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Characterization of a Genetic Locus Essential for Maltose-Maltotriose Utilization in *Staphylococcus xylosus*

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A genetic locus from *Staphylococcus xylosus* involved in maltose-maltotriose utilization has been characterized. The chromosomal region was identified by screening a genomic library of *S. xylosus* in *Escherichia coli* for sucrose hydrolase activity. Nucleotide sequence analysis yielded two open reading frames (*malR* and *malA*) encoding proteins of 37.7 and 62.5 kDa, respectively. MalR was found to be homologous to the LacI-GalR family of transcriptional regulators, and MalA showed high similarity to yeast α-1,4-glucosidases and bacterial α-1,6-glucosidases. Inactivation of *malA* in the genome of *S. xylosus* led to a maltose-maltotriose-negative phenotype. In cell extracts of the mutant, virtually no glucose release from maltose and short maltodextrins was detectable. Inactivation of *malA* in a sucrose-6-phosphate hydrolase-deficient *S. xylosus* strain resulted in the complete loss of the residual sucrose hydrolase activity. The MalA enzyme has a clear preference for maltose but is also able to release glucose from short maltosaccharides. It cannot cleave isomaltose. Therefore, *malA* encodes an α-1,4-glucosidase or maltase, which also liberates glucose from sucrose. Subcloning experiments indicated that *malA* does not possess its own promoter and is cotranscribed with *malR*. Its expression could not be stimulated when maltose was added to the growth medium. Chromosomal inactivation of *malR* led to reduced maltose utilization, although α-glucosidase activity in the *malR* mutant was slightly higher than in the wild type. In the mutant strain, maltose uptake was reduced and inducibility of the transport activity was partially lost. It seems that MalR participates in the regulation of the gene(s) for maltose transport and is needed for their full expression. Thus, the *malRA* genes constitute an essential genetic locus for maltosaccharide utilization in *S. xylosus*.

Maltose-maltodextrin utilization has been studied mainly in gram-negative enteric bacteria (38). The maltose regulon of *Escherichia coli*, which is controlled by a positive regulator MalT (30), consists of a number of genes encoding proteins responsible for uptake and metabolism of maltosaccharides. These enter the periplasmic space through a diffusion pore, the product of *lamB* (38). Uptake of maltose and maltodextrins up to maltoheptaose into the cytoplasm is mediated by a multicomponent, binding protein-dependent transport system encoded by *malE*, *malF*, *malG*, and *malK* (38). Degradation of maltodextrins is achieved by the action of three cytoplasmic enzymes, amylomaltase (21), maltodextrin phosphorylase (34), and maltodextrin glucosidase (29, 43), the gene products of *malQ*, *malP*, and *malZ*, respectively, and a periplasmic α-amylase (24, 36) specified by *malS*, which is required only for growth on long maltodextrins. None of these enzymes is able to cleave maltose itself, but they recognize maltotriose or longer maltodextrins as substrates. Thus, maltose utilization in *E. coli* depends on the formation of maltotriose as the shortest substrate for the degradative enzymes and, equally important, as the inducer for the activator protein MalT (6, 25). A genetic locus containing *malI*, *malX*, and *malY* is involved in the induction process (27, 28), but it seems that at least one more component of the system is still missing (5).

In gram-positive bacteria, work on the maltosaccharide utilization system has been focused on *Streptococcus pneumoniae*. A gene cluster which contains at least seven genes involved in maltodextrin utilization has been identified (40, 41). Within this cluster are the genes encoding amylomaltase and maltodextrin phosphorylase (*malM* and *malP*), proteins for maltotetraose transport (*malXCD*) and utilization (*malA*) (22), and a central regulator (*malR*) (23). In contrast to the positive regulation in *E. coli* by MalT, the maltodextrin utilization system of *S. pneumoniae* seems to be negatively controlled by MalR (23).

