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Enterococcus faecalis MalR acts as repressor of the maltose operons and additionally mediates their catabolite repression via direct interaction with seryl-phosphorylated-HPr

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Abstract

Enterococci are Gram-positive pathogens and leading causes of hospital acquired infections worldwide. Central carbon metabolism was shown as highly induced in *Enterococcus faecalis* during infection context. Metabolism of α-polysaccharides was previously described as an important factor for host colonization and biofilm formation. A better characterisation of the adaptation of this bacterium to carbohydrate availabilities may lead to a better understanding of the link between carbohydrate metabolism and the infection process of *E. faecalis*. Here we show that MalR, a LacI/GalR transcriptional regulator, is the main factor in the regulation of the two divergent operons involved in maltose metabolism in this bacterium. The *malR* gene is transcribed from the *malP* promoter, but also from an internal promoter inside the gene located upstream of *malR*. In the absence of maltose, MalR acts as a repressor and in the presence of glucose, it exerts efficient CcpA-independent carbon catabolite repression. The central PTS protein P-Ser-HPr interacts directly with MalR and enhances its DNA binding capacity, which allows *E. faecalis* to adapt its metabolism to environmental conditions.

Introduction

Enterococci are Gram-positive bacteria widely distributed in the environment (Lebreton *et al.*, 2014). These common commensal microorganisms inhabit the digestive tract of humans and animals. Two species of these normally harmless bacteria, *E. faecalis* and *E. faecium*, have been recognized to be leading causes of hospital acquired infections like urinary tract infection, endocarditis, bacteraemia or septicaemia (Robert C. Moellering, 1992; Edmond *et al.*, 1999; Wisplinghoff *et al.*, 2004; Agudelo Higuita and Huycke, 2014; Ceci *et al.*, 2015; García-Solache and Rice, 2019). Resistance to antibiotics and environmental stresses including desiccation are likely responsible for enterococcal persistence in hospital environments (Bale *et al.*, 1993; Fisher and Phillips, 2009; Kristich *et al.*, 2014). Several virulence factors have been identified and shown to contribute to the pathogenicity of enterococci, but the infection process remains poorly understood (Garsin *et al.*, 2014). This is especially true for the first steps in the process concerning

nutrient mobilisation and proliferation in the infected host. Several studies showed that the metabolism of glycans is an important factor for bacterial host colonisation (Chang *et al.*, 2004; Sonnenburg *et al.*, 2010; Thomas *et al.*, 2011; Koropatkin *et al.*, 2012; Ng *et al.*, 2013; Xu *et al.*, 2019). A global transcriptomic study conducted with *E. faecalis* revealed that during peritoneal infection of mice, 123 bacterial metabolic genes were differentially expressed (Muller *et al.*, 2015). Out of those genes, 74 were upregulated suggesting that they play an important role in host colonisation (Muller *et al.*, 2015). Another recent study showed that a global transcriptional regulator, MafR, is required for the expression of many genes involved in carbon metabolism. Interestingly, a *mafR* mutant caused a lower degree of inflammation in mice, suggesting that the MafR-deficient *E. faecalis* strain was less virulent than its corresponding wild-type (Ruiz-Cruz *et al.*, 2016). Furthermore, two operons involved in maltose metabolism in *E. faecalis* were shown to be upregulated during growth in horse blood (Vebø *et al.*, 2009) and one of these operons has also been reported to be involved in biofilm formation (Hufnagel *et al.*, 2004; Creti *et al.*, 2006).

Maltose is a reducing disaccharide composed of two glucosyl residues connected via an α-1,4 linkage. It represents the simplest form of maltodextrin obtained after hydrolysis of starch or glycogen. Maltose metabolism in *E. faecalis* is achieved through two divergent operons represented in Fig. 1A. As shown in Fig. 1C, the disaccharide is imported by a phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) encoded by *malT* (Le Breton *et al.*, 2005). The main role of the PTS is to catalyse the uptake and concomitant phosphorylation of numerous carbohydrates (Deutscher *et al.*, 2006). It is usually composed of the two general proteins EI and HPr and three or four sugar-specific proteins or domains (EIIA, EIIB, EIIC, and sometimes EIID). The soluble PTS components EI and HPr form a phosphorylation cascade between the PEP phosphoryl donor and the domains EIIA and EIIB. P~EIIB transfers its phosphoryl group to the carbohydrate bound to the corresponding membrane-protein EIIC, and the resulting P-sugar is subsequently released into the cytoplasm (Deutscher *et al.*, 2006) In *E. faecalis*, the resulting maltose-6'-phosphate (M6'P) is dephosphorylated by the MapP protein (Mokhtari *et al.*, 2013) before its cleavage into glucose and glucose-1-phosphate (G1P) by the maltose phosphorylase MalP (Fig. 1C). G1P can be converted into glucose-6-phosphate (G6P) by MalB encoded by the second gene of the *malPBMR* operon before entering glycolysis. Maltose metabolism is well understood in *E. faecalis*, but regulation of the expression of the maltose operons remained unknown in this species. On the other hand, regulation of maltose metabolism

has been studied in the phylogenetically closely related genus *Streptococcus*. Similar to *E. faecalis*, maltose is preferentially imported in through the PTS permease MalT in streptococci (Webb *et al.*, 2007; Shelburne *et al.*, 2008; Bidossi *et al.*, 2012). The M6'P is supposed to be dephosphorylated by a M6'P phosphatase orthologue of the enterococcal MapP. Maltose is then catabolised by combined action of a glucanotransferase (Lacks, 1968; Stassi *et al.*, 1981; Sato *et al.*, 2013) and a glycogen phosphorylase (Martin and Russell, 1987; Sato *et al.*, 2013). Expression of these genes is repressed by the LacI/GalR family transcriptional regulator MalR in *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Streptococcus mutans* (Puyet, 1993; Nieto *et al.*, 1997; Nieto *et al.*, 2001) and subjected to Carbon Catabolite Repression (CCR). The catabolite control protein A (CcpA) was shown to play a role in the regulation of maltose metabolism in streptococci. Indeed, inactivation of CcpA leads to a decrease in basal expression of the maltose genes in *S. pyogenes* (Shelburne *et al.*, 2008) grown in the presence of glucose. To efficiently bind DNA, CcpA first forms a complex with P-Ser-HPr in low G+C Gram-positive bacteria (Jones *et al.*, 1997). The P-Ser-HPr/CcpA complex specifically binds Catabolite-Responsive Elements also named *cre*-boxes to regulate expression of several streptococcal genes including genes involved in metabolism but also in virulence (Iyer *et al.*, 2005; Paluscio *et al.*, 2018).

Phosphorylation on Ser-46 of HPr is catalysed by the ATP-dependent kinase/phosphorylase HprK/P (Deutscher and Saier, 1983) whose kinase activity is stimulated by high concentrations of fructose-1,6-bisphosphate (FBP) and G6P (Deutscher and Engelmann, 1984; Reizer *et al.*, 1988; Kravanja *et al.*, 1999) which is the case in the presence of a rapidly metabolizable carbon source such as glucose. P-Ser-HPr acts as a signaling molecule and has several important regulatory functions including to act as a co-repressor of CcpA (Deutscher and Saier, 1983; Jones *et al.*, 1997).

In this study, we analysed the expression of the *malPBMR* and *malT-mapP* operons (Fig. 1A) in *E. faecalis* OG1RF by RT-qPCR. These operons are induced in the presence of maltose and strongly repressed in the presence of glucose. We showed that CCR is independent of CcpA, but relies on another LacI/GalR family transcriptional regulator, MalR. We confirmed by Electrophoretic Mobility Shift Assay (EMSA) that MalR acts as a direct repressor of the expression of the maltose genes and that addition of maltose reduces its DNA binding capability. By carrying out MicroScale Thermophoresis (MST) we demonstrated a direct interaction between

MalR and P-Ser-HPr. This interaction fine tunes MalR activity and coordinates the cell metabolism in accordance to carbon source availability.

Materials and methods

Bacterial strains and growth conditions. Strains and plasmids used in this study are shown in Table S1 and Table S2, respectively. Briefly, *Escherichia coli* strains used for cloning experiments and tagged-protein purification were grown in Lysogeny Broth (LB) (Bertani, 1951). When appropriate, cultures were supplemented with ampicillin, kanamycin or chloramphenicol with final concentrations of 100, 25 and 10 μg mL^{-1}, respectively.

Enterococcus faecalis OG1RF (Dunny *et al.*, 1978) and its derivative strains (Table S1) were routinely grown in M17 medium containing 0.5% glucose (w/v) (GM17) at 37°C. For growth studies and the determination of transcriptional profiles, we used carbohydrate cured M17 MOPS (ccM17) (La Carbona *et al.*, 2007) supplemented with 0.3% (w/v) of either glucose, maltose, and/or maltotriose according to experimental needs. When appropriate, cultures were supplemented with erythromycin or chloramphenicol with final concentrations of 100 and 10 μg mL^{-1}, respectively.

