy sources a transcript of 1200 b was found, whereas in the presence of xylose also a transcript of more than 7000 b was detected (Fig. 1). This large transcript, which is not found in the presence of glucose, presumably comprises at least two open reading frames (ORF's) in addition to the *xylR* gene, as revealed by DNA sequence analysis. The amino acid sequence of XylQ encoded by the gene immediately upstream of *xylR,* does show considerable

homology to the amino acid sequence of an 88.1 kD hypothetical protein of the *E. coli* genomic region from 81.5 to 84.5 minutes, present in the Swiss-Prot database.

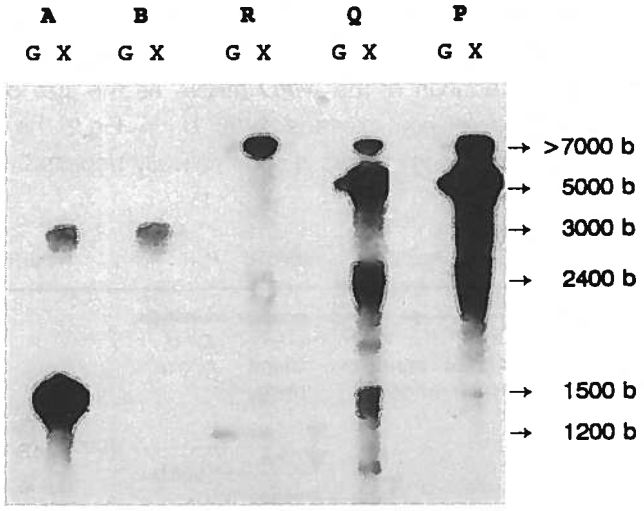

Fig.1 Northern blot analysis of *L. pentosus* RNA, isolated under inducing (X; 1% xylose) and non-inducing (G; 1% glucose) conditions. DNA fragments containing a part of *xylA*, *xylB*, *xylR*, *xylQ*, and *xylP* respectively, were used as a probe. Differences in signal intensities are caused by differently labelled probes.

Upstream of *xylQ* a sequence was found, of which the deduced amino acid sequence showed significant similarity to several transport proteins: XynC of *B. subtilis*, involved in transport of xylose oligomers (Hastrup, personal communication;Hastrup 1988); the melibiose carrier of *E. coli*, MelB (Yazyu et al. 1984); LacS, responsible for transport of lactose into *Streptococcus thermophilus* (Poolman et al. 1989); LacY, lactose transport protein of *Lactobacillus bulgaricus* (Leong-Morgenthaler et al. 1991). Because of the striking homology with these membrane bound proteins it seems likely that this ORF, *xylP*, has a functional role in the transport of xylose into the cell. In *E. coli*, directly upstream of the gene coding for the 88.1 kD hypothetical protein, an ORF was found of which the amino acid sequence also showed homology with transport proteins and therefore also with XylP. From this we conclude that besides the deduced amino acid sequence, also the genetic organisation of the two *E. coli* genes is similar to that of *L. pentosus xylP* and *xylQ*. Whether the products encoded by the two *E. coli* genes

coli genes involved in transport and/or catabolism of D-xylose have been identified sofar. They are located at different sites of the chromosome (Malezka et al. 1982; Davis and Henderson 1987; Sumiya and Henderson 1989; Henderson, personal communication). Beside the 7000 b transcript, a 5000 b and a 2400 b transcript were detected using DNA fragments of *xylP* or *xylQ* as a probe. The latter might be an intermediate in the degradation of the *xylPQ* mRNA as the size of a transcript which includes both genes largely exceeds 2400 b. In Fig.2 the genetic and transcriptional organization of the *xyl* genes is schematically depicted.

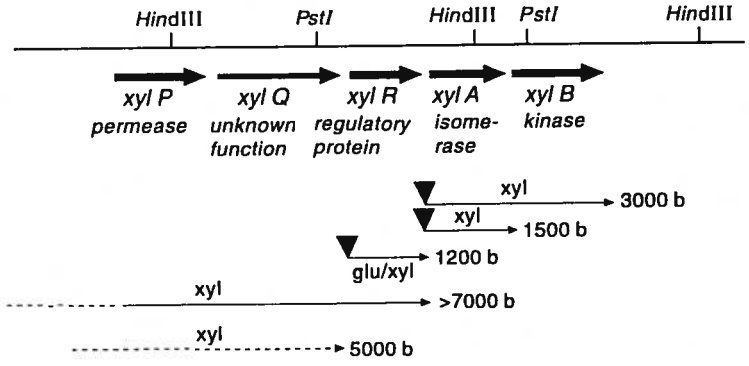

Fig.2 Schematic representation of genetic and transcriptional organization of *xyl* genes from *L. pentosus* MD353. Above: Part of the chromosomal DNA which has been cloned and characterized. The *xyl* genes and their functions are depicted. Under: Transcripts and their sizes found after Northern blot analysis. The growth conditions under which the transcripts were found and the transcript sizes are indicated (xyl: 1% xylose, glu/xyl: 1% xylose or 1% glucose). The position of the 5000 b transcript was estimated. Triangls indicate the mapped 5'-ends.

To investigate whether energy sources other than glucose cause repression of transcription of *xylAB*, we have isolated RNA from cells cultivated in medium containing glucose, ribose, arabinose, xylose, glucose plus xylose, ribose plus xylose, and arabinose plus xylose, respectively. Northern blot analysis showed that in the presence of ribose or arabinose as sole energy source, no transcription of *xylA* takes place. These energy sources repress, as is observed for glucose, transcription of *xylA* when present together with xylose (Fig.3), thus defining a general catabolite repression mechanism.

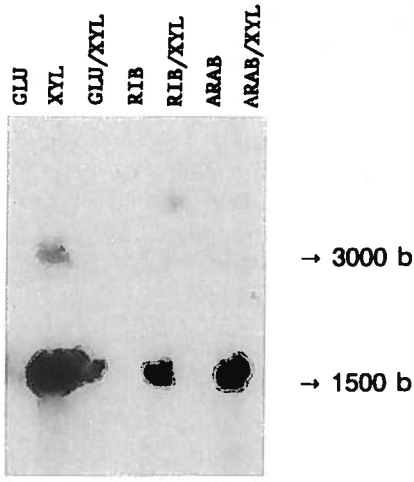

Fig.3 Northern blot analysis of *L. pentosus* RNA isolated after cultivation in the presence of 1% glucose (glu), 1% xylose (xyl), 1% glucose plus 1% xylose (glu/xyl), 1% ribose (rib), 1% ribose plus 1% xylose (rib/xyl), 1% arabinose (arab), and 1% arabinose plus 1% xylose (arab/xyl), respectively. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

Determination of xyl promoters in L. pentosus MD353. The 5'-end of the *xylAB* and *xylR* mRNAs was determined by primer extension analysis. For that purpose, total RNA was prepared from *L. pentosus* cultivated under inducing (1% xylose) and non-inducing (1% glucose) conditions. Only under inducing conditions a primer extension product was obtained, the size of which indicated that the transcription initiation site of *xylA* is 42 bp upstream of the *xylA* translational initiation codon (Fig. 4). The presumed -10 and -35 regions with optimal spacing of 17 bp are TGTAAT and TTTACA, respectively (Fig. 5b). Because of the relatively low amounts of *xylR* mRNA, it was difficult to accurately map the 5'-end. Using an RNA preparation from bacteria cultivated in the presence of xylose a signal, although very weak, could be detected (not shown). From this result we tentatively conclude that the transcription initiation site of *xylR* is located 83 bp upstream of the translational initiation codon, GTG. The presumed -10 and -35 promoter sequences with a spacing of 18 bp are TCACAT and TTGACA, respectively (Fig. 5a). Except for two mismatches, the promoter motif of *xylA* exactly matches the consensus promoter sequence of genes in Gram-positive bacteria (Graves and Rabinowitz 1986) or *E. coli* (Harley and Reynolds 1987) (Fig. 5b). The -10 sequence of the *xylR* promoter contains three mismatches. Nuclease S1 mapping confirmed the results of the primer extension experiments (not shown).

A 5'-end of the *xylB* mRNA was not found, in agreement with the absence of a

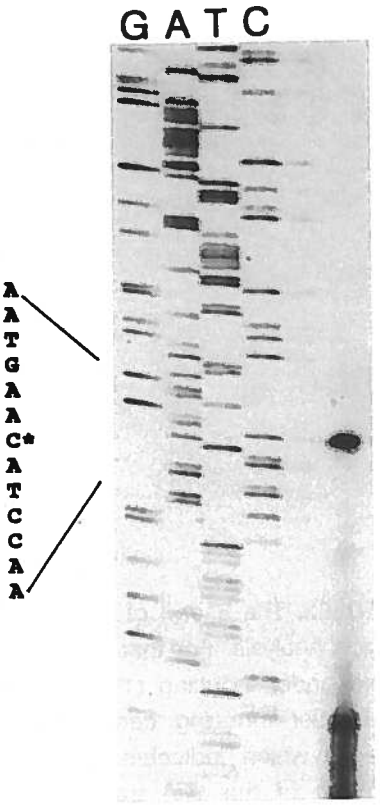

Fig.4 Primer extension analysis of the *xylA* transcript. An apparent 5'- terminus for the xylose-induced transcript was identified by using a radiolabeled primer complementary to the RNA downstream of the translation start site of *xylA*. The same primer was used to prime dideoxynucleotide sequencing reactions from a single-stranded DNA template containing the corresponding fragment of *xylA*. The asterisk indicates the nucleotide at the apparent transcription start site.

Promoterstrength and functionality. As the *xylR* promoter shows less resemblance to the consensus promoter sequence of genes in other Gram-positive bacteria than does the *xylA* promoter, and as there is much less *xylR* mRNA present than *xylA* mRNA, the *xylR* promoter is expected to be a weaker promoter than the *xylA* promoter. The results of the 5'-end mapping of the *xylB* mRNA also suggest that a promoter is not present immediately upstream of *xylB*.

To verify these assumptions, the activity of the promoters was determined. DNA fragments containing the putative promoters and flanking sequences of *xylR, xylA,* and *xylB*, respectively, were fused to the promoterless *cat*-86 gene of the promoterscreening vector pRBE1. Details of constructions are described in Materials and Methods. The resulting plasmids, pRBE2, pRBE3, pRBE4 and pRBE5 were used to transform *L. pentosus*. Chloramphenicol acetyltransferase (CAT) expression was determined after cultivating the transformants in M-medium under inducing (1% xylose) and non-inducing (1% glucose) conditions (Table 2).

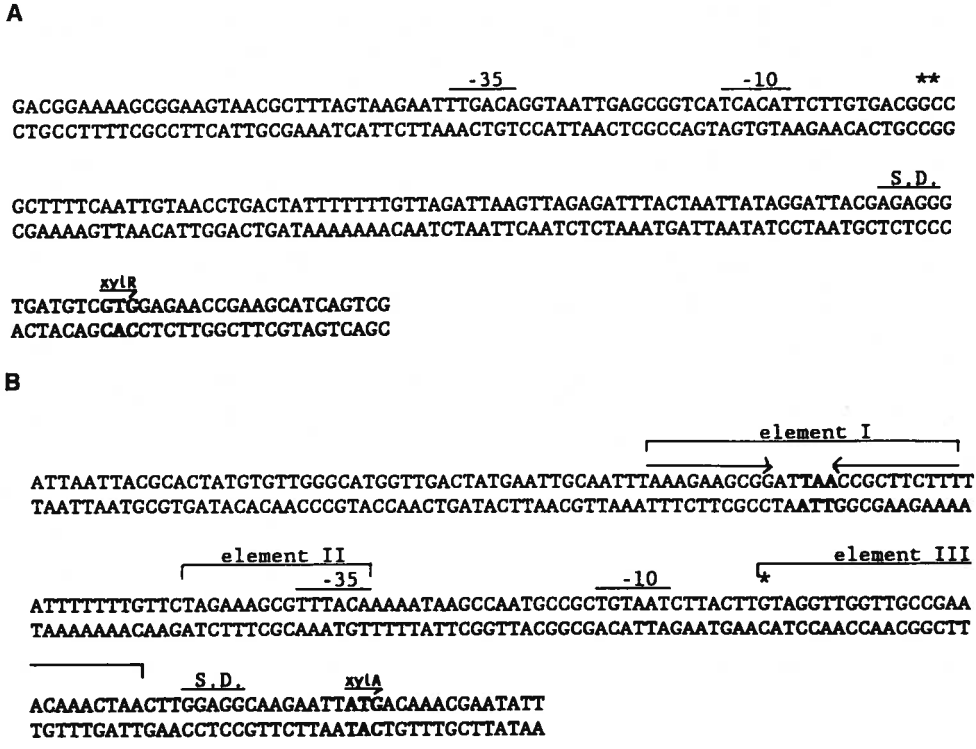

Fig.5A and B Promoter sequences of the *L. pentosus xyl* genes. **A** *xylR* promoter region. Transcriptional start sites determined by primer extension and S1 nuclease experiments are indicated by asterisks. The translational start, Shine- Dalgarno sequence, and -10 and -35 sequences are overlined. **B** *xylA* promoter region. The designations are the same as in **A**. Three *cis*-elements in the *xylR-xylA* intergenic region are depicted. Element I probably serves as a transcription terminator of *xylR* and comprises the translational stopcodon TAA of *xylR* (in bold). Element II might have a function in catabolite repression and element III is expected to be the operator which binds the *xyl*-repressor, *xylR*.

As expected, cells harbouring plasmid pRBE1 (promoterless) and pRBE5 (*xylB* promoter fragment) showed no CAT activity.

The CAT activity of the strains containing the *xylA* promoter (pRBE3 and pRBE4) was 60-80 fold induced in the presence of 1% xylose compared with growth in medium without xylose. The addition of glucose to a xylose-containing medium repressed the promoter activity 15-25 fold. The CAT activity measured with

region) and the same promoter fragment enlarged with 260 bp of the 5'-end of *xylA* (pRBE4) did not differ significantly. These results indicate that the activity of the *xylA* promoter is defined within the *xylR-xylA* intergenic region.

When placed under control of the *L. pentosus xylR* promoter (pRBE2), the *cat* gene was expressed independently of the presence of inducer (Table 2), suggesting that *xylR* is constitutively expressed. Also the promoter of the *xylR* gene of *S. xylosus* was shown to be constitutively expressed (Sizemore et al. 1991). In contrast, *xylR* in *B. megaterium* and *B. licheniformis* was found to be inducible. For the *B. megaterium* gene a nine-fold induction was observed, while that for the *B. licheniformis* gene was two-fold (Rygus et al. 1991; Scheler et al. 1991). Comparison of the CAT-activities showed that under inducing conditions expression from the *L. pentosus xylA* promoter was 10-fold more efficient than from the *xylR* promoter.

Plasmid	Promoter fragment	CAT activity ($U.mg^{-1}$)		
		glucose	xylose	xylose+glucose
pRBE1	-	<0.01	<0.01	<0.01
pRBE2	780 bp *xylR*	0.28	0.27	0.30
pRBE3	105 bp *xylA*	0.03	2.41	0.10
pRBE4	355 bp *xylA*	0.03	1.83	0.13
pRBE5	750 bp *xylB*	<0.01	<0.01	<0.01

Table 2. CAT activities of *L. pentosus* MD353 cells harbouring the indicated plasmids
Average CAT activities of two independent determinations are given (the standard deviation was less than 15%). The energy sources used in the growth medium are indicated.

Based on Northern blot analysis there are at least 10-fold more transcripts coding for XylR under inducing conditions compared to non-inducing conditions. In the presence of xylose nearly all *xylR* RNA is part of a polycistronic messenger RNA of > 7000 b (Fig.1), which is apparently initiated at a promoter which is induced by xylose. Whether expression of *xylR* in *S. xylosus*, which shows the same organization of *xyl* genes as in *L. pentosus*, is subject to a similar control mechanism is presently unknown. If transcription from an inducible upstream promoter had occurred it would have remained undetected with the technique

used to demonstrate constitutivety of expression (Rygus et al. 1991).

Although *xylR* is inducible in both *L. pentosus* and *B. megaterium*, the underlying mechanism for its control must be different. Since the orientation of the *xylR* gene in *B. megaterium* is opposite to that of *L. pentosus* with regard to *xylAB*, it seems highly unlikely that *xylR* in *B. megaterium* is expressed from an inducible upstream promoter. Transcription from such a promoter would have to traverse the *xylAB* operon in opposite direction before reaching *xylR*. Further research is needed to clarify why the mechanism of control of expression of *xylR* in the two organisms is different and how these control mechanisms operate.