We are interested in carbohydrate utilization and carbohydrate-specific gene regulation in the gram-positive bacterium *Staphylococcus xylosus*. The organism was isolated originally from human skin (35) but is also found on farm animals, in dairy products, and in fermented sausages. Because of its nonpathogenic nature, *S. xylosus* may serve as a suitable host for studies of staphylococcal gene regulation in the laboratory. In an attempt to characterize the components of the sucrose utilization system of *S. xylosus*, a gene mediating sucrase activity was isolated from a genomic library of *S. xylosus* DNA in *E. coli*. It was distinct from the previously characterized gene for the sucrose-6-phosphate hydrolase of that organism (4). The genetic locus isolated in this way turned out to be essential for maltose utilization in *S. xylosus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage DNAs. *S. xylosus* C2a, a derivative of *S. xylosus* DSM20267 (35) that had been cured of its endogenous plasmid pSX267 (8), was used for the isolation of chromosomal DNA. HB101(*lac*$^+$) (3), a spontaneous *lacY1* revertant of *E. coli* HB101 [*supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 leuB6 rpsL20 xyl-5 mtl-1 galK2 lacY1*], was used to screen the *S. xylosus* library and for subcloning experiments. M13 cloning (16) for sequencing was performed in *E. coli* TG1 [*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)]. The *S. xylosus* library (4) had been constructed with pBR322 (42). Subcloning for testing *malA* expression was carried out with the shuttle vector pRB473, a derivative of pRB373 (2) that confers chloramphenicol resistance. Subcloning for sequencing was done with M13mp18 or M13mp19 (49). Gene inactivation in *S. xylosus* by homologous recombination was carried out with plasmid pBT6, a derivative of the shuttle vector pBT2 (4), whose replication is temperature sensitive in gram-positive bacteria. To select integration into the

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chromosome, an erythromycin resistance cassette (*ermB*) from transposon Tn*551* (19) flanked by various restriction sites was used. *S. xylosus* TX53, a mutant with a deletion within the sucrase (*scrB*) gene (3), was used to construct the double mutant strain TX151.

Growth media, DNA manipulations, transformation, and transfection. DNA manipulations, plasmid DNA isolation, Southern blot analysis, transformation and transfection of *E. coli*, and preparation of media and agar plates for bacterial growth were done by standard procedures (32). Chromosomal DNA from *S. xylosus* was prepared by the method of Marmur (15). Construction of the genomic library of *S. xylosus* in *E. coli* and screening for sucrase activity have been described previously (4). Plasmids were introduced into *S. xylosus* and *S. carnosus* by protoplast transformation (7). DNA sequencing was done by the dideoxy-chain termination method (33). Analytical PCR (18) analysis was performed with *Taq* polymerase (Boehringer Mannheim GmbH). PCR for cloning was carried out with Vent polymerase (New England Biolabs). The primer used to construct the *malR* deletion hybridized to nucleotide positions 424 to 442 (see Fig. 2). *S. xylosus* was grown in B medium, which consists of 1% peptone (catalog no. 140; Gibco, BRL), 0.5% yeast extract, 0.5% NaCl, and 0.1% K_2HPO_4. For induction experiments, 0.5% the respective sugar was added. Fermentation of carbohydrates by *S. xylosus* was monitored on agar plates (17) containing 0.5% the respective sugar.

Determination of α-glucosidase and sucrase activities. Sucrase activity in bacterial extracts prepared by disrupting the cells with glass beads was quantified by measuring the release of glucose from sucrose as described previously (4). The amount of glucose was determined by means of a commercially available test kit (Boehringer Mannheim). Protein concentrations in cell extracts were determined by the method of Bradford (1). Sucrase activity is expressed in nanomoles of glucose released per minute per milligram of protein.

For the determination of α-glucosidase activity, either maltose and longer maltodextrins or substrate analogs of various chain lengths containing a *p*-nitrophenyl (*p*-NP) group were applied. For this purpose, cells were also opened by glass beads. With maltosaccharides (20 mM), the release of glucose was measured. With the *p*-NP compounds (10 mM), the appearance of yellow color was monitored at 405 nm. Enzyme activities are expressed in nanomoles of glucose released per minute per milligram of protein or nanomoles of *p*-NP produced per minute per milligram of protein.

Kinetic parameters of glucose release from substrates with different chain lengths were determined by using 0.12 mg of cellular protein. The release of nitrophenol from *p*-NP-α-glucoside was monitored accordingly. The reactions followed Michaelis-Menten kinetics. K_m and V_{max} were determined on double-reciprocal plots.