Construction of mutants. The genome sequence assembly of *E. faecalis* OG1RF (GCF_000172575.2) was obtained from the NCBI Reference Sequence Database (O'Leary *et al.*, 2016). Primers used in this study are listed in Table S3. The *malR* null mutant was obtained by introducing two stop codons at the beginning of the open reading frame (stop corresponding to the parental amino acids 57 and 60) in order to produce a small truncated and presumably non-functional protein. The *ccpA* mutant was constructed by deleting nucleotides 49 to 899 of the parental gene, presenting approximately 85% of the entire sequence. PCR products were obtained with the Phusion polymerase (ThermoFisher Scientific) and appropriate primers using the genomic DNA of *E. faecalis* OG1RF as template. The desired amplicons were then cloned into the pMAD vector (Arnaud *et al.*, 2004). The modified alleles were used to replace the wild type alleles by the double crossing over method. Molecular constructs and chromosomal structures of mutants were systematically validated by Sanger based DNA sequencing (Eurofins).

Complementation of mutants. To complement the *malR*$^-$ mutant, the corresponding wild type gene together with its internal promoter located within the *malM* gene was cloned into the pRB473 plasmid (Brückner *et al.*, 1993) and electroporated into *E. coli* EC1000. After extraction and purification, the recombinant plasmid was used to transform *E. faecalis malR*$^-$. *E. faecalis* cells containing pRB473-derived plasmids were always grown in the presence of 2 µg mL^{-1} of chloramphenicol.

Purification of His-tagged proteins. To purify MalR, CcpA and HPr, the corresponding genes were first cloned into expression vectors, pQE30 (Qiagen) for HPr (*ptsH* gene of *Bacillus subtilis*) (Galinier *et al.*, 1997), pET28a(+) (Novagen) for CcpA of *E. faecalis* (Suárez *et al.*, 2011) and pET28b(+) (Novagen) for MalR (Table S2). For the amplification of the *malR* gene, *E. faecalis* OG1RF genomic DNA was used as template together with appropriate primer pairs containing specific restriction sites (Table S3). The DNA fragments were digested with appropriate restriction enzymes and cloned into the above mentioned plasmid cut with the same enzymes. The resulting plasmids were transformed into *E. coli* BL21(DE3) (New England Biolabs) or M15[pREP4]. After sequence verification, a transformant for each gene was grown in Terrific Broth (Tartof and Hobbs, 1987) supplemented with kanamycin and/or ampicillin according to the resistance carried by the plasmids. Cells were grown until the medium reached an OD_{600} above 0.6. Synthesis of the different proteins was induced with 0.5 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Eurobio) for 4 h at room temperature under vigorous agitation. Tagged proteins were then purified using the kit Protino Ni-NTA (Macherey-Nagel) under nondenaturing conditions following the supplier's recommendations. Concentrations of different proteins were determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific) with a BSA standard curve.

Part of HPr was phosphorylated *in vitro* using 10 µg of purified EI from *B. subtilis* (Galinier *et al.*, 1997) for 60 min at 37°C in a buffer (50 mM Tris/HCl pH 7.4, 5 mM PEP, 5 mM $MgCl_2$) to obtain P~His-HPr. P-Ser-HPr was obtained *in vitro* using 10 µg of purified HprK/P(V267F) from *L. casei* (Monedero *et al.*, 2001) for 60 min at 37°C in a buffer (50 mM Tris/HCl pH 7.4, 5 mM ATP, 5 mM $MgCl_2$, 1 mM FBP), HprK/P protein was inactivated 10 min at 65°C. Buffer exchanges (20 mM NaH_2PO_4/Na_2HPO_4 pH 7.4, 250 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol) were performed on phosphorylated proteins using PD-10 Desalting Columns (GE Healthcare). Phosphorylation and absence of unphosphorylated HPr was verified on Coomassie-stained gels after PAGE under non-denaturing conditions. The desired phosphorylated form represents

therefore more than 90% of total HPr protein. This proportion was taken into account in the corresponding experiments.

***Isolation of RNA and transcript analysis*.** Overnight cultures were grown in fresh ccM17 medium supplemented with appropriate carbon sources and harvested at mid-exponential phase (OD_{600} about 0.6) by centrifugation. Total RNA was isolated using Direct-zol™ RNA Miniprep (Zymo Research) following manufacturer's recommendations. The integrity of RNA was verified on a bleach agarose gel (Aranda *et al.*, 2012) using 2 µg of RNA. One µg of total RNA was used to perform a Retro-Transcription (RT) step with the QuantiTect Reverse Transcription Kit (Qiagen). RT-qPCR analysis of cDNA was performed with the GoTaq® qPCR Master Mix (Promega). Primers used for RT-qPCR are shown in Table S4. Relative transcript abundances were determined by a quantification method using a standard genomic DNA curve and the housekeeping *gyrA* gene as reference.

***β-Glucuronidase assays*.** To determine β-glucuronidase (Gus) activity, exponentially growing *E. faecalis* OG1RF carrying pNZ273 (Platteeuw *et al.*, 1994) and pNZ273-derived plasmids were harvested and re-suspended in the same volume of phosphate buffer (50 mM Na_2HPO_4-NaH_2PO_4 pH 6.6, 10 mM KCl, 1 mM $MgSO_4$). OD_{600} was measured to standardize the amount of bacteria. Cells were chemically disrupted by adding lysis buffer (6.5% (v/v) chloroform, 0.002% (v/v) SDS). The solution was vigorously mixed for 1 min and incubated at 37°C for 5 min. The Gus substrate (*p*-nitrophenyl-β-D-glucuronide) was added to a final concentration of 627.5 µg mL^{-1} to start the reaction. After incubation at 37°C for 10 min, the reaction was stopped by the addition of Na_2CO_3 at 250 mM. After removing cellular debris by centrifugation, *p*-nitrophenol concentration was calculated by measuring the OD_{420}. The β-glucuronidase activity was determined in arbitrary units (AU) by the following formula: $Gus = 100 \times {OD_{420}}/{OD_{600}}$.

Rapid Amplification of cDNA ends by polymerase chain reaction (5' RACE PCR). The transcription start site of the *malR* gene was determined by 5'RACE PCR using the kit 5'/3' RACE Kit, 2nd Generation (Roche Applied Science). The transcription start site corresponds to the last nucleotide before the poly-A tail in the DNA sequence determined after Sanger (Eurofins) using the Race primers in Table S3.

Electrophoretic Mobility Shift Assay (EMSA). DNA fragments containing the *malP* or *malT* promoter or an internal fragment of the *gusA* gene (used as negative control) were first cloned into

the pGEM-T Easy plasmid (Promega). DNA fragments were amplified by PCR using a primer labelled with fluorescent cyanine-5 at the 5'end (Table S3) and appropriate pGEMT derived plasmids as template. To obtain a DNA fragment containing the P*2mal* region, genomic DNA of *E. faecalis* was used as template with a primer linked to cyanine-5 (Table S3). Labelled DNA (5 nM) was incubated with MalR or CcpA protein in a final volume of 20 µl in binding buffer (20 mM Tris/HCl, pH7.4, 20 mM KCl, 10 mM $MgCl_2$, 10 mM FBP, 1 mM DTT, 0.1 mM EDTA, 5% (v/v) glycerol, 50 ng μL^{-1} poly(dI-dC), 0.01 mg mL^{-1} bovine serum albumin) at room temperature for 20 min before the assay mixture was loaded on a 5% acrylamide gel and separated under native conditions in TBE 0.5X buffer under constant voltage (140 V) in a dark-chilled room. Fluorescence was read with a ProXpress apparatus (PerkinElmer) with the following settings (top illumination 625 nm, top reading 680 nm, 100 µm resolution, 120 s exposure time). Relative abundances of the different bands were quantified by the OptiQuant Image Analysis Software version 4.0 (Packard Instrument Company).

Synthesis of phosphorylated carbohydrates. M6'P and maltotriose-6''-P were obtained as previously described (Thompson *et al.*, 2001). G6P and FBP were purchased at Sigma-Aldrich.