Why expression of a negative regulator of the *xylAB* operon, XylR is induced by xylose is not understood. However, induction of expression of a negative regulator has been observed also for other genes in other organisms. The *gntR* gene, which encodes a transcriptional repressor for the *gnt* operon in *B. subtilis*, is the promoter-proximal gene of the operon. Expression of the *gnt* operon is induced by gluconate (Fujita and Fujita 1987). Also the *nagC* gene in *E.coli,* which is believed to encode the repressor of the *nag* operon, is induced by the inducer of the operon, N-acetyl-glucosamine. Under non-inducing conditions a *nagC* gene transcript with a size corresponding to that of the gene is found, suggesting that the *nagC* gene, which is the third of the four genes of the *nag* operon, is constitutively expressed. In the presence of the inducer, greatly increased amounts of a *nagC* gene transcript were found comprising sequences of upstream as well as downstream genes, indicating that transcription of *nagC* is inducible (Plumbridge, 1989).

Comparison of the amino acid sequences of XylR and NagC shows that the proteins are structurally related (similarity:46%;identity:22%). Both proteins contain a helix-turn-helix motive, characteristic for DNA-binding proteins. The structural similarity of the two proteins taken together with the observation that expression of the genes encoding the proteins is inducible, might indicate that the mechanism of control of expression is similar for both genes.

How might these control mechanisms operate? One possible explanation for the increased expression of a repressor-encoding gene upon induction is that the product of the gene may have a dual function, as has been demonstrated for the repressor of the *ara* operon in *E. coli*, AraC. For example, XylR might function as a repressor in the absence of xylose, but be an activator when xylose is present. A conformational change or modification of the protein triggered by the inducer might change the protein from a repressor into an activator, or vice versa (Schleif 1987). Moreover, it is also possible that XylR is playing a combined role showing

repressor function for the *xyl* operon and activator function for a different operon. For example the *fruR* gene of *E. coli* and *S. thyphimurium* codes for a protein that represses the *fru* operon, while at the same time it is an activator of the *pps* gene, which codes for phosphoenolpyruvate synthase (Jahreis et al. 1991).

Repression and catabolite repression of the xyl operon. Sofar, three elements with palindromic structure have been observed between *xylR* and *xylA* of *L. pentosus* MD353 (Fig. 5b). As previously described, the first element (I), which can be folded into a possible stem-loop structure followed by a thymidine-rich region, probably serves as a rho-independent transcription termination signal of *xylR* (Lokman et al. 1991; Friedman et al. 1987; Platt 1986). The sequence of element II, 68 bp upstream of the *xylA* translational initiation codon, overlaps with the -35 region of the *xylA* promoter. This sequence shows homology with sequences found at a similar position upstream of the *xylAB* operon of *S. xylosus*, *B. megaterium, B. licheniformis* and *L. brevis* (Sizemore et al. 1992; Rygus and Hillen 1992; Scheler et al. 1991; Batt, personal communication). Moreover, the sequences of the *L. pentosus* element show considerable similarity to that of a 34 bp element present in the coding sequence for xylose isomerase in *B. subtilis* which was shown to mediate glucose repression (Jacob et al. 1991). Fig. 6 shows the nucleotide sequences of the *L. pentosus* element together with that of elements found in *xyl* operons of other organisms and the sequence of the proposed consensus sequence for glucose repression in *B. subtilis* (Weickert and Chambliss 1990). Because of the clear homology between the sequence upstream of the *L. pentosus xylAB* operon and the consensus sequence for glucose repression, it is tempting to believe that the *L. pentosus* element is mediating catabolite repression as well.

Based on sequence similarity with the operator of the *xylAB* operon of *B. subtilis*, element III could function as an operator. To establish whether one or more of these sequences has a functional role in repression of the *xylAB* operon, we have performed titration experiments with *L. pentosus* harbouring plasmids pRBE3 (105 bp *xylR-xylA* intergenic region) or pLP3537-17 (*xylA* promoter followed by 795 nucleotides of the *xylA* ORF and preceded by a *xylR* sequence with a deletion, see Materials and Methods). Transcription of *xylA* of *L. pentosus* transformants and of the wild-type strain was determined under non-inducing and inducing conditions. Surprisingly, a more than 5-fold increase of the amount of *xylA* mRNA was found in the transformants compared with untransformed *L. pentosus* in the presence of xylose, suggesting the titration of a *trans*-acting negative factor (Fig. 7).

L. pentosus (xyl)	a G A A A G C G T T T A C A
L. brevis (xyl)	c G A A A A A C G C T T g C A
B. licheniformis (xyl)	T G A A A G C G A T T A a t
B. megaterium (xyl)	T G A A A G C G C a A A C A
B. subtilis (xyl)	T G g A A G C G T a A A C A
S. xylosus (xyl)	T G T A A G C G T T A A C A
B. subtilis (consensus)	T G W A A N C G N T N W C A

Fig.6 Sequence comparison of a potential *cis*-active sequence (element II in Fig.5B) mediating glucose repression of the *xylAB* operon in *L. pentosus* with target sequences for catabolite repression of xyl genes in *L. brevis*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, and *S. xylosus*. The sequences are compared to a consensus sequence for catabolite repression which was based on a *cis*-acting element in the *B. subtilis amyE* gene (Weickert and Chambliss 1990). Bold capital characters indicate the identity of the *xyl* character with the consensus sequence.

The effect was equal for both transformants and was not found for transformants harbouring plasmids pRBE1 or pLP3537 (vectors without insert of the intergenic region; not shown). These results suggest that in wild-type *L. pentosus* even under inducing conditions a repression factor is binding to specific sequences of the *xylR-xylA* intergenic region. An explanation why *xylAB* is not fully expressed under inducing conditions in wild-type *L. pentosus* might be that the concentration of inducer is kept at a sub-optimal level. If an excess of xylose isomerase and/or xylulose kinase would be deleterious for cell metabolism, some control of expression of *xylAB* under inducing conditions would be necessary to keep the level of inducer below a certain level. This assumption is supported by the finding that overproduction of the *xylA* gene product in *E. coli* resulted in a Xyl^- phenotype (Stevis and Ho 1987). If other genes involved in xylose catabolism, e.g. *xylP*, would be controlled by a similar operator-like element, as suggested by our experiments, expression of such genes might indirectly affect the transcription of *xylA* by controlling the concentration of inducer. For example, when an excess of XylP is formed by titration of repression factors, more xylose might be transported into the cell which could lead to more efficient expression of *xylA*.

If our assumption is correct that XylR is an activator when xylose is present and a repressor in its absence, then the observation that in wild-type *L. pentosus xylA* is not fully induced in the presence of xylose raises a problem. The apparent paradox that XylR partially functions as a repressor in the presence of inducer may be explained by assuming that the concentration of the true inducer (xylose or a

derivative of xylose) is insufficient to drive the equilibrium between repressor and activator to completion. For example, the genes involved in transport of xylose (*e.g. xylP*) may not be fully induced, or genes encoding proteins that modify the repressor may be suboptimally expressed (Amster-Choder et al. 1989).

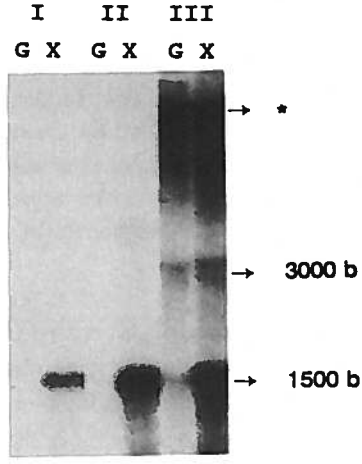

Fig.7 Transcription analysis of *xylA* under inducing (X; 1% xylose) and non-inducing (G; 1% glucose). I, *L. pentosus* MD353, II, *L. pentosus* transformants harbouring plasmid pRBE3, III, *L. pentosus* transformants harbouring plasmid pLP3537-17. The asterisk indicates a hybridisation signal derived from the plasmid. Cells were cultivated overnight in the presence of glucose, washed twice with medium without sugar followed by a 1:3 inoculation into medium with the appropriate sugar. After 2.5 hours of incubation at 37°C cells were harvested and RNA was isolated, electrophoresed and blotted. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

In the presence of glucose a weak transcription signal could be detected only after a long exposure time for the pLP3537-17 transformant (Fig. 7) This could mean that *L. pentosus* cultivated in glucose-containing medium contains such an excess of repression factor(s) that even the presence of multiple elements on the multicopy vector is insufficient to titrate the factor(s). The observation that there is a, although very weak, titration effect with the multicopy vector pLP3537-17 (30-50 copies) but no effect with the low-copy vector pRBE3 (10-20 copies) in the presence of glucose supports this view. The observation that in the presence of glucose under non-inducing conditions, no constitutive expression of *xylA* occurs, when multiple copies of the *xylR-xylA* intergenic region are present, suggests that glucose repression does not involve XylR, or involves other factors besides XylR.

In summary, it appears that at least two elements (II and III) in the *xylR-xylA* intergenic region are involved in binding repression factors. Taking into account the sequence similarity of element III with the operator of the *xylAB* operon of *B.subtilis* as well as the similarity of the XylR proteins of the two organisms, we assume that

element III is the operator of the *xylAB* operon of *L. pentosus*, the target site for XylR. Experiments are in progress to delineate the sequences responsible for the titration effects in the presence of xylose and to determine the role of XylR in repression.

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CHAPTER 4

Characterization of the *Lactobacillus pentosus ccpA* gene and its involvement in catabolite repression

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SUMMARY

An 875 bp fragment of the *ccpA* gene of *Lactobacillus pentosus* missing approximately 45 bp at the 5'-end and 90 bp at the 3'-end was isolated by PCR, using *L. pentosus* chromosomal DNA as template and primers based on the *Bacillus megaterium ccpA* gene. Sequence analysis confirmed that the PCR product originated from the *L. pentosus ccpA* gene, since the deduced amino acid sequence shows significant similarity to the CcpA's of *Bacillus subtilis, B. megaterium, Staphylococcus xylosus* and *Lactobacillus casei*. RNA analysis indicated that *L. pentosus ccpA* is part of an operon of 10 kb which is constitutively expressed. Analysis of *xylA* transcription in *L. pentosus* MD363 wild-type and a *ccpA* disruption mutant showed that catabolite repression depends on CcpA. Furthermore, the results indicate that in contrast to glucose, fructose is not involved in catabolite repression of the *L. pentosus xylAB* operon. The global regulatory role of CcpA was established by demonstrating that glucose repression of the chloramphenicol acetyltransferase gene under control of the α-amylase promoter of *L. amylovorus*, is reduced with 62% in the *L. pentosus ccpA* mutant strain.

INTRODUCTION

The presence of rapidly metabolizable carbon sources in the growth medium

other carbon sources (Magasanik, 1970; Magasanik and Neihardt, 1987). This general phenomenon is termed catabolite repression (CR). CR in enteric Gram-negative bacteria involves a positive regulatory system and is very well understood (for a review see Saier, 1991). In contrast, the elucidation of the mechanism of CR in Gram-positive bacteria has started only recently. For example, the expression of several *Bacillus subtilis* operons, like *amyE* (Nicholson *et al.*, 1987; Weickert and Chambliss, 1990), *gnt* (Miwa and Fujita, 1990; Miwa and Fujita, 1993), *xyl* (Jacob *et al.*, 1991), *hut* (Oda and Furukawa, 1992), and *bglS* (Krüger et al., 1993), is subject to catabolite repression. Nicholson *et al.* (1987) isolated *B. subtilis* mutants that were able to express the *amyE* gene in the presence of glucose. The mutations which were found close to the transcription startpoint of the gene exert an effect at the transcriptional level. The sequence of the region harbouring the mutations showed structural similarity to operator regions of the *E. coli gal* and *lac* operons. In addition, a *trans*-acting gene product homologous to the *E. coli* LacI and GalR repressors was identified and shown to be involved in CR of the *amyE* gene as well (Henkin *et al.*, 1991). This repressor-like protein was designated CcpA and was believed to mediate CR by interaction with the *cis*-acting sequence described above.

The *cis*-acting sequence, called Catabolite Responsive Element (CRE), was also detected in the *xyl* operon of *B. subtilis*, *Staphylococcus xylosus,* and *B. megaterium* (Jacob *et al.*, 1991; Sizemore *et al.*, 1992; Rygus and Hillen, 1992). Also in the latter two organisms a *ccpA* gene was identified (R. Brückner and F. Götz, personal communication; Hueck *et al.*, 1995). Mutation analysis of *ccpA* and CRE have demonstrated their involvement in CR of the *xyl* operon in *Bacillus*. Recently, it has been established that a third factor, HPr, is involved in CR in *B. subtilis*. HPr is a component of the phosphoenolpyruvate:sugar phosphotransferase system and can be phosphorylated both at a histidine residue (His-15) by enzyme I in the presence of phosphoenolpyruvate (PEP), and at a serine residue (Ser-46) by a fructose-1,6-diphosphate-activated protein kinase. HPr(Ser-P) interacts with CcpA (Hueck and Hillen, 1995; Deutscher *et al.*, 1995; Deutscher *et al.*, 1994) and binding of this protein-complex to CRE's of the *B. subtilis gnt* operon and *B. megaterium xyl* operon has been demonstrated (Fujita *et al.*, 1995; E. Küster and J. Deutscher, personal communication). However, in contrast, Ramseier *et al.*, (1995) showed that binding of *B. megaterium* CcpA to several CREs was inhibited by the presence of HPr(Ser-P). Moreover, binding of CcpA itself to CRE has also been demonstrated (Kim *et al.*, 1995).

Transcription of the *xyl* genes of *Lactobacillus pentosus* is also repressed by glucose (Lokman *et al.*, 1994). Disruption of the repressor encoding gene, *xylR*, of *L.*

pentosus did not abolish glucose repression of the *xyl* genes, suggesting that repression factors other than XylR are involved (Lokman *et al.*, 1996). Previously, an element homologous to the consensus sequence for glucose repression in *B. subtilis* was identified (Lokman *et al.*, 1994). The position of this element, overlapping the -35 sequence of the *xylA* promoter, was similar to that of the CRE present in the *xyl* operon of *S. xylosus* (Sizemore *et al.*, 1992). Based on these similarities we assumed that the element in the *L. pentosus xyl* operon also is mediating catabolite repression and, therefore, that *L. pentosus* might also harbour a *ccpA* gene.

In this paper we report on the identification of the *ccpA* gene in *L. pentosus* and its involvement in CR of the *xyl* genes and of the α-amylase promoter of *L. amylovorus*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *L. pentosus* MD353 DNA (Lokman *et al.*, 1991) was used for the isolation of a part of the *ccpA* gene by PCR using primers based on the *B. megaterium ccpA* gene (Hueck *et al.*, 1994b). *L. pentosus* MD363 (kindly provided by Dr. M.A. Daeschel) was used for the construction of a *ccpA* mutant strain. *E. coli* JM109 was used for construction of recombinant DNA plasmids.

Integration vector pEI2/ccpA was made by cloning an 875 bp *Pst*I-*Hin*dIII PCR fragment, containing the major part of the *L. pentosus ccpA* gene, into plasmid pEI2 (Posno *et al.*, 1991). Plasmid pRB-αamy was constructed by cloning a 2 kb chromosomal DNA fragment from *L. amylovorus*, containing a part of the α-amylase gene with promoter sequences (Fitzsimons *et al.*, 1994), into the promoter-screening vector pRBE1 (Lokman *et al.*, 1994).

Lactobacillus strains were routinely cultivated at 37 °C in MRS medium (Difco) or in M-medium as described by Lokman *et al.*, (1994). Energy sources were used at a final concentration of 1% (w/v): glucose, fructose, xylose, or a combination of 1% xylose with 1% of the other sugars. For plating, media were solidified with 1.5 % agar.

DNA and RNA isolation. *Lactobacillus* chromosomal DNA isolation was performed as described by Lokman *et al.* (1991). RNA was isolated from cells that had been precultivated for one night in 10 ml M-medium supplemented with 1% glucose. The cells were harvested, washed twice with medium without sugar ($M^{(-)}$), and resuspended in 1 ml of $M^{(-)}$ (4 C°). Finally, 10 ml medium supplemented with 1% of the appropriate sugar was inoculated with 330 μl of the washed cells. Induction times

of 2 or 24 hours were used. RNA isolation from these cultures was performed as described by Pouwels *et al.* (1994).

Chloramphenicol acetyltransferase assay. Cells were cultivated in 10 ml M-medium supplemented with 1% glucose or 1% galactose, and harvested at an OD_{595} of 0.4. Preparation of bacterial extracts and determination of chloramphenicol acetyltransferase activity were as described by Lokman *et al.* (1994).