Measurements of maltose transport. Transport of maltose was measured by using whole cells grown in B medium supplemented with 0.5% maltose or without the addition of carbohydrate. Cells were harvested at an optical density at 578 nm of 1.5, washed in 0.1 M phosphate buffer, (pH 6.6), and resuspended in the same buffer to a final density of 10^8 cells per ml. After addition of [^{14}C]maltose (100 μM, 13 mCi/mmol) to 1 ml of the cell suspension, the cells were incubated at 25°C, and 0.15-ml samples were taken at intervals, collected on cellulose nitrate disks (pore size, 0.45 μm), and washed with 5 ml of phosphate buffer. Filters were dried at 80°C for 30 min, and the radioactivity was determined by liquid scintillation counting. Uptake rates are expressed in nanomoles of maltose accumulated per minute per milligram cell protein.

Nucleotide sequence accession number. The nucleotide sequence is stored in the EMBL database under accession number X78853.

RESULTS

Cloning of a gene mediating sucrase activity. During the characterization of *scrB*, the gene encoding the sucrose-6-phosphate hydrolase of *S. xylosus*, we detected a residual sucrose hydrolase activity in a *scrB* null mutant (4). To clone the gene encoding this minor sucrase of *S. xylosus*, aliquots of a genomic library ligation, which had been prepared to clone the *scrB* gene (4), were plated to yield about 5,000 *E. coli* transformants. The raffinose fermentation screen to detect sucrase activity (31) was applied, and five red colonies were detected. Four of the clones contained plasmids with the *scrB* sucrase gene, but one transformant harbored a plasmid carrying another region of the *S. xylosus* genome. The plasmid (pRA5), which had an insert of about 20 kb, was reintroduced into *E. coli* HB101(*lac*$^+$). A sucrase$^+$ phenotype was observed for all transformants. Two overlapping fragments of pRA5, a 5.5-kb *Nhe*I-*Sst*I fragment in plasmid pSH1 and a 4.0-kb *Xba*I-*Hin*dIII fragment in pSH3 (Fig. 1), that mediated sucrase activity were identified. The latter fragment was shortened by about 0.5 kb with *Bal* 31 from the *Xba*I end. The resulting blunt end-*Hin*dIII fragment was cloned into pRB473 to yield pSH5 (Fig. 1).

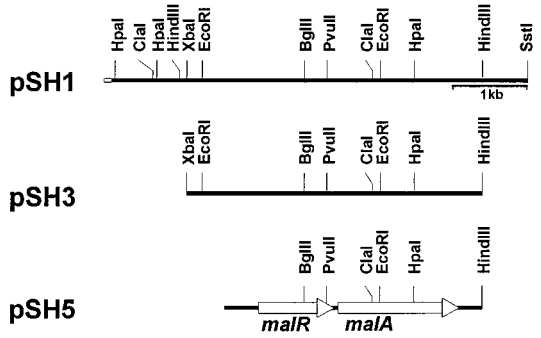

FIG. 1. Physical map of the *malRA* region. Restriction maps of the insertions in plasmids pSH1, pSH3, and pSH5 are shown. Plasmid pSH1 contains a 5.5-kb *Sau*3AI-*Sst*I fragment of *S. xylosus* C2a chromosomal DNA. It was excised as a *Nhe*I-*Sst*I fragment from the pBR322 derivative pRA5 and inserted into the *Xba*I-*Sst*I sites of the vector pRB473. The open bar at the left end of the fragment represents pBR322 DNA. Plasmid pSH3 consists of the *Xba*I-*Hin*dIII fragment of pSH1 cloned into pRB473, and pSH5 was generated by insertion of a 3.5-kb blunt end-*Hin*dIII fragment into the *Sma*I-*Hin*dIII sites of pRB473. Arrows indicate the positions, sizes, and orientations of the ORFs *malR* and *malA*. Map units are given in kilobases.

Further subcloning, using the restriction sites shown in Fig. 1, resulted in white colonies on MacConkey raffinose plates. Therefore, the insertion of 3.5 kb in pSH5 comprised the shortest fragment mediating sucrase activity in *E. coli.*