Thermal Shift Assay (TSA). Sypro™ Orange (Sigma-Aldrich) was used as dye, fluorescence (Ex: 515-535 nm, Em: 560-580 nm HEX™ filter) was monitored in a temperature gradient of +0.5°C per minute from 25°C to 80°C in a CFX96™ Real-Time PCR Detection System (Bio-Rad). Temperatures of hydrophobic exposure (T*h*) were determined with Boltzmann regression on the sigmoid part of each curve using GraphPad Prism 7 software. The variation of T*h* (ΔT*h*) was calculated by subtracting T*h*s from T*h* determined without a ligand. Proteins were used at a final concentration of 2 µM and interaction assays were carried out in a solution containing 10 mM NaH_2PO_4/Na_2HPO_4 pH 7.6, 125 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 7.5% (v/v) glycerol with a final concentration of Sypro™ Orange dye of 11X. Apparent dissociation constants (K_D) were calculated using Differential Scanning Fluorimetry assuming a single binding event with the GraphPad Prism 7 software for windows, GraphPad Software, La Jolla California USA, www.graphpad.com with the following formula $Y = Bot + ((Top - Bot) * (1 - \left(\frac{P - K_D - X + \sqrt{(P + X + K_D)^2 - 4P * X}}{P}\right))$ as previously described, (refer to Vivoli *et al.*, 2014 for more details).

DNase I footprint analysis. The P*malP* region was amplified as described above with primers pU (FAM) and pR (Table S3). The PCR product labelled with the FAM fluorophore was first incubated with 1 µM of His-MalR in binding buffer (20 mM Tris/HCl, pH7.4, 20 mM KCl, 10 mM $MgCl_2$, 10 mM FBP, 1 mM DTT, 5% (v/v) glycerol, 62.5 ng μL^{-1} poly(dI-dC), 0.01 mg mL^{-1} bovine serum albumin) in a final volume of 65 µL at room temperature for 15 min. Different dilutions (10 µL) of RQ1 DNase I (Promega) (from 1/200 to 1/1600) were added to the mixture and incubated for 5 min at room temperature. The digestion reaction was stopped by addition of 2 mM of EDTA. The DNA fragments were finally purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Aliquots of the digested PCR products (10 µL) were added to 10 µL of formamide and 0.1 µL of GeneScan-500 ROX size standard (ThermoFisher Scientific). Analysis of the DNA fragments was performed by the sequencing platform of the Cochin Institut (Eurofins) and computed using the PeakScanner software v1.0 (ThermoFisher Scientific).

Microscale Thermophoresis (MST) Binding Assays. His-tagged MalR and CcpA were labelled with the NT-647-NHS dye using the Monolith NT™ Protein Labeling Kit RED-NHS (Nanotemper Technologies). Serially diluted unlabelled proteins tested for interaction and 5 nM of labelled MalR or CcpA were incubated for 15 min at room temperature in binding buffer (20 mM Tris/HCl pH 7.4, 20 mM KCl, 10 mM $MgCl_2$, 10 mM FBP, 1 mM DTT, 0.1 mM EDTA, 5% (v/v) glycerol, 0.005% (v/v) Tween20) in a final volume of 20 µL. Samples were then loaded into NT.115 premium coated capillaries (Nanotemper Technologies). Binding experiments were performed in a Monolith NT.115Pico apparatus (Nanotemper Technologies) with the following parameters: LED power 5%, MST Power medium. MST traces were analysed between 4.00 and 5.00 seconds after turning on the IR-Laser. Two measurements were carried out with HPr and P~His-HPr and three with P-Ser-HPr. For each experiment, the unlabelled proteins were produced from independent preparations. Results were obtained with the MO.Control software version 1.6 and computed with MO.Affinity Analysis software version 2.3 to determine the fraction of the formed complex. Apparent dissociation constants (K_D) were calculated using non-linear fitting assuming one specific binding site with the GraphPad Prism 7 software for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com with the following formula $Y = {B_{Max} * X}/{K_D + X}$ (where B_{Max} is the maximum theoretical specific binding, here B_{Max}=1).

Multiple alignments. Phyre2 (Kelley *et al.*, 2015) was used to generate a model of the MalR tridimensional structure. The PDB entry 1ZVV, corresponding to the CcpA-Crh-DNA complex from *Bacillus subtilis* was listed with the highest scoring template. The resulting alignment was used as template to perform a multiple alignment with the primary sequence of CcpAs and the RbsR protein using Clustal Omega (Sievers *et al.*, 2011).

Results & Discussion

Inactivation of malR prevents repression of the maltose genes. The genes involved in maltose metabolism in *E. faecalis* are organized in two divergent operons beginning with the *malP* and *malT* genes, respectively (Fig. 1A) (Le Breton *et al.*, 2005; Mokhtari *et al.*, 2013). The *malR* gene, which encodes a transcriptional regulator of the LacI/GalR family, is the last gene of the *malPBMR* operon. In light of its genomic location and its similarity to members of the LacI/GalR family, MalR was predicted to be the regulator of *mal* gene expression in *E. faecalis.* To study the role of MalR in this regulation, we first constructed a markerless *malR* null mutant by inserting two stop codons at the beginning of the *malR* gene (OG1RF_*10680*). Next, the expression levels of the maltose operons were measured by RT-qPCR using the first gene of each operon as target (Fig. 2). When the parental strain OG1RF was grown on glucose, these genes were only weakly expressed. In the presence of maltose or maltotriose, expression of the genes was induced. When glucose is also present in the medium, these genes are repressed independently of the presence of maltose or maltotriose. In a *malR*$^{-}$ mutant, both *malP* and *malT* genes were constitutively expressed. This result suggests that MalR is the repressor of the *mal* operons.

The maltose genes are subjected to CcpA-independent but MalR-dependent CCR. As shown in Fig. 2, induction of the *malP* and *malT* genes was repressed when OG1RF was grown on glucose and either maltose or maltotriose, demonstrating that these genes are subjected to CCR. Interestingly, repression by glucose was independent of CcpA (OG1RF_*11453*), the major global CCR regulator in *Firmicutes*, because expression of *malP* and *malT* was still repressed by glucose in a *ccpA* deletion mutant (Δ*ccpA*) (Fig. 2). In the *malR*$^{-}$ mutant, both *mal* operons were highly expressed under all tested growth conditions, even when grown on glucose. The MalR protein therefore appears to be essential for efficient CCR of these operons. To confirm that the observed deregulation was due to the introduced mutations in the *malR* gene, a wild type copy of the *malR* gene with its internal promoter was inserted into plasmid pRB473. While transformation of the *malR* mutant with the empty plasmid had no noticeable effect on maltose gene expression, a plasmid carrying the *malR* gene restored repression nearly to wild-type level (Fig. S1). The MalR protein is therefore necessary for CCR of the maltose genes exerted by glucose and presumably other efficiently metabolizable carbon sources.

Despite the presence of two imperfect palindromes closely related to *cre*-boxes determined for CcpA of *E. faecalis* (WTGWAARCGYWWWCW where W=A or T, R=A or G and Y=C or T)

(Opsata *et al.*, 2010) upstream of *malT* and *malP* (CGCAATCGGTTTCG and TGCAATCGGTTGCG respectively), CcpA/P-Ser-HPr does not bind to the P*2mal* region *in vitro* (Fig. S2A). This result and the observed failure of the Δ*ccpA* mutant to cause a relief from CCR suggest that CcpA is not involved in CCR of the *E. faecalis* maltose genes.

MalR presents affinity for maltose. As described above, the *mal* operons appear to be repressed by glucose and induced by maltose and maltotriose. The two glucans are imported into the cell through a PTS MalT and therefore enter the cells in phosphorylated form (Le Breton *et al.*, 2005). By analogy with well characterized LacI/GalR type transcriptional regulators, MalR should bind to its operator under repressing conditions and be released in the presence of the inducer (Swint-Kruse and Matthews, 2009). To determine the nature of the inducer, we carried out Thermal Shift Assays (TSA) with His-tagged MalR and either glucose, maltose, maltotriose or the corresponding phospho-compounds by using the Sypro™ Orange fluorophore (Vivoli *et al.*, 2014; Grøftehauge *et al.*, 2015). As shown in Fig. 3, the temperature of hydrophobic exposure (T*h*) of the MalR protein rose with increasing concentrations of maltose, suggesting that MalR presents binding sites for maltose. In contrast, even at very high concentrations of M6'P, only a slight increase of T*h* was observed, demonstrating that MalR has only weak affinity for M6'P. By assuming a single binding site for maltose, non-linear regression correctly fitted the data points and allowed to determine an apparent dissociation constant (K_D) of 2.2 ± 0.4 mM. This relatively low affinity of the repressor for its inducer should require an efficient import system in order to fully induce the *mal* genes. Moreover, since M6'P is not an efficient ligand for MalR, the phosphatase MapP (Mokhtari *et al.*, 2013) might be crucial for induction of these genes because it dephosphorylates M6'P to maltose. No significant variation of T*h* over the whole concentration range tested was observed in the presence of glucose, maltotriose and their phospho-derivatives, suggesting that MalR does not bind these molecules (Fig. S3). These data indicate that maltose is the inducer of the two *mal* operons in this bacterium. In contrast to some bacteria, which hydrolyse M6'P formed during PTS-catalyzed transport of maltose to glucose and G6P by a 6-P-α-glucosidase (Yip *et al.*, 2007), M6'P transported in *E. faecalis* via the PTS permease MalT is re-converted to maltose by MapP (Mokhtari *et al.*, 2013). The unusual dephosphorylation step might have been implemented during evolution in order to keep *malT* expression under control of the MalR/maltose complex.