Other techniques. Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, Southern- and Norternblot analyses were performed according to Sambrook *et al.* (1989). Nucleotide sequencing was performed by BaseClear with a LI-COR infrared automated DNA sequencer.

RESULTS

Identification of the *L. pentosus MD353 ccpA* gene. Initially we attempted to isolate the *ccpA* gene from a library of *L. pentosus* chromosomal DNA in *E. coli* using an internal DNA fragment of the *B. megaterium ccpA* gene (Hueck *et al.*, 1994b, Hueck *et al.*, 1995) as a probe. Under heterologous conditions one strong and one very weak hybridizing band were detected. Since the *ccpA* gene shows similarity to several other repressor encoding genes we assumed that the weaker hybridizing band corresponded to a different gene and that the strong one reflected the *ccpA* gene. Despite several attempts we were unsuccessful in cloning of DNA from the strongly hybridizing band on a multi-copy plasmid in either *E. coli* or *Lactobacillus*. In both strains, cloning of the fragment caused plasmid instability. A similar phenomenon was encountered when the *L. casei ccpA* gene was cloned on a multi-copy plasmid (G. Perez-Martinez, personal communication). We therefore decided to isolate only a part of the *ccpA* gene by PCR. Based on the nucleotide sequence of the *B. megaterium ccpA* gene, two oligonucleotides were prepared. To make the PCR fragment suitable for cloning, restriction sites were included. With these heterologous primers a PCR fragment with the expected size (875 bp) was synthesized, using *L. pentosus* MD353 chromosomal DNA as a template. The nucleotide sequence of the PCR fragment is shown in Fig.1. The deduced amino acid sequence of the PCR product exhibits sequence similarity with CcpA of *B. subtilis* (75%), *B. megaterium* (74%), *S. xylosus* (67%), and *L. casei* (83%), confirming that the synthesized product originated from the *ccpA* gene of *L. pentosus* (Fig.2). Assuming that the length of *L. pentosus ccpA* and

L. casei ccpA is similar, the *L. pentosus* PCR product is missing 45 bp at the 5'-end and 90 bp at the 3'-end of the *ccpA* gene. As a consequence the *L. pentosus* CcpA PCR product lacks the region corresponding to the first α-helix of the DNA-binding domain predicted for the amino-terminal region of *B. subtilis* CcpA (Henkin *et al.*, 1991).

```
HindIII
AAGCTTGTGTCGATGGCAACGGTTTCACGGGTGGTTAATGGGAATCCTAACGTTAAACCA   60
     V  S  M  A  T  V  S  R  V  V  N  G  N  P  N  V  K  P

GCAACGCGCAAGAAAGTTTTAGCCGTCATTGAACGGTTGGACTATCGGCCAAATGCCGTT  120
A  T  R  K  K  V  L  A  V  I  E  R  L  D  Y  R  P  N  A  V

GCACGTGGACTAGCAAGTAAGCGCTCAACGACGGTCGGCGTCATTATCCCAGATGTCACG  180
A  R  G  L  A  S  K  R  S  T  T  V  G  V  I  I  P  D  V  T

AACATTTACTTCGCCTCACTGGCACGTGGGATCGATGACATTGCGATGATGTACAAGTAT  240
N  I  Y  F  A  S  L  A  R  G  I  D  D  I  A  M  M  Y  K  Y

AACATCATTTTGACCAACTCAGACGATGCTGGGGAACAAGAAGTCAACGTGTTAAATACG  300
N  I  I  L  T  N  S  D  D  A  G  E  Q  E  V  N  V  L  N  T

TTGATGGCTAAGCAAGTCGATGGGGTTATTTTTATGGGGAACCATATCGATGATAAGCTC  360
L  M  A  K  Q  V  D  G  V  I  F  M  G  N  H  I  D  D  K  L

CGTGCAGAATTCAAACGGGCTAAGGCACCCGTTGTGTTGGCTGGGACAGTTGATCCTAAC  420
R  A  E  F  K  R  A  K  A  P  V  V  L  A  G  T  V  D  P  N

AACGAAACGCCTAGCGTTAACATTGACTATGCAGCTGCCGTTGAAGAAGCGGTCACGAAC  480
N  E  T  P  S  V  N  I  D  Y  A  A  A  V  E  E  A  V  T  N

TTAATTGGCCGTGGTCACAAGAAGATTGCTTTGGCACTCGGTTCACTCTCACAATCAATC  540
L  I  G  R  G  H  K  K  I  A  L  A  L  G  S  L  S  Q  S  I

AACGCTGAATACCGGTTGACTGGTTACAAGCGGGCGTTGACGAAGGCTAAGATTCCATTT  600
N  A  E  Y  R  L  T  G  Y  K  R  A  L  T  K  A  K  I  P  F

GACGATGCGCTGGTTTATGAAGCGGGCTACTCATACGATGCCGGCCGTAAGTTACAACCG  660
D  D  A  L  V  Y  E  A  G  Y  S  Y  D  A  G  R  K  L  Q  P

GTCATTGCTGATAGCGGTGCGACTGCCGTCTTTGTTGGTGACGATGAAATGGCTGCCGGC  720
V  I  A  D  S  G  A  T  A  V  F  V  G  D  D  E  M  A  A  G

ATTATCAATGCAAGCATGGAAACTGGCATCAATGTGCCTGATGATTTGGAAGTCGTTACG  780
I  I  N  A  S  M  E  T  G  I  N  V  P  D  D  L  E  V  V  T

AGTAACGATACGATCATCACGCAGATTACGCGTCCAGCCATCACCTCAATCACGCAACCA  840
S  N  D  T  I  I  T  Q  I  T  R  P  A  I  T  S  I  T  Q  P

                                PstI
CTTTATGATATTGGTGCAGTAGCGATGCGCTGCAG                            875
L  Y  D  I  G  A  V  A  M
```

Fig.1. Nucleotide sequence of the 875 bp PCR fragment of *L. pentosus ccpA* and the deduced amino-acid sequence (accession number Z80342). The *Hin*dIII and *Pst*I restriction

```
                H       T       H
Bm   ..VNVTIYDV AREASVSMAT VSRVVNGNPN VKPSTRKKVL ETIERLGYRP NAVARGLASK  60
Bs   .MSNITIYDV AREANVSMAT VSRVVNGNPN VKPTTRKKVL EAIERLGYRP NAVARGLASK
Lp   .......... .....VSMAT VSRVVNGNPN VKPATRKKVL AVIERLDYRP NAVARGLASK
Lc   MEKQTITIYA CREANVSMAT VSRVVNGNPN VKPATRKKVL EVIERLDYRP NAVARGLASK
Sx   ..MTVTIYDV AREARVSMAT VSRVVNGNQN VKPETRDKVN EVIKKLNYRP NAVARGLASK
Cons ---------- -----VSMAT VSRVVNGN-N VKP-TR-KV- --I--L-YRP NAVARGLASK

Bm   KTTTVGVIIP DISNIFYAEL ARGIEDIATM YKYNIILSNS DQNQDKELHL LNNMLGKQVD 120
Bs   KTTTVGVIIP DISSIFYSEL ARGIEDIATM YKYNIILSNS DQNMEKELHL LNTMLGKQVD
Lp   RSTTVGVIIP DVTNIYFASL ARGIDDIAMM YKYNIILTNS DDAGEQEVNV LNTLMAKQVD
Lc   KTTTVGVIIP DVTNMFFSSL ARGIDDVATM YKYNIILANS DENNQKEVTV LNTLLAKQVD
Sx   RTTTVGVIIP DISNVYYSQL ARGLEDIATM YKYHSIISNS DNDPSKEKEI FNNLLSKQVD
Cons --TTVGVIIP D--------L ARG--D-A-M YKY--I--NS D-----E--- -N----KQVD

Bm   GIIFMSGNVT EEHVEELKKS PVPVVLAASI ESTNQIPSVT IDYEQAAFDA VQSLIDSGHK 180
Bs   GIVFMGGNIT DEHVAEFKRS PVPIVLAASV EEQEETPSVA IDYEQAIYDA VKLLVDKGHT
Lp   GVIFMGNHID DKLRAEFKRA KAPVVLAGTV DPNNETPSVN IDYAAAVEEA VTNLIGRGHK
Lc   GLIFMGHELT DSIRAEFSRS KTPVVLAGSI DPDEQVGSVN IDYVAAVEEA TRQLLESGNK
Sx   GIIFLGGTIS EEIKDLINKS SVPVVVSGTN GKDEGISSVN IDFESAAKEI TEHLIEKGAK
Cons G--F------ ---------- --P-V----- -------SV- ID---A---- ---L---G--

Bm   NIAFVSGTLE EPINHAKKVK GYKRALTESG LPVRDSYIVE GDYTYDSGIE AVEKLLEEDE 240
Bs   DIAFVSGPMA EPINRSKKLQ GYKRALEEAN LPFNEQFVAE GDYTYDSGLE ALQHLMSLDK
Lp   KIALALGSLS QSINAEYRLT GYKRALTKAK IPFDDALVYE AGYSYDAGRK LQPVIADSG.
Lc   RVALATGSLT HPINGQFRLK GYKQALEKAG VAYDESLIFE NEPSYQAGLA LFDKLQKVG.
Sx   SFAFVGGDYS KKAQEDV.LV GLKDVLVQHE LELDEQLIFN GNETYKDGLR AFESLATA..
Cons --A---G--- ---------- G-K--L---- ---------- ----Y--G-- ----------

Bm   KPTAIFVGTD EMALGVIHGA QDRGLNVPND LEIIGFDNTR LSTMVRPQLT SVVQPMYDIG 300
Bs   KPTAILSATD EMALGIIHAA QDQGLSIPED LDIIGFDNTR LSLMVRPQLS TVVQPTYDIG
Lp   .ATAVFVGDD EMAAGIINAS METGINVPDD LEVVTSNDTI ITQITRPAIT SITQPLYDIG
Lc   .ATAVIAGDD ELAVGLLDGA IDKGVKVPDD FEIITSNNTK LTEMTRPQLT SIDQPLYDIG
Sx   KPDAILSISD EQAIGLVHAA QDAGVNVPND LQIVSFNNTR LVEMVRPQLS SVIQPLYDIG
Cons ---A-----D E-A-G----- ---G---P-D --------T- -----RP--- ---QP-YDIG

Bm   AVAMRLLTKY MNKETVDSSI VQLPHRIEFR QSTK*.                          336
Bs   AVAMRLLTKL MNKEPVEEHI VELPHRIELR KSTKS*
Lp   AVAM...... .......... .......... ......
Lc   AVAMRLLTKM MNKEEIEEKT VMLGFDILKR GSTK*.
Sx   AVGMRLLTKY MNEEDIDEPN VILPHRIEYR GTTK*.
Cons AV-M------ ---------- ---------- ------
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Fig.2. Alignment of amino-acid sequences of *L. pentosus* MD353 *ccpA* gene product (Lp) with CcpA's of *L. casei* (Lc) (G. Perez-Martinez, unpublished), *B. subtilis* (Bs) (Henkin *et al.*, 1991), *B. megaterium* (Bm) (Hueck *et al.*, 1994) and *S. xylosus* (R. Brueckner, unpublished results). The consensus sequence (Cons) represents identical residues in the respective proteins. The predicted α-helix-turn-α-helix region is indicated (H-T-H).

Expression of the *ccpA* gene. To analyze transcription of the *L. pentosus ccpA* gene, RNA was isolated from *L. pentosus* MD353 cells cultivated in the presence of 1% glucose or 1% xylose, as described in Materials and Methods. The induction time was 2 hours. Transcription of the *ccpA* gene was analyzed on Northern blots using the *ccpA* PCR fragment as a probe (Fig. 3). Under both conditions, a transcript of about 10 kb in size was detected, suggesting that the *L. pentosus ccpA* gene is part of an operon which is constitutively expressed. Furthermore, in the presence of glucose a 1 kb transcript was formed. The size of this transcript is in agreement with that of the *ccpA* gene. In addition, a very weak hybridization signal could be detected in the presence of xylose that was slightly larger than that of the 1 kb transcript in glucose medium. The same results were obtained when *ccpA* transcription of *L. pentosus* MD363 was analyzed (not shown).

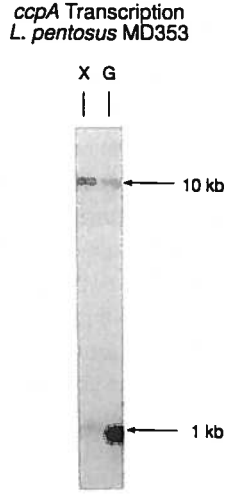

Fig.3. Northern blot analysis of *L. pentosus* MD353 RNA, isolated from cells cultivated in the presence of 1% xylose (X) and in the presence of 1% glucose (G). The 875 bp PCR fragment containing a part of the *L. pentosus ccpA* gene was used as a probe.

Disruption of the chromosomal *ccpA* gene. To analyze the function of the *ccpA* gene in more detail we have disrupted the gene on the chromosome. Our first approach was to disrupt the gene by using the temperature-sensitive shuttle plasmid pIN15E, as described for the disruption of the *L. pentosus xylR* gene (Lokman *et al*, 1996). For unknown reasons we were not successful in disrupting the *ccpA* gene in this way and therefore we have used the suicide *E. coli* vector pEI2 (Posno *et al.*, 1991). This vector, a derivative of pUC19 in which an erythromycin gene was cloned, is not able to replicate in Gram-positive bacteria. For direct integration into the chromosomal *ccpA* gene the PCR fragment was cloned into pEI2, yielding plasmid

transformation with autonomously replicating vectors (Leer *et al.*, 1993) we decided to use the better transformable *L. pentosus* MD363. Southern blot and Northern blot analyses had demonstrated that *L. pentosus* MD353 and *L. pentosus* MD363 are indistinguishable with respect to organisation and expression of the *xyl* genes (not shown). In total 10 erythromycin resistant colonies appeared after transformation. Southern blot analysis showed that in all transformants the *ccpA* gene was disrupted (not shown). A schematic representation of the integration is depicted in Fig. 4.

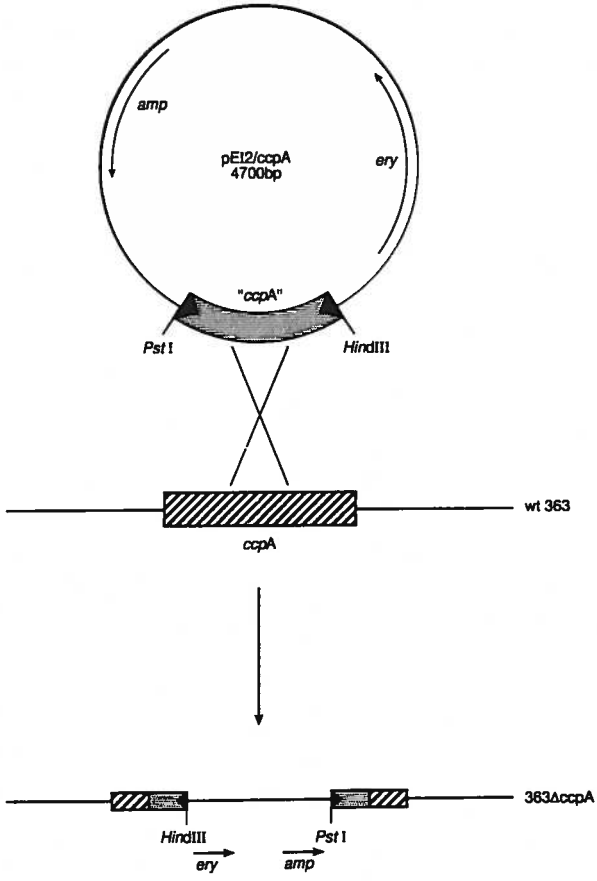

Fig.4. Schematic representation of the integration of plasmid pEI2/ccpA into the *L. pentosus* chromosome. On the top: plasmid pEI2/ccpA harbouring the 875 bp PCR "ccpA" fragment presumably missing 45 bp at the 5'-end and 90 bp at the 3'-end, the ampicillin resistance gene (*amp*) for selection in *E. coli* and the erythromycin resistance gene (*ery*) for selection in *Lactobacillus*. In the middle: part of the chromosomal DNA of *L. pentosus* containing the *ccpA* gene. Bottom: Chromosomal map after integration of pEI2/ccpA by a single cross-over into the chromosome. Two truncated copies of the *ccpA* gene are present.

The disruption mutant contained two truncated copies of the *ccpA* gene, one presumably missing 90 bp at the 3'-end and one presumably missing 45 bp at the 5'-end. The copy missing a part of the 3'-end is not expected to be functional anymore since in *Bacillus megaterium* a C-terminal deletion of 7 amino acids resulted in an

inactivated protein (A. Kraus, personal communication). The second truncated gene is deprived from its promoter and is missing a part of the putative HTH-motif for DNA binding, therefore functionality of this copy can also be excluded. The mutant is designated 363ΔccpA.

Growth behaviour of 363ΔccpA. The growth behaviour of the *ccpA* mutant was analyzed in M-medium with 1% glucose, 1% xylose, or 1% glucose plus 1% xylose, and was compared to that of the wildtype strain. Under the three conditions, the growth rate of the *ccpA* mutant was the same as that of wildtype bacteria, but the length of the lag-phase had almost doubled (not shown).

Effect *ccpA* disruption on *xylA* expression. RNA of *L. pentosus* MD363 wildtype and the *ccpA* mutant, 363ΔccpA, was isolated after two hours cultivation in the presence of glucose, xylose, or glucose plus xylose, as described in Materials and Methods. Northern blots were hybridized with a 600 bp *xylA* DNA fragment under homologous hybridization conditions. Fig. 5 shows clearly the strong repression of wild-type *xylA* transcription when glucose is present together with xylose. This repression is completely relieved in the *ccpA* mutant. Furthermore, in the *ccpA* mutant a significant increase in *xylA* transcription, compared to wildtype bacteria, was observed in the presence of xylose or glucose plus xylose. Neither in wildtype nor in the *ccpA* mutant, *xylA* transcription was detected in the absence of xylose, suggesting that xylose is required for *xylA* expression.

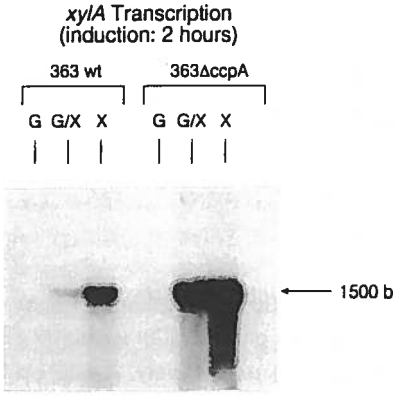

Fig.5. Northern blot analysis of RNA from *L. pentosus* MD363 and 363Δ*ccpA*, isolated after two hours cultivation in the presence of 1% glucose (G), 1% glucose plus 1% xylose (G/X), 1% xylose (X) respectively. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

To determine whether the CcpA-mediated repression of *xylA* is specific for glucose, we have also analyzed RNA that was isolated from cells cultivated for 24 hours in the presence of fructose, glucose, xylose, fructose plus xylose, or glucose plus xylose, respectively. In Fig. 6 the difference between the effects of glucose and fructose in the medium is clearly demonstrated. In the wild-type strain equal amounts of *xylA* transcript could be detected in the presence of xylose or xylose plus fructose. Apparently, fructose does not repress *xylA* transcription whereas glucose does. In the *ccpA* mutant, some repression by glucose was observed after 24 hours incubation in medium containing glucose plus xylose. This repression was abolished when fructose was used instead of glucose. In the presence of fructose but absence of xylose no transcription of *xylA* could be detected, confirming the conclusion that xylose is required for the induction of *xylA*.

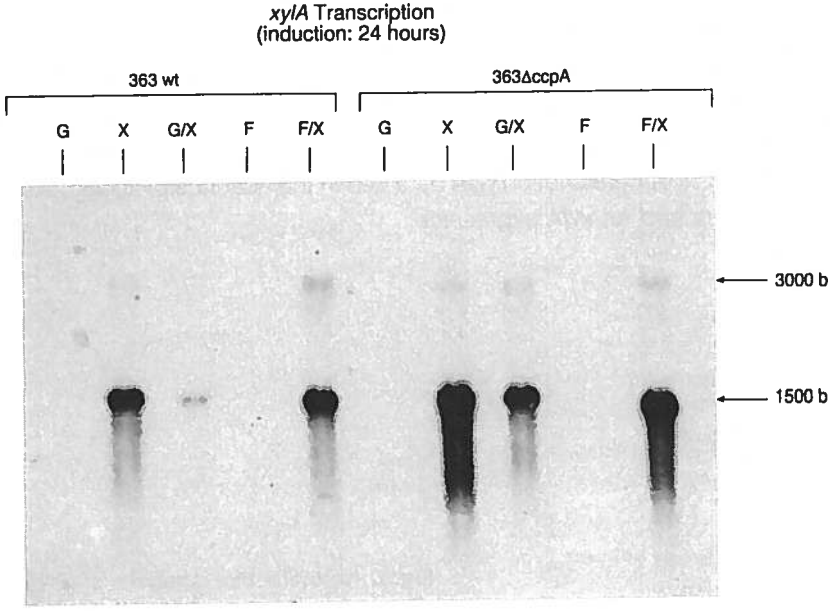

Fig.6. Northern blot analysis of RNA isolated from *L. pentosus* MD363 and 363Δ*ccpA* after 24 hours cultivation in the presence of 1% of the indicated sugars: glucose (G), xylose (X), glucose plus xylose (G/X), fructose (F), fructose plus xylose (F/X) respectively. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

Effect *ccpA* disruption on the α-amylase promoter of *L. amylovorus*. Expression of the α-amylase gene is repressed by glucose but not by galactose or cellobiose, both in *L. amylovorus* and in *L. casei* (J. Jore and B. Chassy, personal communication). To verify whether CcpA is involved in glucose repression of the α-amylase promoter, we have determined the effect of disruption of the *ccpA* gene on expression of the α-amylase promoter of *L. amylovorus*. For this purpose we have introduced plasmid pRB-αamy in *L. pentosus* MD363 and in the *ccpA* mutant 363ΔccpA. Plasmid pRB-αamy contains a part of the *L. amylovorus* α-amylase gene, including its own promoter, followed by a promoterless chloramphenicol acetyltransferase gene (CAT). In Fig. 7 the promoter region of the α-amylase gene of *L. amylovorus* is depicted. As found for the *L. pentosus xylA* promoter, the -35 sequence partly overlaps with a sequence (CRE1) that shows homology with the consensus sequence for glucose repression (Cons CRE) proposed by Weickert and Chambliss (1990). A second putative CRE was identified downstream of the -10 promoter sequence (CRE2).