Sequence analysis of the cloned DNA. The nucleotide sequence of the 3.5-kb insertion was determined on both strands. It consisted of 3,536 bp and contained two fairly large open reading frames (ORFs) on one strand (Fig. 2). The ATG at position 439 (Fig. 2), which is preceded by a potential Shine-Dalgarno sequence (39), constitutes the most likely start site for ORF1. The deduced polypeptide has a length of 337 amino acids with a calculated molecular mass of 37.7 kDa. The probable translation initiation region for ORF2 is found around position 1450. Starting with the ATG at 1454, the deduced protein has 549 amino acids and a molecular mass of 64 kDa. Similarity searches in databases revealed that the ORF1 gene product belongs to the LacI-GalR family (47) of transcriptional regulators. The characteristic amino-terminal helix-turn-helix motif of this protein family is clearly present in the deduced ORF1 protein (Fig. 2). The polypeptide encoded by ORF2 showed a high degree of similarity to α-1,6-glucosidases from bacilli and α-1,4-glucosidases from yeasts. It shares 52% identity with oligo-1,6-glucosidases from *Bacillus cereus* (46) and *Bacillus thermoglucosidasius* (45) and 41% identity with α-1,4-glucosidase from *Saccharomyces cerevisiae* (14) and maltase from *Saccharomyces carlsbergensis* (11). Although the genetic locus had been detected by its ability to mediate sucrose hydrolase activity, no similarity with sucrose-specific proteins was detected.

Inactivation of ORF2 in the chromosome of *S. xylosus*. To elucidate the function of ORF2 in *S. xylosus*, an insertion mutant was constructed. For that purpose, the erythromycin resistance gene *ermB* of Tn*551* (19) was cloned between the *Bgl*II and *Cla*I sites of pSH3 (Fig. 1), deleting the 3′ end of ORF1 and two-thirds of ORF2, including its translation initiation region. The resulting insertion (Fig. 3A) was moved as an *Xba*I-*Hin*dIII fragment into the shuttle plasmid pBT6, which is a derivative of pBT2 (4) containing a multiple cloning site. Like pBT2, it shows temperature-sensitive replication in gram-positive hosts. The ORF2-inactivated plasmid, pAE1, was introduced into *S. xylosus* C2a, and erythromycin-resistant colonies were obtained after plasmid curing at a high temperature

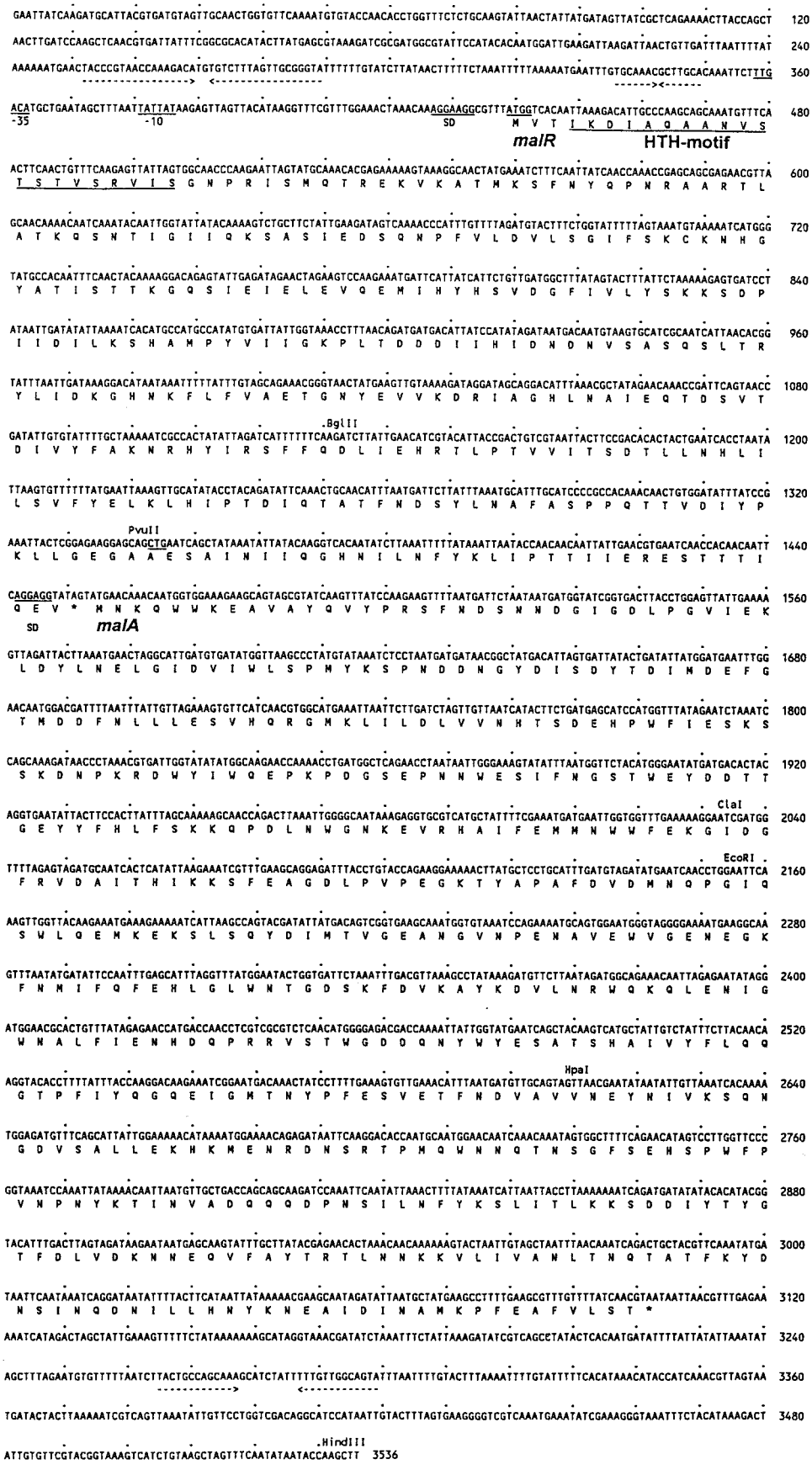
-35
-10
SD
malR
HTH-motif
PvuII
BglII
SD
malA
ClaI
EcoRI
HpaI
HindIII