MalR exhibits affinity for FBP. CcpA binds the glycolytic intermediate FBP, which promotes the formation of the P-Ser-HPr/CcpA complex and therefore play a role in CCR in *Firmicutes*

(Deutscher *et al.*, 1995; Schumacher *et al.*, 2007). We noticed that including FBP in the EMSA binding buffer improved the resolution of the bands shifted by MalR (Fig. S2B). Because MalR was shown to be responsible for CCR of the *mal* genes, we determined the affinity of MalR for FBP by the same technique used before for MalR and maltose. Indeed, the temperature of hydrophobic exposure (T*h*) rose with increasing concentrations of FBP (Fig. 3), thus supporting an interaction with MalR. An apparent K_D of 6.6 ± 1 mM could be determined assuming a single binding site. In resting *Lactococcus lactis* cells, the FBP concentration was found to increase from 2 mM to 25 mM, when glucose was added (Thompson and Torchia, 1984). Similarly to what was observed for CcpA, the interaction with FBP might promote the formation of the MalR/P-Ser-HPr complex during growth on glucose. FBP is therefore expected to play a dual role in maltose CCR in *E. faecalis* by stimulating the kinase function of HprK/P (Deutscher and Engelmann, 1984) and potentially the formation of the MalR/P-Ser-HPr complex.

MalR acts as a repressor by direct binding to the promoter regions of malP and malT. In order to prove the direct implication of MalR in the regulation of the maltose operons, we performed EMSA. Three fluorescently labelled DNA fragments containing either P*malP*, P*malT* or both promoters (the latter DNA fragment is named P*2mal*) (Fig. 4A) were incubated in the presence of MalR. Two distinct shifted bands are visible when the entire intergenic region is used as the template (Fig. 4B, lanes 2 to 5) suggesting a direct specific interaction between MalR and the DNA. In addition, the intensity of the shifted bands decreased when maltose was present in the reaction buffer (Fig. 4B, lanes 4 and 5). The MalR/DNA interactions are considered specific because a control DNA was not shifted under the same conditions (Fig. 4C, lanes 7 to 9), indicating that MalR acts as a transcriptional repressor and that maltose functions as the inducer of the *mal* operons.

The appearance of two shifted bands suggested the presence of two distinct binding sites of MalR in the P*2mal* DNA fragment. The upper shifted band might correspond to a complex of DNA with MalR bound to both sites and the lower band to a complex of MalR bound to a single site. Based on our results that MalR regulates the *malP* and *malT* genes, we hypothesized that there is a specific MalR operator site for each promoter. Indeed, MalR shifted the two DNA fragments containing either only P*malP* or only P*malT* (Fig. 4C), thus confirming our hypothesis that each promoter region contains a distinct MalR operator. The shifted band seems to be more intense and the protein-DNA complex more stable in the case of the *malP* promoter compared to the *malT*

promoter (Fig. 4C, lanes 2 and 5), which might reflect a higher affinity of MalR for P*malP* than for P*malT*. A similar difference in binding affinities of MalR to its operator sites present upstream of operons implicated in maltose metabolism has been noticed for *S. pneumoniae* (Nieto *et al.*, 2001). This difference in repression could be of physiological importance for the bacterium since a too strong repression of the maltose transporter and the M6'P phosphatase genes would slow or even prevent induction of the maltose operons in the presence of the inducer.

***MalR interacts with P-Ser-HPr in vitro*.** When searching for MalR homologues in *E. faecalis* using BLASTp (Altschul *et al.*, 1990), CcpA was listed first because it exhibits 30% identity and 50% similarity to MalR. The finding that in a *malR*$^-$ mutant the *mal* genes are relieved from CCR and the observed significant similarity between MalR and CcpA prompted us to study a direct interaction between MalR and P-Ser-HPr. We carried out MST experiments to detect interactions of MalR with HPr and its two phospho-forms as described in Experimental Procedures. Purified His-tagged MalR was first covalently linked to the NT-647-NHS fluorophore. The amount of NT.647 labelled MalR was maintained constant, while the concentration of the various non-labelled HPr forms varied from 9.76 nM to 20 µM. The known interaction of His-tagged CcpA with P-Ser-HPr was used as a positive control (Fig. 5A). As expected, no interaction signal was detected with NT.647-MalR and HPr or P~His-HPr (Fig. 5A). In contrast, an interaction signal was observed with NT.647-MalR or NT.647-CcpA when P-Ser-HPr was used at concentrations exceeding 1 µM (Fig. 5A). Assuming a single binding site and a specific interaction, a typical binding isotherm curve was obtained. The approximate apparent dissociation constants for the P-Ser-HPr/CcpA and P-Ser-HPr/MalR complexes were in the same order (K_D around 10-20 µM), suggesting a similar affinity for the co-factor. The measured K_D of the CcpA/P-Ser-HPr interaction appears at least two fold higher in our experiments than the K_D previously determined for CcpA/P-Ser-HPr of *Bacillus subtilis* (Jones *et al.*, 1997) but was still in the same order of magnitude.

To get an indication whether MalR uses an interface for the interaction with P-Ser-HPr similar to that of CcpA and RbsR, which have been shown to interact with P-Ser-HPr, a multiple sequence alignment was carried out with MalR, CcpA from *B. subtilis*, *Bacillus megaterium* and *E. faecalis* and RbsR from *B. subtilis*. The crystal structure of the *B. megaterium* CcpA/P-Ser-HPr complex had revealed two CcpA regions which interact with P-Ser-HPr. The first region extends from amino acids 69 to 99 (Fig. 5B) and includes three aspartic acids D69, D84 and D99 of subunit II,

which interact with R17 of P-Ser-HPr (Schumacher *et al.*, 2004). While all three Asp are conserved in RbsR, none of them are conserved in MalR. D84 is replaced by a conservative exchange with E90 and the other two Asp are replaced by Arg and Lys. In addition, just upstream from the first conserved Asp in RbsR and CcpA, MalR carries an insertion of six amino acids (Fig. 5B). It is therefore likely that the structure of this region of MalR is significantly different from that in CcpA proteins and RbsR and other negatively charged amino acids of this region might be involved in the interaction with R17 of P-Ser-HPr. The second region of CcpA involved in the interaction with P-Ser-HPr extends from amino acids 296 to 307 in subunit I. D296 forms hydrogen bonds with unphosphorylated H15 and the main chain of A16, R303 and K307 interact with the phosphate group bound to S46 in P-Ser-HPr (Fig. 5C) (Schumacher *et al.*, 2004). D296 is conserved in RbsR and replaced with E297 in MalR. In MalR and RbsR, R303 is replaced with a Lys, whereas R307 corresponds to a Glu in MalR and a Gly in RbsR. In summary, the alignment suggests that the RbsR interface involved in the interaction with P-Ser-HPr largely resembles that of CcpA, whereas the interface of MalR is probably significantly different. It will therefore be interesting to determine the structure of the MalR/P-Ser-HPr complex in order to find out how this regulator formed its P-Ser-HPr binding surface. The six additional amino acids present in MalR upstream of R75 probably played an important role in this adaptation process (Fig. 5B).

The results obtained from MST experiments established that MalR forms a complex with P-Ser-HPr, which we suspected to be responsible for the observed CcpA-independent CCR of the *mal* genes. We tried to support this hypothesis by carrying out *in vivo* experiments with an *E. faecalis* OG1RF-derived *ptsH1* (*ptsH*S46A) mutant (Eisermann *et al.*, 1988; Deutscher *et al.*, 2006). In this mutant, the *mal* genes should be relieved from CCR, because the HprK/P-dependent phosphorylation of HPr is abolished by the replacement of S46 with a non phosphorylable alanine. Unfortunately, numerous attempts to obtain the *ptsH1* mutant failed, suggesting that preventing the phosphorylation of HPr at S46 does not seem to be possible in *E. faecalis*. A similar behaviour has already been reported for *S. pneumoniae* where the formation of P-Ser-HPr appears to be essential, although the exact cause remains unknown (Fleming *et al.*, 2015).