```
                          *                                   *     *
Cons CRE      TGWAANCGNTNWCA                                TGWAANCGNTNWCA

                        -35                                 -10
TTTTCGATTTTTATGAAAACGGTTGCAAAGAAGTTAGCAAAAATATATAATTTCTTTTGAAATTGTTCACTTGGC
                    CRE1                                          CRE2

CAAGCTGCAGTTTCAATATTTTAATAAAGGGGGCAGTAAAAAGTG
                              RBS          START α-Amy
```

Fig.7. Promoter region of the *L. amylovorus* α-amylase gene. The -35 and -10 sequences are overlined. The ribosome binding site (RBS), translation startcodon, and putative catabolite responsive elements (CRE) are underlined. Asterisks indicate deviations from the consensus CRE sequence proposed by Weickert and Chambliss (1990). W: A or T.

CAT activity was analyzed after cultivation of wildtype 363 and 363ΔccpA, both harbouring plasmid pRB-αamy, in the presence of glucose or galactose. The results are depicted in Table 1. Strong repression (89%) was observed when the *L. pentosus* transformant harbouring pRB-αamy was cultivated in the presence of glucose. This repression was reduced to 27% when the *ccpA* gene was deleted. This result clearly

transcription of the *cat* gene directed by the promoter sequences of the α-amylase gene of *L. amylovorus*.

	CAT activity ($U.mg^{-1}$)		
Strain	Glucose	Galactose	Repression (%)
L.pentosus/pRBE1	<0.01	<0.01	-
L.pentosus/pRBαamy	0.03	0.26	89
*L.pentosus*Δ*ccpA*/pRBαamy	0.16	0.22	27

Table 1. Chloramphenicol acetyltransferase (CAT) activities of *L. pentosus* MD363 and strain 363ΔccpA harbouring the indicated plasmids. Average CAT activities of two independent determinations are given. The energy sources used in the growth medium are indicated.

DISCUSSION

The presence of a *ccpA*-like gene in *L. pentosus* MD353 was first demonstrated in a Southernblot of chromosomal DNA using a *B. megaterium ccpA* fragment as a probe. Part of the *ccpA* gene was subsequently isolated by PCR using primers based on the *B. megaterium ccpA* gene. Sequence analysis confirmed that the isolated fragment was indeed derived from the *ccpA* gene since the deduced amino acid sequence of the 875 bp PCR product was highly homologous to CcpA's of *B. subtilis*, *B. megaterium*, *S. xylosus* and *L. casei*. Transcription analysis of the *ccpA* gene showed that in the presence of glucose or xylose the gene is part of an operon (10 kb) and is constitutively expressed. Miwa *et al.* (1994) demonstrated that the CcpA

protein in *B. subtilis* is constitutively synthesized as well. Moreover, in *B. megaterium* and *B. subtilis*, *ccpA* is also part of an operon. Downstream of *ccpA* in these two bacilli two open reading frames (ORF) have been identified that show homology to the *motA* and *motB* genes of *B. subtilis*, respectively (Hueck *et al.*, 1994b). Disruption of the two *Bacillus* ORF's has demonstrated that they are not involved in catabolite repression (C. Hueck, personal communication). Since *mot* genes are involved in flagellar rotation and *L. pentosus* lacks flagella, it is unlikely that the other genes of the operon represent *mot* genes. In the presence of glucose as well as in the presence of xylose a *ccpA* transcript of approximately 1 kb was formed in *L. pentosus*. In the presence of glucose this transcript is slightly smaller indicating either the use of different transcription start- or termination points, or a different processing of the 10 kb transcript. The small difference in size of the *ccpA* mRNA in different media might suggest either control in expression levels of *ccpA* or a different function under different conditions. A dual function for the CcpA protein of *B. subtilis* has also been reported. Beside the repressor function, *B. subtilis* CcpA is involved in activation of transcription of the acetate kinase gene, in the presence of glucose (Grundy *et al.*, 1993). Since much more 1 kb transcript was formed in the presence of glucose than in the presence of xylose, we assume that CcpA translated from this smaller transcript, is the active catabolite repressor protein. Transcript analysis of *ccpA* in other organisms has not been reported sofar.

To verify the hypothesis that a part of the *L. pentosus* MD353 *ccpA* gene had been cloned and that CcpA is involved in catabolite repression, we disrupted the *ccpA* gene of *L. pentosus* MD363. Growth analysis of two independent mutants demonstrated that the duration of the lag-phase had almost doubled, but the doubling time was similar to that of wild-type bacteria (not shown). In contrast, greatly impaired growth was observed for a *B. megaterium ccpA* deletion mutant in the presence of a variety of energy sources, such as glucose, fructose, glucitol, and glycerol (Hueck *et al.*, 1995). *B. subtilis ccpA* mutants exhibited a complete growth defect on minimal medium with glucose as sole carbon source. This defect was partially restored by addition of citrate or other tricarboxylic acid (TCA) cycle intermediates (Wray *et al.*, 1994). Hueck and Hillen (1995) proposed that the growth defects indicate that CcpA might specifically influence the upper part of the glycolytic pathway which may, in turn, lead to CR. Interestingly, growth of a *B. megaterium ccpA* deletion strain on xylose, which is degraded *via* the pentose phosphate cycle, was almost identical to that of wildtype bacteria (Hueck and Hillen, 1995). Since *L. pentosus* can ferment glucose either by glycolysis or by the pentose phosphate cycle (Kandler, 1983), the absence of growth defects of *ccpA* mutants in glucose and xylose medium suggest that in this

Analysis of *xylA* transcription in the *L. pentosus ccpA* mutant showed the involvement of CcpA in CR. Similar amounts of *xylA* transcript were detected in the *ccpA* mutant strain in the presence of glucose plus xylose or xylose only. In contrast, the presence of glucose strongly repressed *xylA* transcription in wild-type *L. pentosus* (Fig. 5). Furthermore, compared to the wild-type strain, *xylA* transcription of the *ccpA* mutant was increased in the presence of xylose, indicating that CcpA is partially repressing *xylA* transcription even under inducing conditions. It is interesting to note that the amount of 1 kb *ccpA* mRNA is greatly reduced when cells have been cultivated in xylose containing medium.

In the presence of glucose or fructose no *xylA* transcript could be detected after 24 hours of cultivation of the *ccpA* mutant, confirming the conclusion that xylose is required for transcription of *xylA* (Lokman *et al.*, 1996). After 24 hours of induction some repression was observed by glucose in the *ccpA* mutant (compare xylose *vs* xylose plus glucose, (Fig. 6). This repression is specific for glucose as no repression was observed when fructose was present together with xylose. In *B. subtilis* some repression by fructose was observed but it was greatly reduced compared to glucose repression (factor 2 *vs* 18). Fructose repression was relieved in a *B. subtilis ptsH* mutant in which serine on position 46 was replaced by alanine, whereas only a minor reduction in glucose repression was observed in this mutant. Mutation in the *B. subtilis xylR* gene indicate that full glucose repression not only requires CcpA and HPr, but also XylR (Dahl and Hillen, 1995). In *B. subtilis* the XylR mediated effect was shown to be specific for glucose and did not occur with fructose or glycerol (Kraus *et al.*, 1994; Dahl and Hillen, 1995). The results with the *L. pentosus ccpA* mutant also suggest the involvement of an additional factor in CR exerted by glucose. Involvement of XylR in catabolite repression, as proposed for *B. subtilis* is not to be expected since no effect of glucose on *xylAB* transcription was found in an *L. pentosus xylR* deletion mutant (Lokman *et al.*, 1996). That fructose showed no repression of *xylA* transcription, in contrast to glucose, may at first sight seem surprising, as other PTS sugars beside glucose are expected to show catabolite repression of *xyl* gene expression in *L. pentosus*. Fructose was considered a PTS sugar in *L. pentosus*, based on a comparison with other Gram-positive bacteria (Thompson, 1988). In addition, *L. pentosus*, being a facultative heterofermentative organism, expresses the enzymes of the Embden-Meyerhof pathway constitutively. The lack of repression by fructose may, however, be explained by assuming that the enzymes of the fructose PTS are only induced under anaerobic conditions, as was observed for *L. brevis* (Saier *et al.*, 1996a). .

Beside expression of the *xyl* operon, CcpA also regulates expression of the α-

amylase gene of *L. amylovorus*. Inspection of the nucleotide sequence of the promoter region of α-amylase has revealed the presence of two potential CRE's, one overlapping the -35 sequence (CRE1) and a second one (CRE2), immediately downstream from the -10 sequence (Fig. 7). In CRE2 two bases differ from the consensus sequence whereas in CRE1 only one mismatch was identified. Weickert and Chambliss (1990) showed with site-directed mutagenesis of the catabolite repression operator sequence of the *B. subtilis* α-amylase gene that most double substitutions severely reduce the repression ratio. Moreover, the C and G on position 7 and 8 of the consensus CRE are strongly conserved in all CRE's of Gram-positive bacteria identified sofar (Hueck *et al.*, 1994a). Therefore we assume that CRE1 is the *cis*-active sequence mediating catabolite repression of the *L. amylovorus* α-amylase gene in the presence of glucose.

Disruption of the *L. pentosus ccpA* gene reduced glucose repression of the chloramphenicol acetyltransferase gene under control of the α-amylase promoter of *L. amylovorus*, from 89% to 27% (Table 1). Also in a *B. subtilis ccpA* mutant α-amylase expression is 50% repressed in the presence of glucose. Furthermore, in *B. subtilis* catabolite repression of inositol dehydrogenase and histidase was only partially affected by a *ccpA* mutation, implying the presence of other CR control mechanisms, beside the one involving CcpA (Henkin *et al.*, 1991). The presence of a second catabolite control protein, CcpB, was recently described (Saier *et al.*, 1996b). This protein exhibits 30% sequence identity with CcpA. CcpA and CcpB were suggested to function in parallel in response to HPr(ser-P) concentrations, to allow the catabolite repression phenomenon to be more sensitive to environmental conditions.

In summary, this report clearly demonstrates that catabolite repression in *L. pentosus* and *L. amylovorus* involves CcpA. However, the specific role of CcpA in catabolite repression remains to be established.

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CHAPTER 5

Regulation of the *Lactobacillus pentosus xylAB*-operon by XylR

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SUMMARY

Expression of the *xyl* operon of *Lactobacillus pentosus* is regulated at the transcriptional level by XylR and by factors involved in catabolite repression. Introduction of a plasmid carrying the *xyl* operator or disruption of the chromosomal *xylR* gene of *L. pentosus* demonstrated that XylR is functional as a repressor in the presence and absence of xylose. Disruption of *xylR* resulted in constitutive expression of *xylA*, and showed that glucose repression does not require XylR. The *xylR* mutant displayed a prolonged lag phase and increased generation time compared to wild-type bacteria. For *L. pentosus* wild-type or *xylR* mutant bacteria no correlation was found between the growth rate and *xylA* transcription, in contrast to *Lactobacillus casei* harbouring a plasmid with *xylAB*, suggesting that the uptake of xylose is regulated in *L. pentosus* but not in *L. casei*. The role of XylR in regulation of xylose utilization will be discussed.

INTRODUCTION

Xylose utilization in bacteria involves the transport of xylose into the cell, the isomerization to xylulose, the conversion of xylulose to xylulose-5-phosphate, followed by further degradation resulting in lactate and acetate as final products. At least five

are encoded by the xylose regulon, comprising *xylP*, *xylQ*, *xylR*, *xylA* and *xylB*, which encode a putative xylose permease, a protein with unknown function, the regulatory protein of the regulon, xylose isomerase and xylulose kinase, respectively (Lokman *et al.*, 1991 and 1994). Expression of the *xyl* genes is induced by xylose and repressed by glucose, which is regulated at the transcriptional level. The deduced amino acid sequence of XylR is homologous to that of XylR of *Bacillus subtilis* (Kreuzer *et al.*, 1989), *Bacillus megaterium* (Rygus *et al.*, 1991), *Bacillus licheniformis* (Scheler *et al.*, 1991), and *Staphylococcus xylosus* (Sizemore *et al.*, 1991). In these organisms the repressor function of XylR has been unambiguously demonstrated (Kreuzer *et al.* 1989, Kauder *et al.*, 1993, Scheler and Hillen, 1994, Sizemore *et al.*, 1992). Recent studies have established that XylR of *B. subtilis* is not only involved in repression of the *xyl* operon in the absence of the inducer xylose, but also in catabolite repression (Dahl and Hillen, 1995; Kraus *et al.*, 1994).