FIG. 2. Nucleotide sequence of the *malRA* locus and deduced amino acid sequences of its two gene products. Three inverted repeats are indicated by broken arrows. The first and the third inverted repeats are followed by a stretch of T's resembling rho-independent terminators. The inverted repeat at positions 336 to 349 shows high similarity to the catabolite-responsive element (12). A potential promoter region upstream of *malR* (−10, −35) and the potential Shine-Dalgarno (SD) sequences of *malR* and *malA* are underlined. An amino acid sequence in MalR resembling the helix-turn-helix motif of the LacI-GalR transcriptional regulator family is underlined and indicated as HTH-motif. Nucleotides at positions 441 and 1340 represent the fusion sites for the *malR* deletion in *S. xylosus* TX152.

(40°C) as described previously (4). The predicted chromosomal arrangement (Fig. 3A) produced by a double-crossover event was confirmed by Southern blot and PCR analyses (data not shown). The phenotype of one representative clone, TX150, was further analyzed.

As a first approach, utilization of sucrose, isomaltose, maltose, maltotriose, and longer maltodextrins up to a chain length of seven glucose units was monitored on indicator plates (17). Wild-type *S. xylosus* C2a was able to utilize sucrose, maltose, and, less efficiently, maltotriose, but it could not use longer maltodextrins or isomaltose. The ORF2 insertion mutant TX150 could not ferment maltose and maltotriose, whereas sucrose utilization was not affected. Therefore, ORF2 is essential for maltose-maltotriose utilization in *S. xylosus*. It was designated *malA*.

Substrate specificity of the MalA enzyme. The phenotype of the *malA* mutant suggested that the gene encodes an α-1,4-glucosidase rather than an α-1,6-glucosidase. To confirm this result, cell extracts of the wild-type *S. xylosus* C2a and the *malA* mutant TX150 were tested for sugar-hydrolyzing activities. As summarized in Table 1, glucose is produced from maltose, and to a lesser extent from maltotriose and maltopentaose, in crude extracts of wild-type cells, but it could not be detected in TX150 extracts incubated with the same substrates. Even after 24 h of incubation, no glucose was produced (data not shown). Therefore, the observed cleavage of maltose and maltodextrins in wild-type cells must be due to the MalA enzyme. The extracts were also tested with α-1,4-glucosides of different chain lengths containing a *p*-NP group as substrates. In this case, nitrophenol release was observed only with *p*-NP-α-glucoside (15 nmol of nitrophenol per min per mg of protein) but could not be detected with *p*-NP-α-maltoside or *p*-NP-α-maltopentaoside. Isomaltose was not cleaved by *S. xylosus* cell extracts. These results identify MalA as an α-1,4-glucosidase, which is consistent with the maltose-maltotriose-negative phenotype of the *malA*-insertion mutant TX150.