The presence of P-Ser-HPr enhances the DNA binding affinity of MalR in vitro. Since MalR interacts with P-Ser-HPr, we tested whether addition of the co-factor during EMSA would increase the DNA binding affinity of MalR. Indeed, in the presence of P-Ser-HPr and 25 nM MalR, the repressor binds DNA more efficiently (compare Fig. 4B, lanes 2 and 6). The upper

shifted band is stronger and the free DNA amount is lower. Moreover, in the presence of P-Ser-HPr, the maltose-mediated inhibition of MalR/DNA interaction is less efficient (Fig. 4B, compare lanes 4 and 5 to lanes 8 and 9). This finding might reflect a binding competition between P-Ser-HPr and maltose for MalR or a P-Ser-HPr induced conformational change of MalR, which might lower its affinity for maltose. Of note, no enhancement of MalR DNA binding was observed with other physiologically relevant forms of HPr (unphosphorylated HPr and P~His-HPr). In addition, HPr and its two P-derivatives do not bind to P*2mal* (Fig. S2C). The specific P-Ser-HPr-mediated increase of the DNA binding affinity of MalR is probably responsible for CcpA-independent CCR of the two *mal* operons. The utilization of glucose increases the amount of P-Ser-HPr (Deutscher and Saier, 1983; Monedero *et al.*, 2001; Ludwig *et al.*, 2002) and hence MalR affinity for its operator sites. Regarding the strong repression of the *mal* operons in the Δ*ccpA* mutant grown on glucose, two conditions might be responsible. First, the elevated amount of P-Ser-HPr in the Δ*ccpA* mutant (Leboeuf *et al.*, 2000; Ludwig *et al.*, 2002) and, second, the absence of competition between MalR and CcpA for P-Ser-HPr.

Determination of MalR binding sites. The above data clearly show that *E. faecalis* MalR binds to DNA fragments harbouring the *malP* or *malT* promoter regions. Therefore, we screened them for imperfect palindromic sequences with a central AANC motif (where N can be any nucleotide), which is typical for operators of LacI/GalR type transcriptional regulators (Weickert and Adhya, 1992; Swint-Kruse and Matthews, 2009). A single short inverted repeat sequence was found in each promoter region (Fig. 4A). To confirm these putative operator sequences experimentally, DNase I footprinting experiments were carried out. Due to the higher affinity of MalR for the *malP* promoter, we performed these assays using the P*malP* region as template. The predicted MalR binding site was expected to span positions 185 to 198 of this region. The protected region in Fig. 4D coincides with the predicted positions. Interestingly, these motifs show strong similarity to the MalR operators identified in *S. pneumoniae* (Nieto *et al.*, 1997) and to the *E. faecalis* consensus *cre*-boxes, the binding sites of CcpA, which also belongs to the LacI/GalR family (Opsata *et al.*, 2010). The resulting MalR consensus sequence of *E. faecalis* is YGCAATCGGTTKCG (where Y can be C or T, K can be T or G). In the *malP* promoter, this sequence is located between the -10 and the putative -35 box, whereas in the *malT* promoter it is located between the transcription start site and the ribosome binding site (RBS) of *malT* (Fig. 4A). These positions are consistent with the repressor function of MalR, which may either sterically

hinder transcription initiation of RNA polymerase (at *malP*) or form a roadblock (at *malT*). Given that MalR was identified as the main regulator of the *mal* system, an in-depth characterisation was carried out.

The malR gene is expressed from two promoters. The *malR* gene has previously been shown to be expressed through the *malPBMR* operon (Le Breton *et al.*, 2005). However, the presence of a putative promoter has been determined by DNA sequence analysis at the end of the *malM* gene, upstream of *malR*. To test the function of this presumed promoter and to map its transcriptional start site (+1), we performed a Rapid Amplification of cDNA 5' Ends by Polymerase Chain Reaction (5' RACE PCR) on cells grown on maltose. The results confirmed that this promoter is indeed functional and that its +1 position is located 201 bp upstream of the predicted start codon of *malR* (Fig. 1B). A probable -10 box (TATTAT) is located upstream of the +1 extending from -8 to -13.

To further characterize the *malR* promoter P_R, we performed transcriptional fusion assays. A genome region of OG1RF containing P_R was cloned into plasmid pNZ273 upstream of a *gusA* reporter gene encoding the enzyme β-D-glucuronidase (Fig. 6) (Platteeuw *et al.*, 1994). After transforming *E. faecalis* OG1RF, β-D-glucuronidase activity was determined at 6 h after inoculation in ccM17 medium supplemented with glucose or maltose. The β-D-glucuronidase activity obtained with the P_R-*gusA* construction was about five-fold higher compared to control plasmids (empty pNZ273 and P_R inserted in the opposite direction) independently of the carbon source used by the bacteria (Fig. 6). These results confirmed that the promoter present upstream of the *malR* gene is functional and, in view of the low β-D-glucuronidase activity, allows a low and constitutive expression of *malR*. MalR auto regulates the expression of its gene from P_P. The additional constitutive promoter P_R at the end of *malM* allows the synthesis of a constant basal level of repressor, which prevents the cell from wasting energy in the absence of maltose.

The *malR* gene has previously been studied in *E. faecalis* T9, a strong biofilm-producing strain, and was originally named *bopD* (Hufnagel *et al.*, 2004). The authors showed that the *bopABCD* operon is implicated in biofilm formation especially in the presence of glucose and maltose (Creti *et al.*, 2006). They also noticed a residual expression of the *bopD* gene in a mutant with an insertion of a transposon in the upstream gene *bopB* (here named *malB*) of the *bopABCD* operon (Hufnagel *et al.*, 2004). This residual expression can now be explained by the internal promoter P_R identified in our study. Previous attempts to construct a *malR*$^-$ (*bopD*) mutant in the *E. faecalis* T9

strain had failed (Creti *et al.*, 2006). Here we obtained the *malR*$^{-}$ mutant in the OG1RF strain. Furthermore, in the three *E. faecalis* strains, JH2-2 (also named TX4000), EnGen0241 and EnGen0299 the well conserved MalR (>99% sequence identity) contain a non-conservative replacement of threonine-33 in the Helix-Turn-Helix (HTH) domain by a proline (T33P). We evidenced that the JH2-2 strain exhibits constitutive expression of the maltose genes even in the absence of the inducer similar to the *malR*$^{-}$ mutant of OG1RF (data not shown), suggesting that the MalR protein of JH2-2 might be non-functional. Altogether, these data suggest that MalR is not essential in most strains but might have additional functions than the regulation of maltose genes in other *E. faecalis* isolates.

Conclusion

In this study we demonstrate that *E. faecalis* MalR functions not only as repressor of the two *mal* operons, but that it is also involved in their CCR by probably using P-Ser-HPr as co-repressor. MalR therefore allows *E. faecalis* to specifically respond to the presence of maltose either as sole carbon source or together with rapidly metabolizable carbohydrates. Adaptation of bacteria to environmental conditions is crucial for efficient fitness. In oligotrophic milieu and under harsh competition, optimization of carbohydrate utilization can be essential for survival. The central sensor of CCR, P-Ser-HPr, is known to play a major role in metabolic regulation in *Firmicutes* (Deutscher, 2008) by functioning as co-repressor of the global regulator CcpA or by directly inhibiting non-PTS transporters (Ye *et al.*, 1996; Dossonnet *et al.*, 2000; Viana *et al.*, 2000; Monedero *et al.*, 2008; Homburg *et al.*, 2017). P-Ser-HPr was also shown to interact *in vitro* with the *B. subtilis* LacI/GalR type transcriptional regulator RbsR (Müller *et al.*, 2006), but no physiological function was reported for the complex. We demonstrate in our study that MalR interacts with P-Ser-HPr, but we were not able to establish an *in vivo* role of the complex in CCR, because the *ptsH1* mutant could not be obtained. Nevertheless, it is likely that the two proteins also form a complex in the cell to prevent induction of the *mal* genes in the presence of a preferred carbon source such as glucose. The implication of P-Ser-HPr in the formation of complexes with transcriptional regulators other than CcpA might be a more frequent CcpA-independent CCR mechanism in Gram-positive bacteria than presently known. For example, in *Listeria monocytogenes,* glucose caused CcpA-independent repression of the maltose and maltodextrin genes, which was relieved in an HprK/P mutant (Gopal *et al.*, 2010). The authors hypothesized an inducer exclusion mechanism but did not rule out another yet unknown

mechanism. In view of our results, the MalR protein of *L. monocytogenes*, a LacI type repressor, might also form a complex with P-Ser-HPr in order to exert CcpA-independent CCR.

Despite some similarities, there are specific differences between CcpA and MalR. With the exception of a few examples (Kim *et al.*, 1995), CcpA requires P-Ser-HPr to efficiently interact with *cre* sites. In contrast, MalR binds efficiently and specifically to its operators already in the absence of the co-repressor P-Ser-HPr. Both transcription regulators bind FBP, the presence of this glycolytic intermediate promotes the formation of the CcpA/P-Ser-HPr complex. However, MalR binds in addition unphosphorylated maltose. It is therefore likely that MalR has two distinct binding sites, one for FBP (phosphorylated and negatively charged), the exact role of which remains to be elucidated, and one for unphosphorylated maltose which diminished MalR affinity for its operator and which is required for induction of the *mal* operons. The FBP might play a dual role in maltose CCR in *E. faecalis* by stimulating the kinase function of HprK/P (Deutscher and Engelmann, 1984) and the formation of the MalR/P-Ser-HPr complex.