Based on the sequence homology with the Xyl repressors described above and the presence of an operator-like element downstream of the *L. pentosus xylA* promoter, we assumed that *L. pentosus* XylR also functions as a repressor protein (Lokman *et al.*, 1991). However, some differences in the expression of *xylR* between *L. pentosus* and the other bacterial species have been noticed. Northern blot analyses e.g. showed that *L. pentosus xylR* is constitutively expressed from its own promoter, whereas under inducing conditions it is also part of a larger transcript. Beside *xylR*, this transcript comprises *xylP* and *xylQ*. Based on a comparison of the amounts of transcript, there is at least ten-fold more XylR under inducing conditions than under non-inducing conditions (Lokman *et al.*, 1994). Moreover, in the presence of xylose introduction of multiple copies of a DNA fragment, containing the *xylR-xylA* intergenic region with the putative *xyl*-operator and flanking sequences into *L. pentosus* resulted in a five- to ten-fold increase of *xylA* expression. This suggests that a negative regulator, probably XylR, even under inducing conditions binds to the operator (Lokman *et al.*, 1994). On the other hand, the presence of XylR under inducing conditions might also suggest a dual function, a repressor in the absence and an activator in the presence of xylose (Lokman *et al.*, 1994). In *S. xylosus* the *xylR* gene is also constitutively expressed, whereas in *B. megaterium* and *B. licheniformis xylR* is inducible by xylose (Sizemore *et al.*, 1991; Rygus *et al.*, 1991; Scheler *et al.*, 1991).

We have extended our studies of the role of XylR in regulation of expression of the *xylAB* operon, by analysing the effect of XylR on the growth of *L. pentosus* in xylose medium, and determining the transcription of *xylAB* at various growth stages. We have performed similar studies in a *Lactobacillus* strain which cannot ferment xylose, *L. casei*, after introduction of the *xyl* genes.

MATERIALS AND METHODS

Bacterial strains and media. *L. pentosus* MD353 (Lokman *et al.*, 1991) was used as a host for the titration experiments and *L. pentosus* MD363 (Lokman *et al.*, 1996) was used for the construction of a *xylR* mutant strain. *E. coli* JM109 was used for the construction of integration vectors. *L. casei* ATCC 393 (Chassy and Flickinger, 1987) was used as a D-xylose non-fermenting *Lactobacillus* strain in complementation experiments. *Lactobacillus* strains were routinely cultivated at 37 °C in MRS medium (Difco) or in M-medium as described by Lokman *et al.* (1994). The following energy sources were used at a final concentration of 1% (w/v): glucose, xylose, or a combination of 1% xylose with 1% glucose. For plating, media were solidified with 1.5 % agar.

Plasmids and plasmid constructions. The plasmids used in this study are listed in Table 1. Plasmid pIN15E was constructed by cloning the multicloningsite of pGEM-3, present on a *Hin*dIII-*Sca*I fragment, into the temperature sensitive shuttle plasmid pWH1509E (Schmiedel and Hillen, 1996; Rygus and Hillen, 1992). Integration vector pIN15E-xylR was constructed as follows. PCR was used to obtain a 404 bp *xylR* fragment, missing 178 bp at the 5'-end and 582 bp at the 3'-end of the gene. Since the primers contained a *Pst*I and *Hin*dIII restriction-site respectively, the PCR-product was suitable for direct cloning into pIN15E. The resulting plasmid, pIN15E-xylR, was used to disrupt the chromosomal *xylR* gene by a single cross-over event. As described previously, pLP3537-17 contains the *xylR* gene which harbours a deletion of 220 bp followed by the *xylR-xylA* intergenic region and the first 795 bp of *xylA* (Lokman *et al.*, 1994). Plasmid pLP3537-xylΔR was made by cloning the 3.7 kb *Hin*dIII fragment of pXH37A (Lokman *et al.*, 1991), containing the remaining 3' part of *xylA* followed by the complete *xylB* gene, into pLP3537-17. In this way an intact *xylA* gene was restored. Plasmid pLP3537HP45 was constructed by cloning the 2.4 kb *Pst*I-*Hin*dIII fragment from pXH50A (comprising the *xylR* gene, the *xylR-xylA* intergenic region, and the first 795 bp of the *xylA* gene), into pLP3537 (Posno *et al.*, 1991a; Posno *et al.*, 1991b). Plasmid pLP3537-9 was constructed by cloning a PCR fragment, containing the *xylR-xylA* intergenic region followed by the first 180 nt of *xylA* into pLP3537.

Chromosomal integration. After transformation of *L. pentosus* MD363 by electroporation (Lokman *et al.*, 1994) with pIN15E-xylR, colonies were selected on MRS plates with 5 μg/ml erythromycin at 30 °C. Two individual transformants

harbouring the correct plasmid were used for the chromosomal integration as follows. MRS medium with 2.5 μg/ml erythromycin was inoculated with cells from the respective transformants. Cultures were incubated for 5 hours at the non-permissive temperature of 38 °C, followed by temperature shifts of 2 hours at 42 °C and 1 hour at 38 °C. Dilutions of the cultures were plated on MRS plates containing 2.5 μg/ml erythromycin and were incubated for 16 hours at 38 °C. Southern blot analysis of chromosomal DNA isolated from the putative integrants showed that approximately 80% of the colonies contained the disrupted *xylR* gene.

Plasmids	Selection markers	Source of reference
pWH1509E	Ap^r;Ery^r;Tc^r	Rygus and Hillen, 1992; Schmiedel and Hillen, 1996
pGEM-3	Ap^r	Promega
pIN15E	Ap^r;Ery^r	This study
pIN15E-xylR	Ap^r;Ery^r	This study
pLP3537	Ap^r;Ery^r	Posno *et al.*, 1991a
pLP3537-9	Ap^r;Ery^r	This study
pLP3537-17	Ap^r;Ery^r	Lokman *et al.*, 1994
pLP3537-xyl	Ap^r;Ery^r	Posno *et al.*, 1991b
pLP3537-xylΔR	Ap^r;Ery^r	This study
pLP3537-xyl*	Ap^r;Ery^r	This study
pLP3537HP45	Ap^r;Ery^r	Posno *et al.*, 1991b
pXH37A	Ap^r	Lokman *et al.*, 1991
pXH50A	Ap^r	Lokman *et al.*, 1991

Table 1. Plasmids used in this study (Ap, ampicillin; Ery, erythromycin; Tc tetracycline).

DNA isolation. *Lactobacillus* plasmid DNA isolation was performed according to Posno *et al.* (1991a), and chromosomal DNA isolation was as described by Lokman *et al.* (1991).

RNA isolation. RNA was isolated as described by Pouwels *et al.*, (1994). Two different cultivation procedures were used. Procedure 1, an induction procedure, was also described by Lokman *et al.*, (1996): 10 ml of an overnight culture in MRS was harvested and washed twice with M-medium without sugar ($M^{(-)}$-medium). For induction, 10 ml M-medium with 1% glucose and/or 1% xylose was 1:3 inoculated with the washed cells. After 2 hours incubation at 37 °C cells were harvested and RNA was isolated. Procedure 2: M-medium with 1% xylose was inoculated 1:100 with washed cells of an overnight culture precultivated in the presence of glucose. RNA was

isolated directly at different time points during growth.

Other techniques. Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, DNA and RNA transfer to Hybond N filters and filter hybridizations were performed according to Sambrook *et al.* (1989). Nucleotide sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the T7 DNA polymerase sequencing system of Pharmacia and [α-^{35}S]dATP (Amersham).

RESULTS

Titration experiments in *L. pentosus* MD353. Previously we have demonstrated that in the presence of xylose one or more repression factors are titrated when the *xylR-xylA* intergenic region and flanking sequences is introduced on a multi-copy plasmid in *L. pentosus* MD353. Two elements in the *xylR-xylA* intergenic region are probably involved in binding repression factors. The first element is overlapping with the -35 sequence of the *xylA* promoter and is involved in catabolite repression (Lokman *et al.*, 1994; Lokman *et al.*, 1996). The second element is located downstream of the -10 promoter sequence of *xylA* and is the putative *xyl*-operator, presumably interacting with XylR in the absence of xylose (Lokman *et al.*, 1994). To investigate whether XylR and/or another factor is titrated under inducing conditions, we repeated the titration experiments by transforming *L. pentosus* MD353 with a plasmid containing the *xylR-xylA* intergenic region and *xylR* (pLP3537HP45), and with a plasmid harbouring the intergenic region only (pLP3537-9). RNA was isolated from wildtype bacteria and from these transformants after cultivation in xylose. Transcription of *xylA* was analyzed on a Northern blot using an internal *xylA* DNA fragment as probe. In the presence of xylose an increase in *xylA* transcription was observed with the transformant harbouring the plasmid lacking *xylR*, but not with the *xylR* bearing plasmid (Fig. 1).

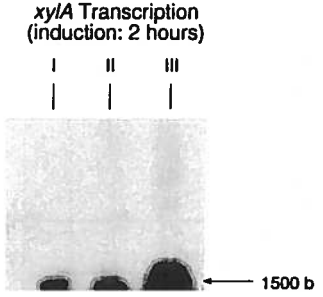


Fig.1. Northern blot analysis of *xylA* synthesized under inducing (1% xylose) conditions (procedure 1, see Materials and Methods). I, *L. pentosus* MD353; II, *L. pentosus* transformant harbouring plasmid pLP3537HP45; III, *L. pentosus* transformant harbouring plasmid pLP3537-9. A 600 bp

This result indicates that in the presence of xylose XylR is the only factor that is titrated, demonstrating that this protein is also active as a repressor under inducing conditions.

Disruption of the chromosomal *xylR* gene. To further study the role of *xylR* in regulating the expression of the *xylAB* operon, we have disrupted the gene on the chromosome. For this purpose, we have used a plasmid which carries a temperature-sensitive origin of replication from pE194ts (Youngman, 1987) and which is able to replicate in *E. coli* and *Bacillus* at temperatures below 35°C (Rygus and Hillen, 1992). The vector and the relevant region of the chromosome before and after integration of the vector, are shown in Figure 2.

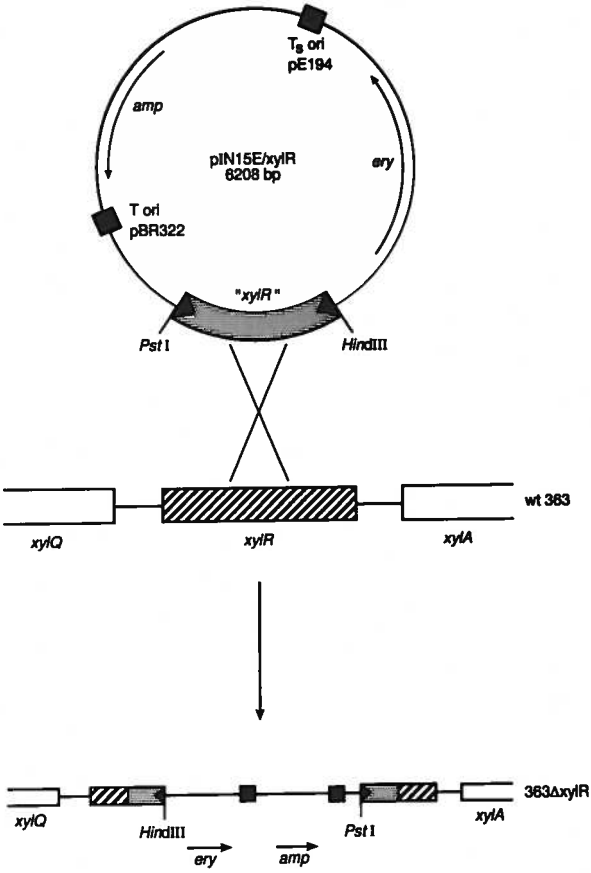


Fig.2. Schematic representation of the integration of plasmid pIN15E-xylR into the chromosome of *L. pentosus* MD363. Top: plasmid pIN15E/xylR harbouring the 404 bp PCR *xylR* fragment, the ampicillin recistance gene (amp) for selection in *E. coli*, the erythromycin resistance gene (ery) for selection in *Lactobacillus*, and the temperature sensitive origin of replication from pE194 (Ts ori). Middle: part of the chromosome of *L. pentosus* containing the *xylR* gene. Bottom:chromosomal map after integration of pIN15E/xylR by a single cross-over into the chromosome. Two truncated copies of the *xylR* gene are present; one missing 178 bp at the 5'-end and one missing 582 bp at the 3'-end.

For *xylR* disruption studies we have used the *L. pentosus* MD363 instead of strain MD353. Southern and Northern blot analysis had demonstrated that with respect to the organization and expression of the *xyl* genes, *L. pentosus* MD363 is the same as *L. pentosus* MD353 (not shown). The resulting mutant, designated 363ΔxylR, contains two truncated *xylR* genes, one missing 582 bp at the 3'-end and the other missing 178 bp at the 5'-end.

RNA analysis of the *xylR* deletion mutant. The effect of *xylR* disruption on *xylA* expression was analyzed using Northern blots with total RNA isolated from cells cultivated in medium containing glucose, xylose, and glucose plus xylose. As shown in Figure. 3, *xylA* transcription of 363ΔxylR also occurs in the presence of glucose, whereas in the wildtype strain no *xylA* transcript could be detected under non-inducing conditions. Furthermore, in the presence of xylose and absence of glucose, at least a five fold increase of *xylA* transcription was observed in the mutant strain. The constitutive expression of *xylA* in 363ΔxylR indicates that *xylR* codes for a repressor protein. The amount of *xylA* transcript in 363ΔxylR in the presence of glucose is less than that in 363wt in the presence of xylose, suggesting that glucose repression does not depend on XylR. Since repression of *xylA* transcription in 363ΔxylR in glucose medium is partly relieved, more transcript was expected in the presence of glucose plus xylose than in the presence of glucose only in the mutant strain. However, under both conditions a similar amount of *xylA* transcript was produced in 363ΔxylR.

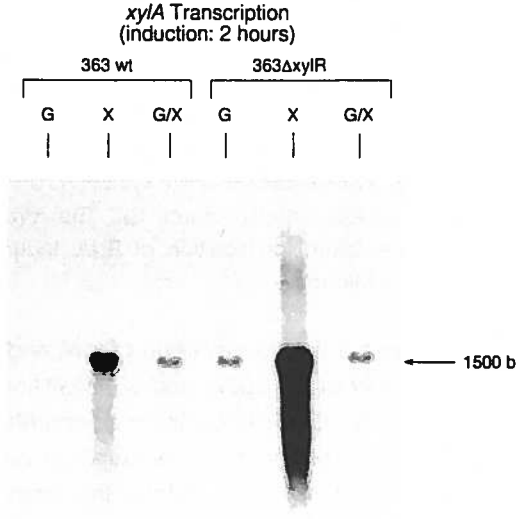


Fig.3. Northern blot analysis of RNA from *L. pentosus* MD363 (363 wt) and the *xylR* disruption mutant (363ΔxylR), isolated after 2 hours incubation in the presence of 1% glucose (G), 1% xylose (X), 1% glucose plus 1% xylose (G/X). Induction was according to procedure 1, see Materials and Methods. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

The effect of XylR on the growth and transcription of *xylAB* in the presence of xylose. To determine whether the observed effect of *xylR* disruption on *xylA* expression has an influence on xylose utilization, we have compared the growth behaviour of 363wt and 363ΔxylR. M-medium with 1% xylose was inoculated (1:100) with cells of an overnight culture in glucose containing medium. The wildtype strain showed a lag phase of ~48 hours whereas that of the *xylR* mutant was ~75 hours (Fig. 4). Although both strains reached the same final cell density, the generation time was increased from 9 hours (363wt) to 13 hours (363ΔxylR). In M-medium with 1% glucose the generation time of mutant and wild type bacteria was the same and no lag period was observed (results not shown).

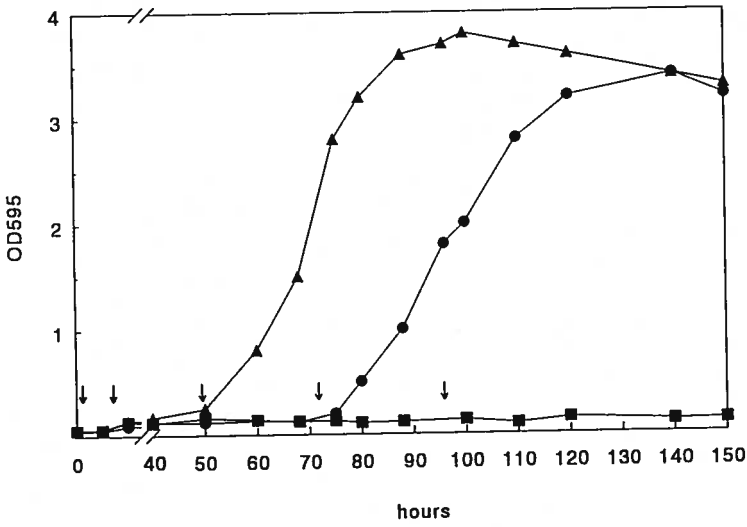


Fig.4. Growth curves of *L. pentosus* MD363 (363wt;▲) and the *xylR* disruption mutant (363ΔxylR;●)in the presence of 1% xylose. As a control *L. pentosus* MD363 was cultivated in M-medium without energy source (■). The arrows indicate the different points at which samples were taken for isolation of RNA. Induction was according to procedure 2, see Materials and Methods.

To determine the transcription of *xylA* and *xylB* at various stages of growth, RNA was isolated from the 363wt and 363ΔxylR cultures after 2, 24, 48, 72, and 96 hours of cultivation in xylose-containing medium. After 2 hours of cultivation, the 1500 b *xylA* and 3000 b *xylAB* transcripts were present in both, 363wt and 363ΔxylR, in a 1:1 ratio (Fig. 5). As was observed before, the amount of transcript was larger in the *xylR* deletion mutant. After 24 and 48 hours of cultivation, when cells are still not growing,

degradation of RNA was observed. As a consequence no *xylA* or *xylAB* transcript could be detected in the wildtype strain, and only weak *xylA* transcription was observed in 363ΔxylR after 24 hours. Transcription of *xylA* was detected again during growth, after 72 hours for the wildtype and after 96 hours for the *xylR* deletion mutant. During the exponential phase of growth at least 10 times more *xylA* transcript was detected in 363ΔxylR than in 363wt. However, the increased *xylA* transcription of 363ΔxylR did not result in better growth in xylose containing medium. Surprisingly, no *xylAB* transcript could be detected during the exponential phase of growth for both, 363wt and 363ΔxylR.