To characterize the substrate specificity of the enzyme more closely, we measured the saturation kinetics of glucose release from different maltosaccharides (Table 2). Maltose and maltotriose showed almost identical binding to the enzyme, but the maximum velocity of glucose release was about twofold higher for maltose. For maltopentaose, affinity was about fivefold lower, but hydrolysis was comparable to the rate for maltotriose. With amylose as the substrate, practically no glucose production was detectable (data not shown). We also determined V_{max} and K_m values for *p*-NP-α-glucoside, the only *p*-NP substrate that is recognized by MalA. Both binding and cleavage of the synthetic substrate are less than for the corresponding carbohydrate maltose; cleavage was strongly (eightfold) reduced. The results of the kinetic measurements indicate that the MalA enzyme has a clear preference for maltose and may be regarded as a maltase. Apparently, MalA showed a relatively low affinity for the tested substrates. The use of crude extracts and reaction conditions that have not been optimized may account for this observation.

Construction of a *malA-scrB* mutant strain. Since the *malA* gene had been identified as a sucrase-encoding gene, we wanted to determine whether MalA was indeed responsible for the residual sucrase activity in the *scrB* mutant (4). For that purpose, an *scrB-malA* double mutant was constructed by introducing the erythromycin resistance gene (*ermB*) at the *malA* locus (Fig. 3A) in the *scrB*-deletion mutant TX53. In extracts of the resulting strain, TX151, virtually no sucrose hydrolase activity could be detected (Table 1). Even in TX150, a reduction in sucrase activity was measurable when the cells where grown without sucrose and the gene for the major sucrase remained uninduced. Thus, MalA is able to cleave sucrose,

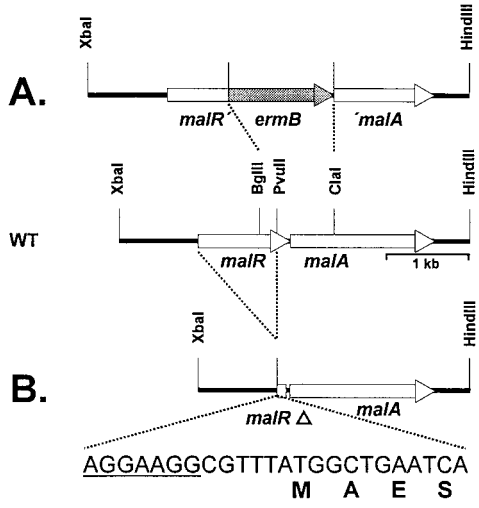

FIG. 3. Genomic organization of the *malRA* region in the deletion mutants TX150 and TX152. The wild-type (WT) *malRA* organization is shown in the middle. (A) Structure of the *malRA* locus in *S. xylosus* TX150. The positions and orientations of genes are represented by arrows. Relevant restriction sites are marked. (B) Structure of the *malRA* locus in *S. xylosus* TX152. The positions and orientations of genes are indicated by arrows. Relevant restriction sites are marked. The DNA sequence of the internally deleted *malR* gene (*malR*Δ) at the start codon-alanine codon junction (position 1339) is also shown, along with the deduced amino acid sequence of the internally truncated protein around the fusion joint.

TABLE 1. Glucose release from carbohydrates by cell extracts of *S. xylosus*

Carbohydrate[a]	Glucose release (nmol of glucose produced/min/mg of protein)[b]			
	S. xylosus C2a	*S. xylosus* TX150 (Δ*malA*)	*S. xylosus* TX53 (Δ*scrB*)	*S. xylosus* TX151 (Δ*scrB* Δ*malA*)
Maltose	356	<1	ND	ND
Maltotriose	165	<1	ND	ND
Maltopentaose	128	<1	ND	ND
Sucrose	267	196	47	<1
Isomaltose	<1	<1	ND	ND

[a] The concentration of carbohydrates in the assays was 20 mM except for sucrose (100 mM).

[b] The assays were performed with aliquots of extracts prepared from 20 ml of cells grown in B medium without additional carbohydrate to an optical density at 578 nm of 2.0. Mean values were taken from three measurements, each with bacterial extracts from two cultures. Standard deviations were in the range of ±15%. ND, not determined.

TABLE 2. Kinetic parameters of α-glucosidase activity in cell extracts of *S. xylosus*

Substrate[a]	V_{max} (nmol/min/mg of protein)[b]	K_m (mM)[b]
Maltose	372	0.9
Maltotriose	176	1.2
Maltopentaose	161	5.1
p-NP-glucoside	21	6.9

[a] Substrate concentrations were 0.1, 0.5, 1.0, 1.5, 2.5, 3.0, 5.0, 7.5, 10.0, 15.0, and 20.0 mM. In the assays with maltosaccharides as substrates, glucose release was measured. In the assays with *p*-NP-glucoside as the substrate, release of nitrophenol was determined.