When searching for MalR binding sites elsewhere in the *E. faecalis* genome, we found two putative degenerated sites located in the promoters of physiologically related genes, which code for proteins required for the uptake and metabolism of maltotetraose and longer maltodextrins (Sauvageot *et al.*, 2017; Joyet *et al.*, 2017). In the closely related *E. faecium* E1162, the maltodextrin genes are positively regulated by an additional LacI/GalR type transcriptional regulator, MdxR (Zhang *et al.*, 2013). No homolog of MdxR is present in *E. faecalis*, suggesting that regulation of maltodextrin gene expression is differently controlled in this species. Investigations are under way to determine whether *E. faecalis* MalR is also implicated in the regulation of the two maltodextrin operons.

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Figures legends

Fig. 1. Presentation of the two operons required for maltose metabolism in *E. faecalis* OG1RF. **A.** Large arrows represent ORFs with their orientation indicating the direction of transcription. Gene names are written in the arrows, predicted or proven functions are indicated above and reference loci below the arrows. Promoters are symbolized by thin bent arrows. **B.** Presented are the nucleotide sequence of the chromosomal region between the *malM* and *malR* genes of *E. faecalis* OG1RF and the sequence data obtained with 5'-RACE-PCR. The first nucleotide downstream from the poly-A tail corresponds to the first transcribed nucleotide and is marked +1. The putative Pribnow box and ribosome binding site (RBS) of *malR* are underlined. **C.** Summary of maltose metabolism in *E. faecalis*. Implicated proteins are framed either with solid lines or, in the case of general PTS proteins, with stroke lines. Circles with minus or plus represent inhibitions/inactivations or activations, respectively.

Fig. 2. Relative expression of *malP* (**A**) and *malT* (**B**) in the parental strain OG1RF and the *malR*$^-$ and Δ*ccpA* mutants. Expression of the housekeeping *gyrA* gene was used as reference to normalize the total quantity of cDNA. Mean values calculated from at least three independent experiments are presented; error bars represent the standard deviations. Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$) determined with Holm-Sidak multiple comparisons versus the reference group OG1RF without assuming equal standard deviations.

Fig. 3. TSA performed with 2 µM of MalR. The temperature-induced exposure of hydrophobic surfaces (ΔT_h) in response to increasing concentrations of various compounds is presented. The mean values of three independent experiments are presented, errors bars indicate the standard deviations. Lines represent non-linear regression fitting data points (solid line for maltose and stroke line for FBP). Horizontal dotted line represents a ΔT_h of 0°C. Dissociation constants were determined using Differential Scanning Fluorimetry assuming a single binding event model.

Fig. 4. EMSA with DNA fragments containing one or both P*mal*. **A.** Schematic presentation of DNA regions used as template for EMSA. The assays were performed with 5 nM of Cyanine-5 labelled PCR products. Positions of the predicted MalR motifs with respect to the putative domestic promoter elements (-10 and -35) are indicated. **B.** EMSA results for MalR binding to P*2mal*. Maltose (final concentration of 5 mM) was added to the samples loaded on lanes 4, 5, 8 and 9 and P-Ser-HPr was added to the samples loaded on lanes 6 to 9. **C.** EMSA results for MalR binding to P*malP* (lanes 1 to 3), P*malT* (lanes 4 to 6) and an internal fragment of the *gusA* gene (lanes 7 to 9). **D.** DNAse I footprinting with a DNA fragment containing the region upstream from *malP* carried out in the absence (upper panel) and presence (lower panel) of MalR. The two read outs were aligned on an internal size standard (500 ROX), the positions of which are indicated by diamonds on the diagonal. The predicted MalR binding motif and the entire protected region are indicated by stroke lines.

Fig. 5A. MST assays were carried out with 5 nM of NT.647-labelled proteins (MalR or CcpA) and different concentrations of either HPr, P-Ser-HPr or P~His-HPr. The fraction of bound labelled protein is shown as a function of the concentration of the various HPr forms ranging from 9.76 nM to 20 μM. The fitting curve of the MalR/P-Ser-HPr interaction is shown in solid line and the fitting curve for CcpA/P-Ser-HPr is shown in disrupted line. Analysis with GraphPad Prism allowed the determination of the apparent K_D by assuming one specific binding site. **B and C.** Alignment of partial primary sequences of different interaction partners of P-Ser-HPr. The numbers of amino acids indicated below the sequences are based on the position in each studied protein. Amino acids identical in MalR and/or RbsR to amino acids of one of the CcpA's are in bold, conserved amino acids are underlined. Amino acids of CcpA which have been shown to interact with P-Ser-HPr are marked with a rectangle. Regions identified as CcpA-specific are underlined in grey, the insertion in MalR is marked in italics. (**B**) D69, D87 and D99 of one subunit of CcpA (*B. megaterium*) interact with R17 of P-Ser-HPr. (**C**) D296 of the other subunit of CcpA (*B. megaterium*) forms hydrogen bonds with unphosphorylated H15 of P-Ser-HPr, while R303 and K307 interact with the phosphate group bound to S46.

Fig. 6. β-glucuronidase activity in *E. faecalis* strains carrying various pNZ273-derived plasmids. Schematic presentation of the region upstream from the *gusA* gene in the pNZ273-derived plasmids and β-glucuronidase activity (in arbitrary units) determined with the corresponding construction. (1) No promoter in front of *gusA*. (2) Presumed P_R inserted upstream from *gusA* in the correct orientation and (3) in the opposite orientation. β-glucuronidase activities were measured after growth of the various *E. faecalis* strains transformed with pNZ273-derivative grown in medium containing 0.3% glucose or 0.3% maltose. The mean values of three independent experiments together with standard deviations are presented. Asterisks indicate significant differences (**: $p<0.01$; ***: $p<0.001$) determined with Holm-Sidak multiple comparisons versus the reference group pNZ273 without assuming equal standard deviations.

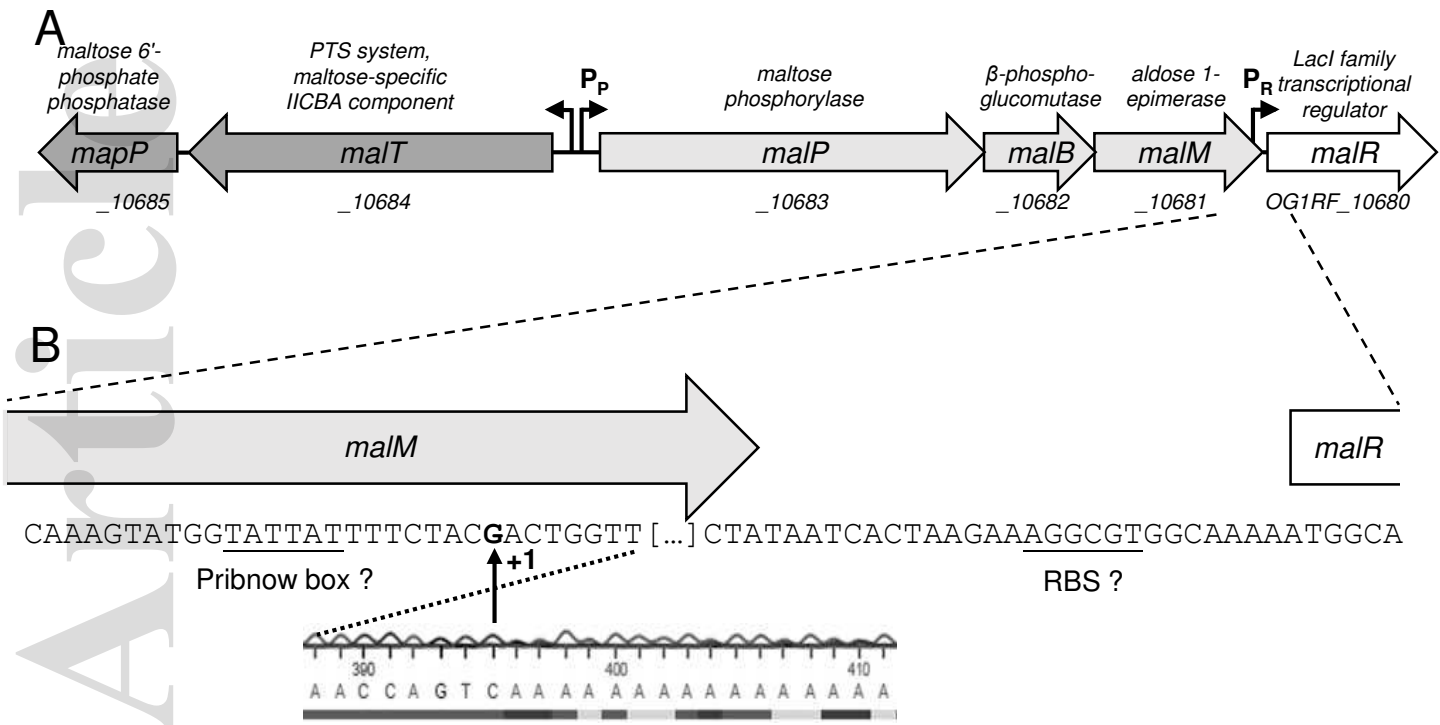
Supplementary Figures

Fig. S1. Relative expression of *malP* (**A**) and *malT* (**B**) in the parental strain OG1RF, the *malR*$^-$ mutant and the complemented *malR*$^-$ mutant carrying the pRB:*malR* plasmid. The *malR*$^-$ strain carrying the empty plasmid pRB473 was used to confirm that the expression profile remains unchanged by the presence of the plasmid. Expression of the housekeeping *gyrA* gene was used as reference to normalize the total quantity of cDNA. Mean values calculated from at least three independent experiments are presented; error bars indicate the standard deviations.