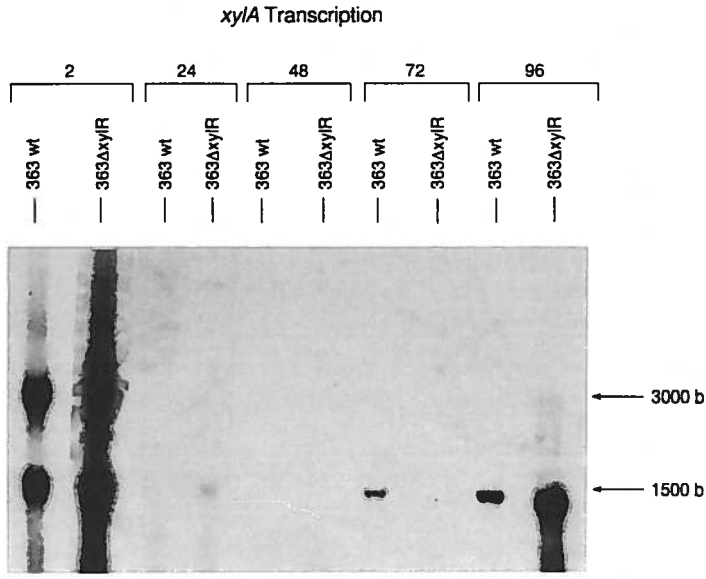


Fig.5. Northern blot analysis of RNA isolated from *L. pentosus* MD363 (363wt) and the *xylR* disruption mutant (363ΔxylR) after 2, 24, 48, 72, and 96 hours of cultivation in the presence of xylose (indicated in Fig.4). Induction was according to procedure 2, see Materials and Methods. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

Effect of low concentrations of glucose on growth of *L. pentosus* in xylose medium. *L. pentosus* ATCC 8041 (referred to in the cited reference as *L. plantarum* NCIMB 8026) is able to grow on D-xylose or D-ribose in the presence but not in the absence of glucose. It was suggested that the inability of *L. plantarum* NCIMB 8026 to grow on pentoses as a sole source of carbohydrate may be caused by the inability

(Westby, 1989; Westby *et al.*, 1993). To verify whether low concentrations of glucose would affect the lag period we have determined the growth behaviour of *L. pentosus* MD363 in the presence of 1% xylose, and 1% xylose plus 0.05% glucose. Figure 6 shows that the long lag phase in the presence of 1% xylose is largely reduced when glucose is also present in the medium. Moreover, as soon as the glucose has been depleted cells stop growing, despite the presence of xylose, implying a role of glucose in xylose uptake or catabolism. The ability to grow, albeit after a long lag period without added glucose (Fig. 4) indicates that an external C_6 carbohydrate is not essential for growth.

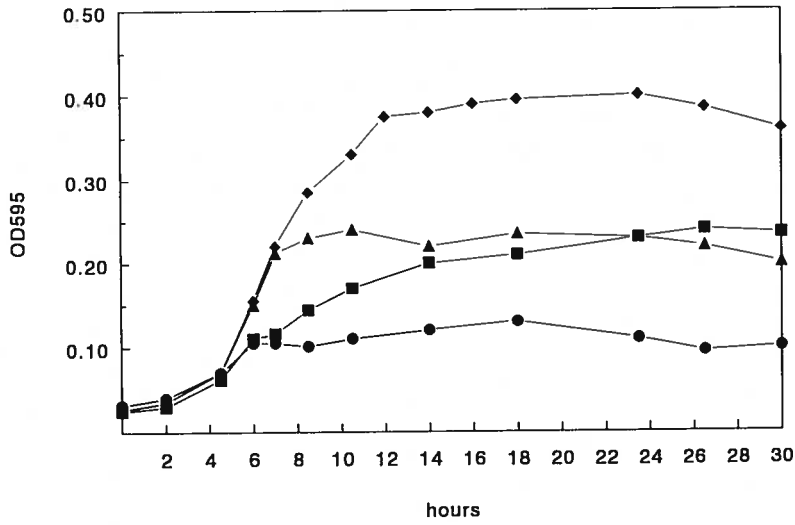


Fig.6. Growth analysis of *L. pentosus* MD363 in M-medium with 0.05% glucose (▲), 1% xylose (■), 0.05% glucose plus 1% xylose (◆), and without carbon source (●).

Functional analysis of *xylR* in *L. casei* 393. To better understand the function of XylR we analyzed *xylA* transcription and growth in the presence of xylose of *L. casei* cells harbouring a plasmid containing *xylRAB* (pLP3537-xyl) or a plasmid with *xylAB* only (pLP3537-xylΔR). Southern blot analysis revealed that *L. casei* 393 does not contain genes for xylose catabolism and is therefore unable to utilize xylose (data not shown). This inability can be complemented by the introduction of plasmid pLP3537-xyl (Posno *et al.*, 1991b). The resulting *L. casei* transformant is designated 393-xyl. As shown in Figure 7, 393-xyl grows very slowly on M-medium with 1% xylose. This is probably caused by the absence in *L. casei* of a xylose-specific transport mechanism.

An *L. casei* transformant harbouring the plasmid with a deleted *xylR*, designated 393-xylΔR, showed faster growth than 393-xyl and was probably able to more efficiently ferment xylose. No difference in growth behaviour was observed between wildtype bacteria and transformants in medium supplemented with glucose (not shown).

Interestingly, one of the *L. casei* 393 transformants harbouring pLP3537-xyl was able to grow much faster on M-medium with xylose. Transformation of *L. casei* 393 with the plasmid isolated from this fast growing transformant, designated 393-xyl*, yielded transformants with the same fast growing phenotype. This indicated that the different growth behaviour was plasmid encoded. Sequence analysis of the *xylR-xylA* intergenic region showed that in pLP3537-xyl* the -10 sequence of the *xylA* promoter has changed from TGTAAT to the optimal -10 sequence TATAAT (Graves and Rabinowitz, 1986).

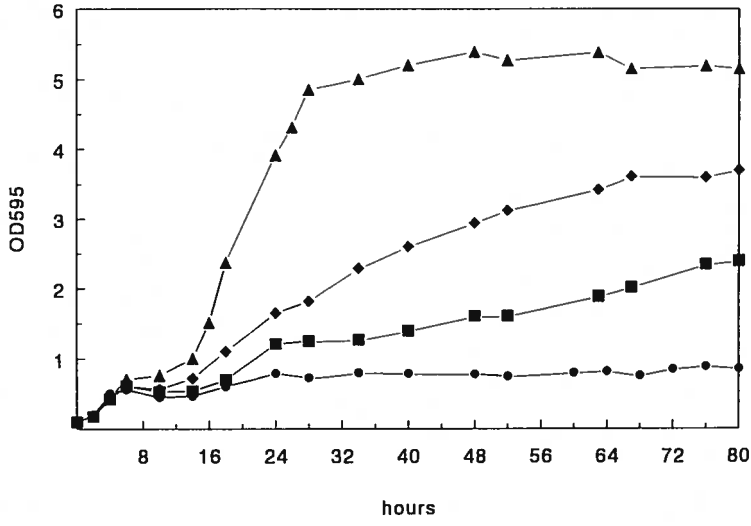


Fig.7. Growth analysis in the presence of 1% xylose of *L. casei* 393 (●) and *L. casei* transformants harbouring plasmid pLP3537-xyl (■), pLP3537-xylΔR (◆), pLP3537-xyl* (▲), respectively.

The results of the growth analysis are in agreement with those obtained with Northern blot analysis of RNA isolated from 393, 393-xyl, 393-xylΔR, and 393-xyl*. Figure 8a shows that the absence of a functional *xylR* leads to a five-fold increase of *xylA* transcription in the presence of xylose. Moreover, in 393-xylΔR weak *xylA*

transcription was observed in the presence of glucose (Fig. 8a). The results with 393-xyl* were comparable to those of 393-xylΔR.

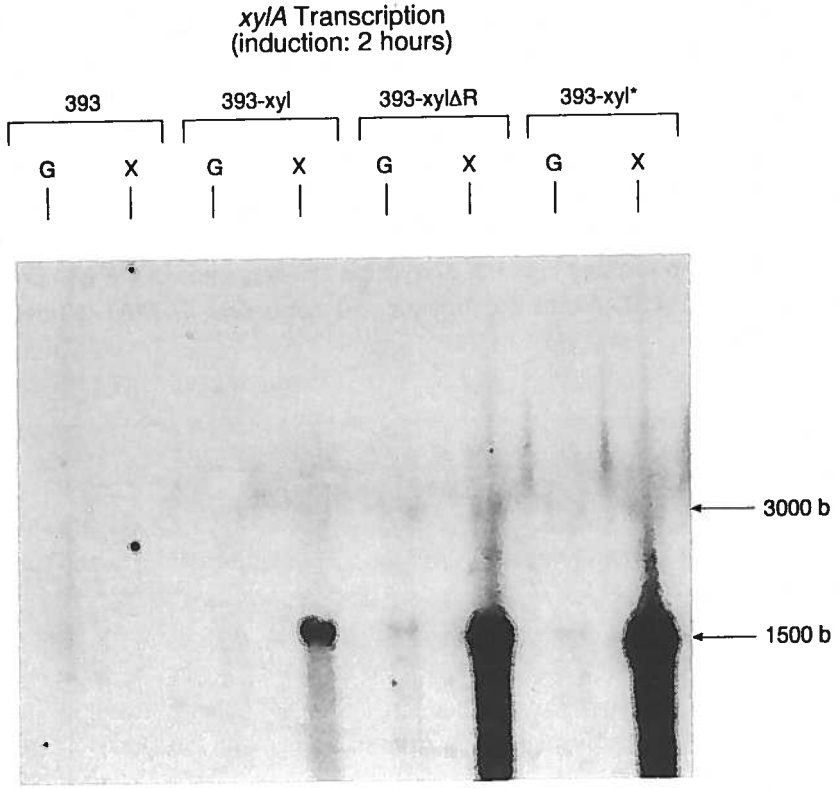
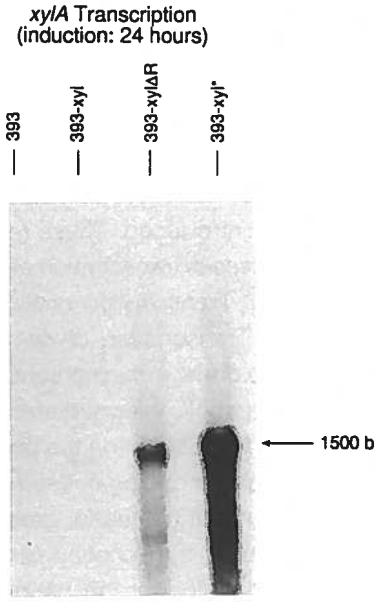


Fig.8a. Northernblot analysis (procedure 2) of RNA isolated after 2 hours cultivation in the presence of 1% glucose (G) and 1% xylose (X) from *L. casei* 393 (393) and *L. casei* transformants harbouring plasmid pLP3537-xyl (393-xyl), pLP3537-xylΔR (393-xylΔR), pLP3537-xyl* (393-xyl*), respectively.

A positive correlation between growth rate and *xylA* transcription is found by inspection of the results at 24 hours after induction by xylose (Fig. 8b). Almost no *xylA* transcript was observed in the transformant harbouring plasmid pLP3537-xyl, which corresponds to the very slow growth in xylose containing medium. The increased growth rate of 393-xylΔR compared to 393-xyl is accompanied by a considerable increase in *xylA* transcription. The highest amount of *xylA* transcript was obtained in *L. casei* transformants harbouring plasmid pLP3537-xyl*, showing the fastest growth (Fig. 8b). The correlation between growth rate and transcription of *xylA* indicates that

XylR is functional rather as a repressor than as an activator in this organism.



8b. Northern blot analysis (procedure 2) isolated after 24 hours cultivation in the presence of 1% xylose from *L. casei* 393 and transformants. Induction was according to procedure 2, see Materials and Methods. A 600 bp *Eco*RI *xylA* fragment was used as a probe.

DISCUSSION

Two elements, present in the *xylR-xylA* intergenic region of *L. pentosus*, appear to be involved in binding of repression factors in such a way that transcription of the *xylAB* operon is tightly regulated. One element is the operator of the *xylAB* operon, the target site for XylR (Lokman *et al.*, 1994). The second element has been implicated in catabolite repression (Lokman *et al.*, 1996). The titration experiments presented in this and in an earlier study (Lokman *et al.*, 1994) clearly show that under inducing (xylose) conditions XylR, but no other repression factor, is titrated when multiple copies of the *xylR-xylA* intergenic region are present. The observation that XylR is titrated under inducing conditions indicates that XylR functions as a repressor under inducing and non-inducing conditions.

Disruption of the chromosomal *xylR* gene of *L. pentosus* MD363 resulted in *xylA* transcription in the presence of glucose and absence of xylose. Similarly, a transcript

compared to the same plasmid comprising *xylR* in glucose medium. These results indicate that *xylR* encodes a repressor protein. No difference was observed between wildtype 363 and 363ΔxylR, in the amount of *xylA* transcript in the presence of glucose plus xylose, suggesting that glucose repression does not depend on a functional *xylR* gene. Similar observations have been made for the *S. xylosus xyl* operon (Sizemore *et al.*, 1992). The results indicate that glucose repression is mediated by a XylR-independent mechanism. One such mechanism involves the carbon catabolite repression protein, CcpA (Lokman *et al.*, 1996).

We have previously observed that transcription of *xylA* in *L. pentosus* is five- to ten-fold enhanced in xylose medium when multiple copies of the *xylR-xylA* intergenic region were introduced. These results have been interpreted by assuming that XylR partially represses transcription of the *xylAB* operon in the presence of xylose (Lokman *et al.*, 1994). Transcription analysis of the *xylR* deletion mutant has confirmed this hypothesis. Transcription of *xylA* in wild-type bacteria takes place at 10-20% of its maximal rate under inducing conditions, possibly because elevated concentrations of one of the catabolic intermediates of xylose are toxic to the cells (Lokman *et al.*, 1994).

XylR exerts an effect on the physiology of the cell as a *xylR* deletion mutant shows a considerably longer lag phase compared with wild-type bacteria. Despite constitutive expression of *xylA* no cellular growth takes place in xylose-containing medium until 96 hours. Evidently, transcription of *xylA* is not the limiting factor for growth under these conditions. The longer lag period of 363ΔxylR may be explained by an increased (constitutive) production of toxic metabolites. Alternatively, XylR may act as a repressor for (an)other gene(s), the function of which is to control expression of the *xyl* genes. A third possibility which has to be considered is that XylR also functions as an activator for the *xyl* genes. Induction of expression of a negative regulator has also been observed for other genes in other organisms (c.f. Discussion Lokman *et al.*, 1994). An activator function has also been demonstrated for the repressors of the *ara* operon and *fru* operon of *E. coli* (Schleif, 1987; Jahreis *et al.*, 1991). Furthermore, in *B. subtilis* the expression of the glucitol dehydrogenase gene (*gutB*) is suggested to be regulated both positively and negatively by GutR (Ye *et al.*, 1994). Several prokaryotic transcriptional activators, like *B. subtilis* LevR (Débarbouillé *et al.*, 1991) or GutR (Ye *et al.*, 1994), contain an ATP binding site. A putative ATP/GTP binding motif was also found in *L. pentosus* XylR (Fig. 9). This ATP/GTP binding motif was not found in XylR proteins that function solely as a repressor (Kreuzer *et al.*, 1989; Rygus *et al.*, 1991; Scheler *et al.*, 1994; Sizemore *et al.* 1991), lending support to the notion that *L. pentosus* XylR might also function as an activator.

When the *xyl* genes are expressed in *L. casei* on a multi-copy plasmid a positive

correlation was observed between the level of expression of *xylA* and the growth rate, suggesting that expression of *xylA* is the limiting factor for growth in this host. In addition, no lag phase is observed in this organism. This contrasts the situation in *L. pentosus* in which *xylA* transcription and growth are not correlated and which shows a considerable lag phase. The highest level of transcription was observed 2 hours after induction, during the lag phase, whereas a much lower level of *xylA* transcription was found during the exponential phase of growth. From 24 hours until the end of the lag phase, no *xylA* transcription could be detected. Moreover, degradation of ribosomal RNA was observed, suggesting that metabolism of the cells has stopped. That no correlation between *xylA* transcription and growth is observed in *L. pentosus*, in contrast to *L. casei*, may be explained by the presence in the former but not the latter organism of a regulator that controls the uptake of xylose into the cells rendering growth dependent on transport. Potential candidates for such regulatory proteins are XylP and/or XylQ that are encoded by the first two genes of the *xylPQR* operon (Lokman *et al.*, 1994).

		Motif A		Motif B	
		* * * * * * * * * * *		* * * *	
244	XylR	L T L L G P N T A G Q S	376	L G M V D	P21940
196	GutR	C L I T G W A G M G K T	284	L L I V D	P39143
174	Rho	G L I V A P P K A G K T	261	I I L L D	P03002
144	LevR	M L L L G P T G S G K S	222	I L F M D	P23914
30	GlnQ	V V I I G P S G S G K S	157	M M L F D	P10346
11	Adk	I F V V G G P G S G K G	89	G F L I D	P00568
6	Ras	L V V V G A G G V G K S	53	L D I K D	P01112
14	Ef-Tu	V G T I G H V D H G K T	76	Y A H V D	P02990
		G T			
consensus		h h h h X X X X G K		h h h h D	
		A S			

Fig.9. Comparison of ATP or GTP binding motifs (A and B) in XylR with those in other regulatory proteins. Residues of XylR that are identical with those in the consensus sequences are marked by asterisks. h indicate residues with hydrophobic side chains. X can be any amino acid. The accession number of each protein in the Swiss Protein Data Bank is given.

xylA mRNA is found as a monocistronic messenger and as a bi-cistronic messenger together with *xylB* RNA, in a ratio of 1:1, two hours after *L. pentosus* is

be detected during the exponential phase of growth or when glucose-grown bacteria were inoculated (1:3) in xylose medium and cultivated for 2 hours before RNA extraction. Under these conditions *xylB* mRNA might be more susceptible to nucleolytic degradation than *xylA* mRNA, or *xylB* might not be transcribed. The palindromic sequence between *xylA* and *xylB* which can form a stem-loop structure might control either the stability of *xylAB* mRNA (Lokman *et al.*, 1991) or its formation by a mechanism involving attenuation. An anti-terminator mechanism of regulation for the *xylAB* operon as described for the *bgl* and *sac* operon of *B. subtilis* is not very likely, since the palindromic structure and flanking sequences between *xylA* and *xylB* showed no homology with the specific RNA binding sequences involved in anti-termination (Le Coq *et al.*, 1995, Débarbouillé *et al.*, 1990, Houman *et al.*, 1990). If *xylB* is not expressed, then the question arises how xylulose is converted into xylulose-5-phosphate. An alternative mechanism for conversion of xylulose to xylulose-5-phosphate was described for mutants of *Klebsiella pneumoniae* (Neidhardt *et al.*, 1987). In this organism D-xylulose can be converted into D-arabitol by D-arabitol oxidoreductase. In the presence of D-arabitol *altK*, which is coding for a kinase and is able to phosphorylate D-xylulose, is induced. Determination of enzyme activities that phosphorylate xylulose may help to resolve these questions.

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CHAPTER 6

SUMMARY AND CONCLUDING REMARKS