Fig. S2. EMSA with PCR products containing either P*2mal* or an internal *gusA* fragment. The PCR products were labelled with 5 nM of Cyanine-5. **A.** EMSA was carried out with DNA fragments containing either P*2mal* (lanes 1 to 5) or an internal *gusA* fragment (lanes 6 to 10), 2 µM P-Ser-HPr and various concentrations of CcpA (1.56 – 12.5 nM). **B.** EMSA was carried out with a P*2mal*-containing PCR fragment and various concentrations of MalR (12.5 - 50 nM) in absence (left panel) or presence (right panel) of 10 mM of FBP. Maltose (5 mM) was added to lanes 4 and 9 and 2 µM of P-Ser-HPr was added to lanes 7 and 9. **C.** EMSA carried out with a P*2mal*-containing PCR fragment and 2 µM of either HPr (lanes 2 and 5), P~His-HPr (lanes 3 and 6) or P-Ser-HPr (lanes 4 and 7) in the presence (lanes 2, 3 and 4) or absence (lanes 1, 5, 6 and 7) of MalR. The control with MalR alone is shown in Fig. 4B lane 2.

Fig. S3. TSA performed with 2 µM of MalR. The temperature-induced exposure of hydrophobic surfaces (ΔT_h) in response to increasing concentrations of various compounds is presented: glucose (**A**), glucose-6-P (**B**), maltotriose (**C**) and maltotriose-6"-P (**D**). The mean values of three independent experiments are presented; errors bars indicate the standard deviations. Horizontal dashed lines represent a ΔT of 0°C. Dissociation constants were determined using Differential Scanning Fluorimetry assuming a single binding event model with GraphPad Prism.

Figure 1



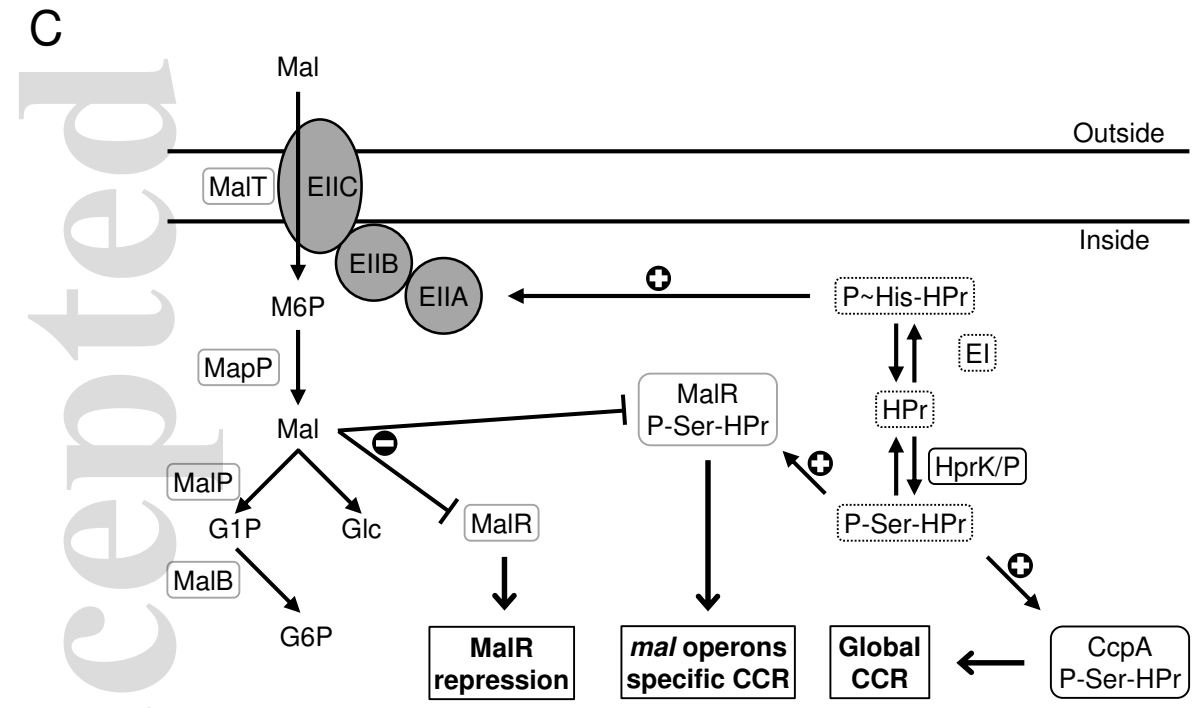


Figure 2

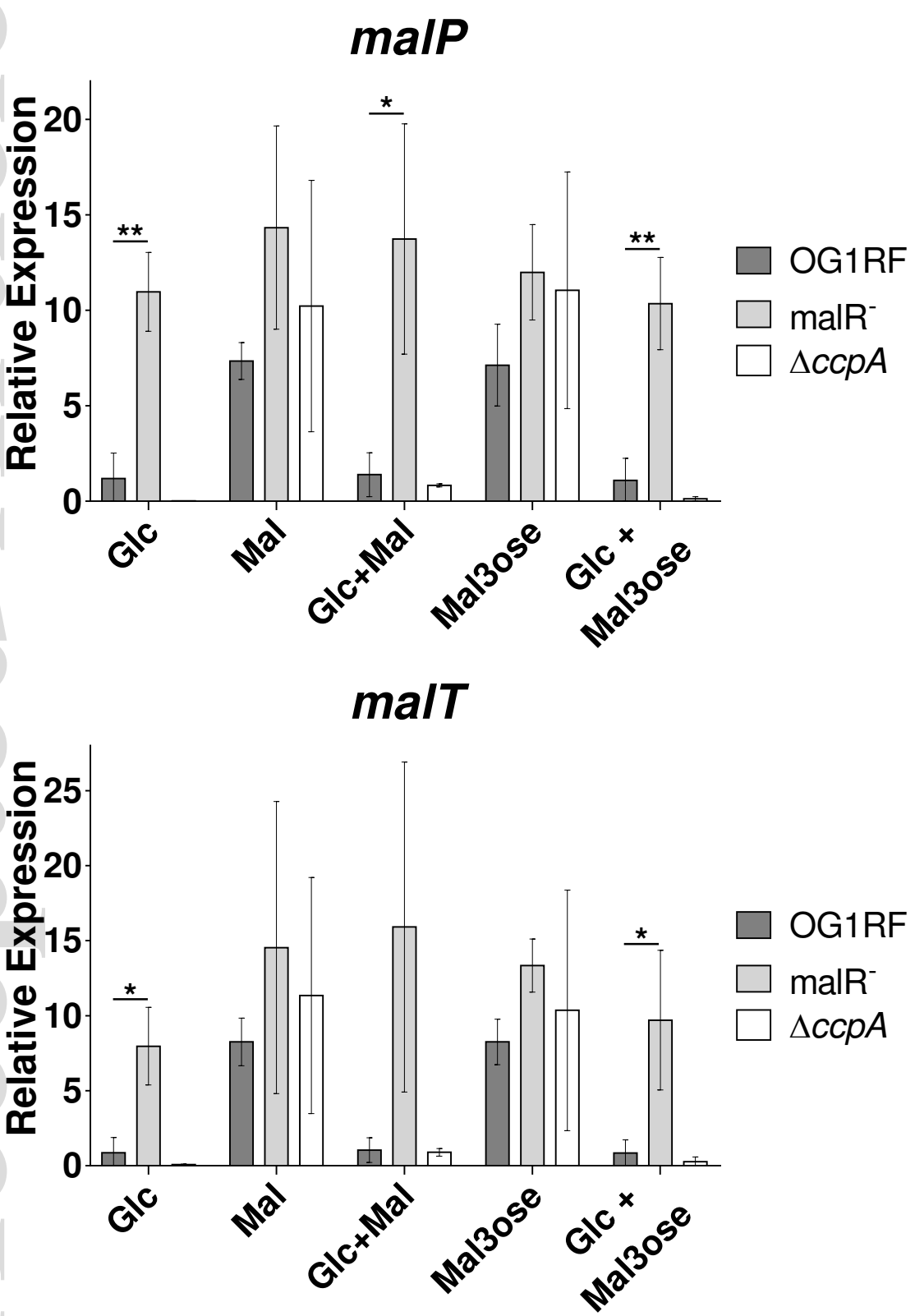


Figure 3

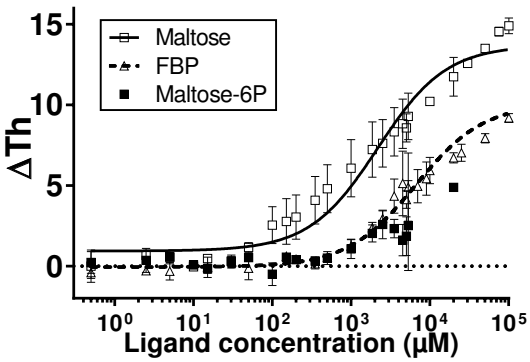


Figure 4

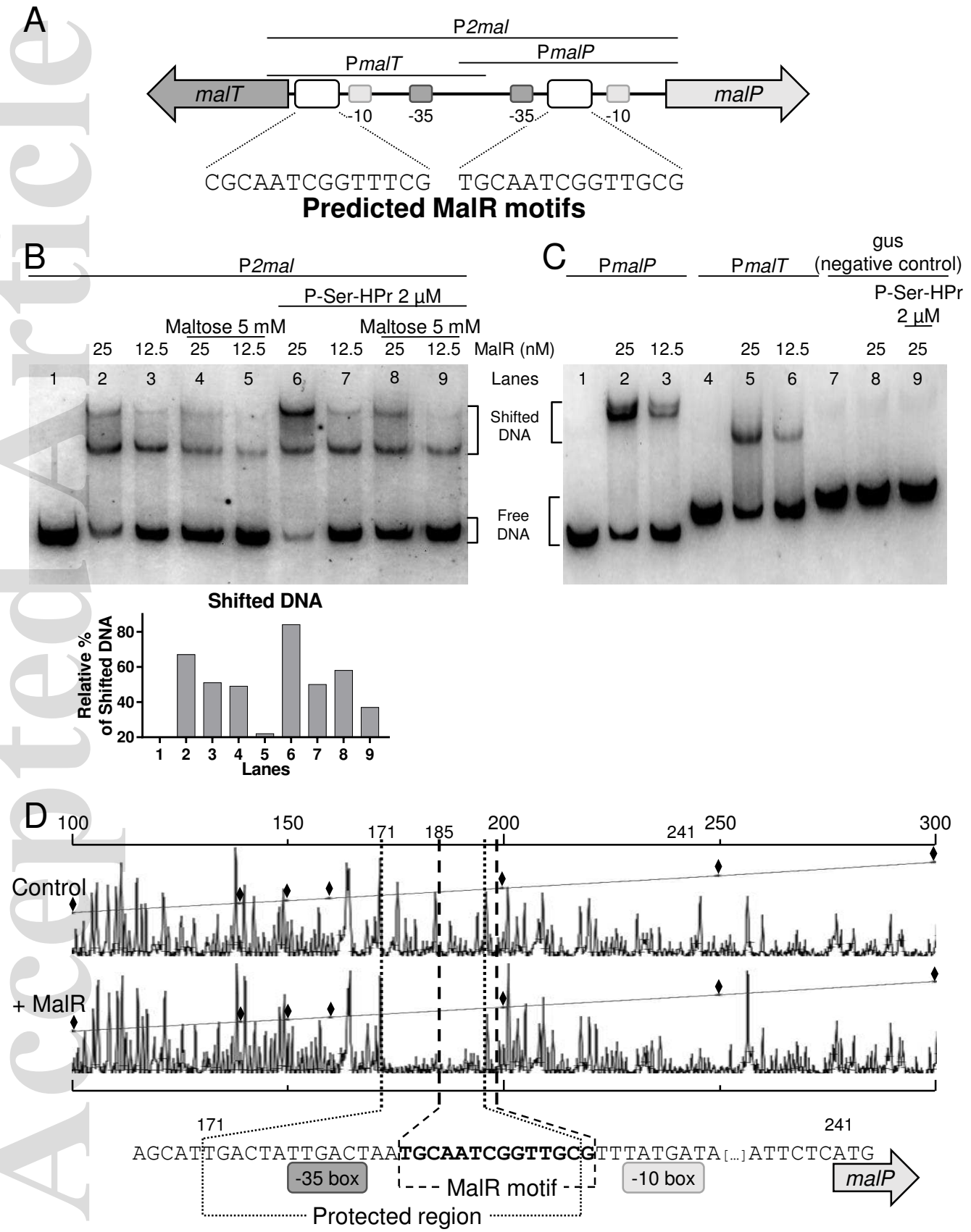
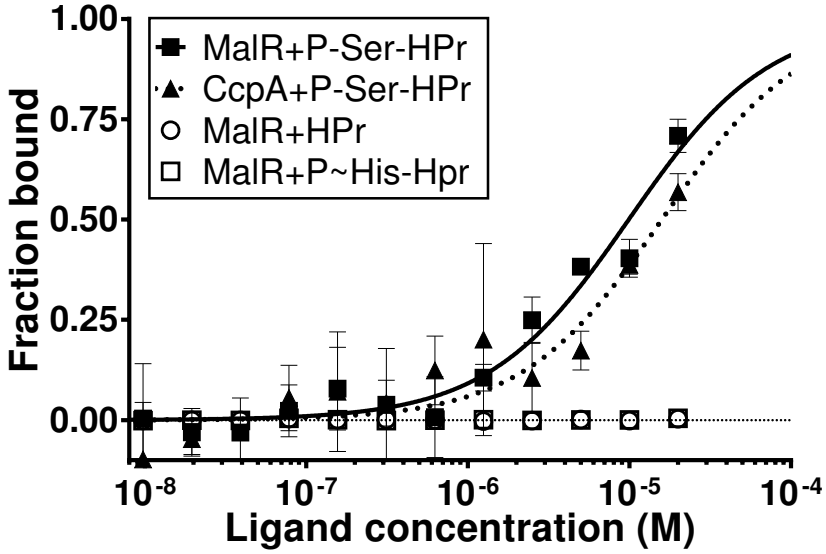


Figure 5

A



B

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CcpA (E. faecalis)      GVIIP------DVSNAFFASLARGIDDVATMYKYNIILANSDGDD
                        67         72                87              102
CcpA (B. subtilis)      GVIIP------DISSIFYSELARGIEDIATMYKYNIILSNSDQNM
                        65         70                85              100
CcpA (B. megaterium)    GVIIP------DISNIFYAELARGIEDIATMYKYNIILSNSDQNQ
                        64         69                84              99
MalR (E. faecalis)      GVILPPLDSKERLGNPFYLEIMEAINEEARLYDMTTAIATAKSFD
                        64         75                90              105
RbsR (B. subtilis)      GLLLP------DITNPFFPQLARGAEDELNREGYRLIFGNSDEEL
                        63         68                83              98
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C

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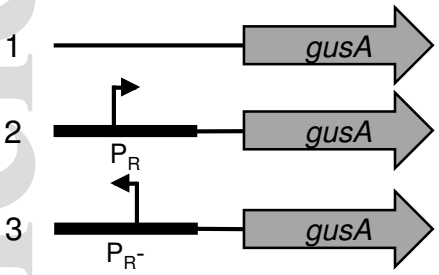
CcpA (E. faecalis)      PRLSSITQPLYDIGAVSMRLLTKLMNKE
                        286        297     304  308  313
CcpA (B. subtilis)      PQLSTVVQPTYDIGAVAMRLLTKLMNKE
                        286        297     304  308  313
CcpA (B. megaterium)    PQLTSVVQPMYDIGAVAMRLLTKYMNKE
                        285        296     303  307  312
MalR (E. faecalis)      PYLTSIDIDTSELGRMAMKKLYELIHEE
                        286        297     304  308  313
RbsR (B. subtilis)      PPLSTIKQPAYDMGKEAAKLLLGIIKKQ
                        276        287     294  298  303
```

Figure 6



	Glucuronidase activity (AU)	
	Glucose	Maltose
PNZ273	3.12 ± 1.81	4.70 ± 3.39
PNZ:P_R	30.09 ± 3.49 (***)	28.77 ± 3.54 (**)
PNZ:P_R-	4.94 ± 3.20	5.49 ± 3.56