During the past years considerable progress has been made in developing the techniques to genetically analyze strains of the genus *Lactobacillus*. However, except for the molecular cloning and characterization of several *Lactobacillus* genes, little is known about the regulation of gene expression. For strain improvement programmes detailed knowledge about how gene expression is regulated in *Lactobacillus* is needed. This thesis describes the regulation of expression of the xylose catabolizing genes of *Lactobacillus pentosus*, which serves as a model system for gene expression in *Lactobacillus*.

Chapter 1 presents some background information about the physical traits and appearance, the taxonomy, and the role in human nutrition and health of lactobacilli. A brief overview is given about the genetics of these bacteria and in addition, some examples of the present research on lactobacilli are presented. The second part of this chapter describes the knowledge about xylose catabolism in *E. coli, Ampullariella*, *S. xylosus*, and *Bacillus* spp.. Genes involved in xylose catabolism and their regulation of expression in these organisms are described. Furthermore, mechanisms involved in catabolite repression in Gram-negative and Gram-positive organisms are discussed.

Chapter 2 reports the cloning and characterization of three genes involved in D-xylose catabolism in *L. pentosus* MD353. The genes are organized in a cluster on the chromosome in the order 5'-*xylR* (encoding the presumed regulatory protein)- *xylA* (encoding D-xylose isomerase)- *xylB*-3' (encoding D-xylulose kinase). The deduced amino acid sequences of *xylR, xylA*, and *xylB* showed considerable similarity to the *B. subtilis* repressor protein involved in the regulation of expression of xylose genes (58%), *E. coli* and *B. subtilis* D-xylose isomerase (68% and 77%, respectively), and to *E. coli* D-xylulose kinase (58%), respectively. In contrast to *B. subtilis xylR*, *L. pentosus xylR* is transcribed in the same direction as *xylA* and *xylB*. The same polarity for *xylR*, *xylA*, and *xylB* was also found in *S. xylosus*. The cloned xylose genes represent functional genes on the basis of the following criteria: (1) The inability of a *L. casei* strain to ferment D-xylose was overcome by introduction of *L. pentosus* xylose genes, and (2) NMR analysis revealed that ^{13}C-xylose was converted into ^{13}C-acetate in *L. casei* transformed with *L. pentosus* xylose genes, but not in untransformed cells.

Promoter analysis and transcriptional regulation of the *L. pentosus* xylose genes

is described in **Chapter 3**. Northern blot analysis showed that the *xyl* genes are induced by xylose and repressed by glucose, ribose, and arabinose. This regulation is mediated at the transcriptional level. Under inducing conditions two *xylA* transcripts were detected, a major transcript of 1.5 kb and a minor transcript of 3 kb. The 3 kb transcript was also derived from *xylB*, suggesting that as in most other organisms studied sofar, *xylA* and *xylB* are transcribed together. Transcription of the *xylR* gene is constitutive. A 1.2 kb transcript was found under inducing and non-inducing conditions, whereas in the presence of xylose also a transcript of more than 7 kb was detected. As a concequence, there was at least 10-fold more *xylR* present, derived form this large transcript under inducing conditions, than under non-inducing conditions. Why expression of a negative regulator of the *xylAB* operon is induced by xylose is not understood, but similar regulation mechanisms have also been observed for other genes in other organisms. The more than 7 kb transcript is derived from at least two additional genes involved in xylose catabolism of *L. pentosus*. Sequence analysis of the region upstream of the *xylR* gene revealed the presence of two open reading frames, designated *xylP* and *xylQ*. The amino acid sequence of XylQ, encoded by the gene immediately upstream of *xylR*, does show considerable homology with a putative 88.1 kDA protein encoded by the *E. coli* genomic region from 81.5 to 84.5 min. The deduced amino acid sequence of *xylP*, upstream of *xylQ* was very similar to that of several transport proteins: XynC of *B. subtilis* (suggested to be involved in transport of xylose oligomers); MelB, the melibiose carrier of *E. coli*; LacS and LacY, the lactose transport protein of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, respectively. Based on these striking homologies, a functional role in transport of xylose is proposed for XylP. The transcription start sites for *xylR* and *xylA* were mapped by primer extension and S1 nuclease experiments. In agreement with the absence of a discrete transcript for *xylB*, no 5'-end of *xylB* mRNA was found. The activity of the promoters was determined by analysing the expression of the chloramphenicol acetyltransferase (CAT) gene under control of the *xylR* and *xylA* promoter, under inducing and non-inducing conditions. Induction by xylose of the CAT gene under control of the *xylA* promoter was 60 to 80-fold, but only 3 to 10-fold in the presence of glucose and xylose. Expression of CAT under control of the *xylR* promoter was constitutive at a level 10-fold less than that observed under control of the *xylA* promoter.

Sequence analysis suggests the presence of two operator-like elements, one overlapping with the promoter -35 region of *xylA* (CRE) and controlling the expression of *xylA* by binding factors involved in catabolite repression (Chapter 4), and a second operator downstream of the promoter -10 region of *xylA*, which may bind the product

of *xylR*, the repressor (Chapter 5). Titration experiments with multiple copies of these elements showed that, under inducing conditions, expression of *xylA* in wild-type *L. pentosus* is suboptimal. Based on the results described above, two hypotheses were proposed: (1) XylR is constitutively expressed because XylR has a dual function: an activator in the presence of xylose and a repressor in its absence. In this respect, XylR might partially function as a repressor under inducing conditions assuming that the concentration of the inducer is insufficient to drive the conversion from repressor into activator to completion. (2) XylR functions as a repressor in the presence of inducer to keep the amount of xylose isomerase and/or xylulose kinase at a sub-optimal level, since high concentrations might be lethal for the cell.

The presence of a CRE overlapping the -35 region of the promoter of *L. pentosus xylA* was based on sequence homologies with the consensus sequence for glucose repression proposed by Weickert and Chambliss, and strongly suggested that catabolite repression in *L. pentosus* is mediated by the Catabolite control protein A, CcpA. In **Chapter 4** the presence of a *L. pentosus ccpA* gene is demonstrated. A part of the *ccpA* gene was isolated by PCR using primers of the *B. megaterium ccpA* gene. Sequence analysis showed that the deduced amino acid sequence was highly homologous to CcpA's of *B. subtilis*, *B. megaterium*, *S. xylosus*, and *L. casei*. RNA analysis indicated that *L. pentosus ccpA* is part of an operon of approximately 10 kb which is constitutively expressed. Moreover, in the presence of glucose a transcript of 1 kb was detected, corresponding to the size of the *ccpA* gene. In the presence of xylose a small amount of a 1 kb transcript was also observed. This transcript was slightly larger than the transcript that was found in the presence of glucose. Analysis of *xylA* transcription in a *L. pentosus* MD363 *ccpA* disruption mutant demonstrates that catabolite repression depends on CcpA. Similar amounts of *xylA* transcript were detected in the *ccpA* mutant strain in the presence of glucose plus xylose or xylose only. In contrast, the presence of glucose strongly repressed *xylA* transcription in *L. pentosus* wild-type bacteria. Furthermore, the obtained results indicate that xylose is required to express the *xylAB* operon and that fructose, unlike glucose, is not involved in catabolite repression of the operon. Beside expression of the *xyl* operon, CcpA also regulates expression of the α-amylase gene of *L. amylovorus*, confirming its presumed global regulatory role in glucose repression.

In **Chapter 5** we have extended our studies on the role of XylR in regulation of expression of the *xylAB* operon. Titration experiments in this and in an earlier study (Chapter 3) clearly show that under inducing conditions XylR, but no other repression factor, is titrated when multiple copies of the *xylR-xylA* intergenic region are present. The observation that XylR is titrated under inducing conditions suggests that XylR

functions also as a repressor under inducing conditions. This was confirmed by transcription analysis of an *xylR* deletion mutant in which transcription of *xylAB* was enhanced 5- to 10-fold in the presence of xylose. Furthermore, disruption of the chromosomal *xylR* gene resulted in constitutive expression of *xylA* and also showed that glucose repression does not depend on a functional *xylR* gene but is mediated by a XylR-independent mechanism. In Chapter 4 we have demonstrated that this mechanism involves CcpA. Similar results were obtained with the xylose non-fermenting *L. casei* strain, transformed with pLP3537-xyl (harbouring *xylR, xylA* and *xylB*) and pLP3537-xylΔR (harbouring *xylA* and *xylB*), respectively. When the *xyl* genes are expressed in *L. casei* a positive correlation was observed between the level of expression of *xylA(B)* and the growth rate, suggesting that expression of *xylA(B)* is the limiting factor for growth in this host. Impaired growth in xylose containing medium was observed for the *L. pentosus xylR* disruption mutant. Lag-phase and generation time were increased significantly compared to *L. pentosus* wild-type bacteria, indicating that either XylR is also involved in activation of the *xyl* operon or that disruption of *xylR* is deleterious for the cell caused by an increase in the amounts of xylose isomerase and/or xylulose kinase. The latter assumption corresponds better to results of the transcription analysis of the *xylR* deletion mutant and to those of the titration experiments, in which it was proven that XylR is also functional as a repressor under inducing conditions. Support of the activator mode of action is the presence of a putative ATP/GTP binding motif in XylR, that is also found in several prokaryotic transcriptional activators. The ability of *L. casei* transformed with *xylAB* to grow on xylose suggests the presence of a secondary non-specific xylose transport system. This transport system might also be present in *L. pentosus*. Furthermore, we have demonstrated that growth and *xylAB* transcription of *L. pentosus* in xylose containing medium are facilitated when limited amounts of glucose are present.

A relationship was also found between xylose utilization and the PTS system. *L. pentosus* PTS mutants, either obtained by selection on 2-deoxyglucose resistance or by chromosomal disruption of the *ptsI* gene were not able to express the *xylAB* operon and could not ferment xylose (our unpublished results). Since xylose is not expected to be a PTS sugar, the results suggest that transport of xylose and/or expression of the *xyl* operon is activated by phosphorylation by a component of the PTS system.

Although the repression functions of XylR and CcpA have been clearly demonstrated, many questions have to be answered. No fully satisfactorily explaination is available for the difference between the well growing *L. casei* transformants harbouring only *xylA* and *xylB* and the disturbed growth of an *L. pentosus xylR*

disruption mutant. Possibly *L. casei* is missing a negative regulator that is present in *L. pentosus*. Another explanation might be that high concentrations of the xylose intermediates are toxic for *L. pentosus* but not for *L. casei*. If high concentrations of xylose intermediates are deleterious for the cell then impaired growth would also be expected for a *xylR-ccpA* double mutant in the presence of glucose, since such a mutant is expected to express similar amounts of *xylA* in the presence of glucose compared to a *xylR* disruption mutant in the presence of xylose. Previously, a transport function for *xylP* was proposed based on the striking homology with several transport proteins. However, recent studies have shown that *xylP* is not the (only) factor involved in transport of xylose since *xylP* disruption mutants were still able to ferment xylose (S. Chaillou, manuscript in preparation). To better understand the regulation of the *xyl* genes, the specific xylose-uptake system has to be identified. A second, as yet poorly understood phenomenon concerns expression of *xylB*. Under some conditions this transcript was not detectable. Whether the stem-loop structure in the *xylA-xylB* intergenic region is involved in regulation of *xylB* transcription has to be established. Determination of the amount of xylulose kinase during growth of the *L. pentosus xylR* deletion mutant may shed light on this question.

How CcpA is controlling the transcription of *xylAB* still has to be established. In chapter 4 it is described that disruption of the *L. pentosus ccpA* gene reduced glucose repression of the chloramphenicol acetyltransferase gene under control of the α-amylase promoter of *L. amylovorus*, from 89% to 27%. Also in *B. subtilis* disruption of the *ccpA* gene did not lead to full expression of the α-amylase gene in the presence of glucose, implying the presence of an additional factor involved in catabolite repression. Saier *et al.*, (1996b, chapter 4), report on the presence of a second catabolite control protein, CcpB. This protein exhibits 30% sequence identity with CcpA. The presence of a second *L. pentosus* catabolite control protein is suggested by Southern blot analysis of *L. pentosus* chromosomal DNA in which not only the identified *ccpA* gene but also an additional band was hybridizing, although much weaker, with the *B. megaterium ccpA* DNA fragment. Furthermore, downstream of the *L. pentosus xylB* gene an ORF was localized that showed 30% identity to CcpA of *L. pentosus* and to the other CcpA's discussed sofar (unpublished results). However, this ORF did not correspond to the weak hybridizing band and showed also homology to several repressor proteins, like the ribose repressor of *E. coli*. Since this *L. pentosus* ORF was followed by an ORF showing homology with the ribose kinase of *E. coli*, we assumed that the first ORF is rather involved in regulation of the ribose operon than in catabolite repression. Therefore further investigations are needed to identify other factors involved in catabolite repression in *L. pentosus*.

Samenvatting

Gedurende de afgelopen jaren is er belangrijke vooruitgang geboekt in de ontwikkeling van technieken die het mogelijk maken *Lactobacillus* stammen genetisch te analyseren en te modificeren. Hoewel, verschillende *Lactobacillus* genen gekloneerd en gekarakteriseerd zijn, is er nog maar weinig bekend over de regulatie van gen-expressie. Ten behoeve van stam verbetering is gedetailleerde kennis nodig over de regulatie van gen-expressie in *Lactobacillus*. Dit proefschrift beschrijft de regulatie van expressie van genen betrokken bij het xylose catabolisme in *Lactobacillus pentosus* als een model systeem voor gen-expressie in *Lactobacillus*. De keuze voor het xylose catabolisme als model systeem was o.a. gebaseerd op het feit dat er al enige kennis bestond over de regulatie van expressie van deze genen in andere organismen, zoals *E. coli* en *B. subtilis*.

In **hoofdstuk 1** wordt achtergrond informatie gegeven over de herkomst van lactobacilli, de taxonomie en de rol die deze bacteriën spelen in voeding en gezondheid van mens en dier. Een kort overzicht wordt gegeven van de genetische kennis van lactobacilli aangevuld met enkele voorbeelden van het huidige onderzoekprogramma bij TNO. Het tweede gedeelte van dit hoofdstuk beschrijft de kennis van het xylose catabolisme in *E. coli*, *Ampullariella,* en verschillende *Bacillus* soorten. De regulatie van de genen betrokken bij het xylose catabolisme in deze organismen wordt besproken. Verder worden de mechanismen die betrokken zijn bij cataboliet repressie in Gram-negatieve en Gram-positieve organismen behandeld.

Hoofdstuk 2 beschrijft de klonering en karakterisatie van drie genen die betrokken zijn bij het xylose catabolisme in *L. pentosus* MD353. De genen zijn gelegen in een cluster op het chromosoom, in de volgorde 5'-*xylR* (coderend voor een regulatie eiwit)- *xylA* (coderend voor het D-xylose isomerase)- *xylB*-3' (coderend voor D-xylulose kinase). De afgeleide aminozuur sequentie van *xylR, xylA* en *xylB* vertoont duidelijke homologie met respectievelijk, het *B. subtilis* repressor eiwit dat betrokken is bij de regulatie van expressie van de xylose genen (58%), *E. coli* en *B. subtilis* D-xylose isomerase (respectievelijk, 68% en 77%) en met het *E. coli* D-xylulose kinase (58%). In tegenstelling tot *xylR* van *B. subtilis* heeft *xylR* van *L. pentosus* dezelfde transcriptie richting als *xylA* en *xylB*. Deze organisatie is ook aangetroffen in *S. xylosus*. De gekloneerde xylose genen vertegenwoordigen functionele genen gebaseerd op de volgende criteria: (1) Een *L. casei* stam die zelf geen xylose kan fermenteren kon gecomplementeerd worden na introductie van de *L. pentosus* xylose genen en (2) NMR analyse laat zien dat in deze getrans-

formeerde *L. casei* cellen ^{13}C-xylose wordt omgezet in ^{13}C-acetaat, in tegenstelling tot niet getransformeerde *L. casei* cellen.

Promoter analyses en regulatie van transcriptie van de *L. pentosus* xylose genen zijn beschreven in **Hoofdstuk 3**. Northernblot analyse laat zien dat de *xyl* genen geïnduceerd worden door xylose en onderhevig zijn aan repressie in aanwezigheid van glucose, ribose en arabinose. Deze regulatie vindt plaats op het niveau van transcriptie. Onder geïnduceerde condities worden twee *xylA* transcripten gevonden; een 1.5 kb transcript, dat in overmaat aanwezig is en een 3 kb transcript. Het 3 kb transcript is ook afkomstig van *xylB* en suggereert dat net als in de meeste andere organismen die tot dusver bestudeerd zijn, *xylA* en *xylB* tezamen getranscribeerd worden. Transcriptie van *xylR* is constitutief, een 1.2 kb transcript werd gedetecteerd onder inducerende en niet-inducerende condities. Bovendien werd er in aanwezigheid van xylose ook nog een groot transcript van meer dan 7 kb gevonden. In vergelijking met het 1.2 kb transcript is er zeker 10-maal zoveel van dit grote transcript aanwezig onder inducerende condities. Dit zou betekenen dat er meer XylR gevormd wordt in de aanwezigheid van xylose. Waarom een negatieve regulator van het *xylAB* operon geïnduceerd wordt door xylose is niet duidelijk, maar vergelijkbare regulatie mechanismen zijn ook wel beschreven voor andere genen in andere organismen. Het meer dan 7 kb transcript omvat de informatie van nog twee genen die betrokken zijn bij het xylose catabolisme in *L. pentosus*. Met sequentie analyse stroom opwaarts van *xylR* konden twee open leesramen aangetoond worden, *xylP* en *xylQ*. De aminozuur sequentie van XylQ, gecodeerd door het gen dat direct stroom opwaarts van *xylR* gelegen is, vertoont duidelijk homologie met een hypothetisch 88,1 kDA eiwit gecodeerd door een gen van het *E. coli* genoom van 81.5 tot 84.5 minuten. De afgeleide aminozuur sequentie van *xylP*, stroom opwaarts van *xylQ* vertoont veel gelijkenis met verschillende transport eiwitten: XynC van *B. subtilis* (betrokken bij transport van xylose oligomeren; MelB, de melibiose carrier van *E. coli*; LacS en LacY, respectievelijk het lactose transport eiwit van *Streptococcus thermophilus* en *L. bulgaricus*. Gebaseerd op deze opvallende homologieën, wordt een functionele rol in het transport van xylose aan XylP toe bedacht. De transcriptie start plaatsen van *xylR en* xylA werden bepaald d.m.v. primer extensie en S1 nuclease experimenten. Een 5'-uiteinde van *xylB* kan niet aangetoond worden, wat in overeenstemming is met de afwezigheid van een discreet *xylB* transcript. De activiteit van de promoters is bepaald door de expressie te analyseren van het chloramphenicol acetyltransferase (CAT) gen onder controle van respectievelijk de *xylR* en de *xylA* promoter. De inductie door xylose van het CAT gen onder controle van de *xylA*

promoter is 60 tot 80-maal, maar slechts 3 tot 10-maal in de aanwezigheid van glucose en xylose. Expressie van CAT onder controle van de *xylR* promoter is constitutief en 10-maal lager dan onder controle van de *xylA* promoter.

Sequentie analyse suggereert de aanwezigheid van twee operator-achtige elementen. De eerste overlapt met de -35 promoter sequentie van *xylA* (CRE) en controleert de expressie van *xylA* door binding van factoren die betrokken zijn bij cataboliet repressie (Hoofdstuk 4). De tweede operator, stroom afwaarts van de -10 promoter sequentie van *xylA* bindt waarschijnlijk het produkt van *xylR*, de repressor (Hoofdstuk 5). Titratie experimenten die zijn uitgevoerd door het introduceren van een groot aantal kopieën van deze elementen laten zien dat onder inducerende condities de expressie van *L. pentosus xylA* sub-optimaal is. Gebaseerd op de boven beschreven resultaten worden twee hypotheses voorgesteld: (1) *xyl*R wordt constitutief tot expressie gebracht omdat XylR een dubbel functie heeft: activator in de aanwezigheid en repressor in de afwezigheid van xylose. Ook kan XylR gedeeltelijk als repressor functioneren in de aanwezigheid van xylose wanneer aangenomen wordt dat de concentratie inducer niet voldoende is om een volledige omzetting te verkrijgen van repressor naar activator. (2) XylR functioneert als een repressor in de aanwezigheid van inducer om de hoeveelheid xylose isomerase en/of xylulose kinase op een sub-optimaal niveau te houden, omdat te hoge concentraties toxisch zouden kunnen zijn voor de cel.

De aanwezigheid van een CRE, overlappend met de -35 promoter sequentie van *L. pentosus xylA*, was gebaseerd op sequentie homologie met de consensus sequentie voor glucose repressie zoals die is voorgesteld door Weickert en Chambliss. Dit suggereert sterk dat cataboliet repressie in *L. pentosus* wordt gecontroleerd door het Cataboliet contole protein A, CcpA. In **Hoofdstuk 4** wordt de aanwezigheid van het *L. pentosus ccpA* gen aangetoond. Een deel van het gen wordt geïsoleerd met PCR, waarbij primers gebruikt worden gebaseerd op het *B. megaterium ccpA*. Sequentie analyse demonstreert dat de afgeleide aminozuur sequentie zeer homoloog is met CcpA's van *B. subtilis, B. megaterium, S. xylosus,* en *L. casei*. Uit RNA analyse blijkt dat *L. pentosus ccpA* deel uitmaakt van een operon van ongeveer 10 kb dat constitutief tot expressie gebracht wordt. Bovendien wordt in de aanwezigheid van glucose een transcript van 1 kb gedetecteerd, wat overeenkomt met de grootte van het *ccpA* gen. In de aanwezigheid van xylose wordt ook een kleine hoeveelheid 1 kb transcript gevormd. Dit transcript is een klein beetje groter dan het transcript dat was gevonden in de aanwezigheid van glucose. Uit de analyse van de transcriptie van *xylA* in een *L. pentosus* MD363 *ccpA* disruptie mutant blijkt dat CcpA betrokken is bij cataboliet repressie. In de

ccpA mutant worden gelijke hoeveelheden *xylA* transcript gevormd in de aan- of afwezigheid van glucose in combinatie met xylose, terwijl in *L. pentosus* wild-type bacteriën glucose sterke repressie veroorzaakt op de expressie van *xylA*. Verder laten de resultaten zien dat xylose nodig is voor de expressie van het *xylAB* operon en dat fructose, in tegenstelling tot glucose, niet betrokken is bij cataboliet repressie van het operon. Naast de expressie van het *xyl* operon, reguleert CcpA ook de expressie van het α-amylase gen van *L. amylovorus*, waarmee het zijn algemene regulerende rol in glucose repressie bevestigt.

In **Hoofdstuk 5** is de regulerende rol van XylR op de expressie van het *xylAB* operon verder onderzocht. Titratie experimenten in deze studie en in een eerdere studie (Hoofdstuk 3) laten duidelijk zien dat onder inducerende condities XylR de enige repressor factor is die getitreerd wordt wanneer een groot aantal kopieën van het *xylR-xylA* intergen gebied aanwezig zijn. Deze waarneming suggereert dat XylR ook een repressor is onder inducerende condities. Dit wordt bevestigd door transcriptie analyse van een *xylR* deletie mutant, waarin de transcriptie van *xylAB* in de aanwezigheid van xylose met een factor 5 tot 10 verhoogd is. Disruptie van het chromosomale *xylR* gen resulteert in constitutieve expressie van *xylA*. Bovendien blijkt uit de resultaten dat glucose repressie onafhankelijk is van een functioneel *xylR* gen. In hoofdstuk 4 is aangetoond dat CcpA betrokken is bij glucose repressie. Vergelijkbare resultaten worden ook verkregen met de xylose negatieve *L. casei* stam, getransformeerd met respectievelijk plasmide pLP3537-xyl (bevat *xylR, xylA* en *xylB*) en plasmide pLP3537-xylΔR (bevat alleen *xylA* en *xylB*). In de *L. casei* transformanten wordt een positieve correlatie gevonden tussen de groei in de aanwezigheid van xylose en de expressie van *xylA(B)*. Dit is in tegenstelling met wat waargenomen is in *L. pentosus*. In dit organisme wordt de groei ernstig verstoord na disruptie van het *xylR* gen, terwijl de transcriptie van *xylA(B)* verhoogd is. In vergelijking met *L. pentosus* wild-type cellen zijn zowel de lag-fase als de delingstijd duidelijk vergroot in de *L. pentosus xylR* deletie mutant. Dit zou kunnen betekenen dat XylR ook betrokken is bij de activatie van het *xyl* operon of dat disruptie van *xylR* schadelijk is voor de cel omdat te hoge concentraties xylose isomerase en/of xylulose kinase gevormd worden. De laatste suggestie is beter in overeenstemming met de resultaten van de transcriptie analyse van de *xylR* deletie mutant en met de titratie experimenten, waarbij is bewezen dat XylR ook in de aanwezigheid van xylose functioneert als een repressor. Steun voor de activator functie is de mogelijke aanwezigheid van een ATP/GTP bindings motief in XylR, wat ook gevonden wordt in verschillende prokaryotische transcriptie activatoren. De waarneming dat *L. casei* getransformeerd met *xylAB* in staat is om op xylose te

groeien, suggereert dat er een tweede niet-specifiek xylose transport systeem aanwezig is. Of dit transport systeem ook aanwezig is in *L. pentosus* zou onderzocht moeten worden. De resultaten hebben verder aangetoond dat groei en *xylAB* transcriptie van *L. pentosus* in xylose medium vergemakkelijkt worden wanneer een kleine hoeveelheid glucose aanwezig is.

Curriculum vitae

Christien Lokman werd op 5 februari 1963 geboren te Haarlem. In 1979 behaalde zij het M.A.V.O diploma aan de Openbare M.A.V.O. te Krimpen aan den IJssel. Na het behalen van het H.A.V.O. diploma aan de Chr. Scholengemeenschap Comenius te Capelle aan den IJssel in 1981 begon zij aan het voorbereidend jaar voor het H.L.O. aan het Van Leeuwenhoek Instituut te Delft. Vanaf 1982 volgde zij de vier jarige opleiding in de richting biotechnologie met als hoofdvakken; biochemie, microbiologie en proceskunde. Tijdens de studie werd een stage doorlopen aan het Laboratorium voor Microbiologie en Hygiëne van de Vrije Universiteit Brussel (Prof. Dr. A. Boeyé en Dr A.A.M. Thomas). Het HLO diploma biologie werd in juni 1986 behaald.

Vanaf 1 juni 1986 is zij werkzaam bij de afdeling Moleculaire Genetica en Gen Technologie van het Instituut Voeding van de Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO). Aldaar heeft zij van 1991 tot 1996 gewerkt aan het onderzoek wat is beschreven in dit proefschrift.

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Chrishin

Nu maar hopen dat ik niemand vergeten ben !