[b] Assays were performed with 0.12 mg of cellular proteins. Values were determined from double-reciprocal plots and represent mean values calculated from three independent measurements with bacterial extracts from one culture of *S. xylosus* C2a.

which explains its initial identification by the sucrase activity screening.

Complementation of the ORF2 (*malA*) mutant. In the *malA-ermB* insertion mutant TX150, part of ORF1 had also been deleted. To confirm that the observed maltose-maltotriose-negative phenotype is caused by the loss of MalA function, a plasmid containing only *malA* was constructed for complementation. The mutant strain also provided the opportunity to test several plasmids for α-glucosidase expression in the natural host.

During the initial subcloning experiments in *E. coli*, a plasmid that contained a *Bgl*II-*Sst*I fragment (pSH6) was obtained. The plasmid did not express *malA*, although the complete coding sequence is present (Fig. 2). Obviously, the *malA* promoter is missing. To provide a promoter for *malA* expression, the *Bgl*II-*Sst*I fragment was moved to pRB474, a derivative of pRB374 (2). In the resulting plasmid, pSH7, *malA* is transcribed from the strong *veg*II promoter of *Bacillus subtilis* (20), which also functions in *S. xylosus*. The mutant strain TX150 was transformed with pSH6, pSH7, and, as controls, pSH1, pSH3, and pSH5 and the vectors pRB473 and pRB474. The transformants were tested for maltose-maltotriose utilization on plates, and cell extracts were assayed for α-glucosidase activity.

Transformants harboring pSH1 (*malRA*), pSH3 (*malRA*), pSH5 (*malRA*), and pSH7 (*veg*II-*malA*) regained the maltose-maltotriose-positive phenotype (Table 3), whereas cells with pSH6 (*malA*) did not. As expected, Mal^+ clones had α-glucosidase activity (Table 3). Even in pSH6-containing cells, *malA* expression was detectable but obviously not sufficient to complement the MalA deficiency. The results of the complementation tests show that the loss of MalA function is indeed responsible for the Mal^- phenotype of TX150 and that the *malA* gene does not possess a promoter within 300 bp upstream of the *malA* reading frame (Fig. 2).

TABLE 3. α-Glucosidase activity in *S. xylosus* TX150 (Δ*malA*) mediated by plasmids containing different parts of the *malRA* region

Plasmid	*mal* region	Phenotype (maltose utilization)[a]	α-Glucosidase activity (nmol of nitrophenol produced/min/mg of protein)[b]
pSH1	*malRA*	+	37
pSH3	*malRA*	+	38
pSH5	*malRA*	+	35
pSH6	*malA*	−	1
pSH7	*veg*II-*malA*	+	421
pRB473		−	<1
pRB474		−	<1

[a] Evaluated by the appearance of red color caused by acid production on sugar indicator plates containing 0.5% maltose.

[b] Determined with *p*-NP-glucoside as the substrate at a concentration of 10 mM. Mean values were taken from two measurements, each with cell extracts from two cultures. Standard deviations were in the range of ±10%.

Inactivation of ORF1 (*malR*) in the chromosome of *S. xylosus*. Since the ORF1 gene product showed similarity to the LacI-GalR regulator family, we anticipated that the gene product would take part in maltose regulation; it was thus designated *malR*. To test its regulatory function, a *malR* null mutant strain was constructed.

Since the results of the subcloning experiments indicated that *malA* is cotranscribed with *malR*, we designed a strategy to minimize polar effects of *malR* disruption on *malA* expression. A DNA fragment containing the genomic region in front of *malR* up to the *Xba*I restriction site (Fig. 1) was amplified from plasmid pSH3 as the template by PCR. One primer was complementary to vector sequences beyond the *Xba*I site (Fig. 1), and the other was complementary to positions 424 to 442 in *malR* (Fig. 2), covering the translation initiation region and the start codon. After the PCR product was cut with *Xba*I, the resulting *Xba*I-blunt end fragment was cloned into pSH1 (Fig. 1), which was cleaved with *Xba*I and *Pvu*II, to yield plasmid pDR1. At the PCR fragment-*Pvu*II junction within *malR*, the sequence ATGGCTG was created (Fig. 3B), as determined by DNA sequencing. This results in the fusion of sequences encoding 36 amino acids at the carboxy terminus of MalR to the *malR* start codon (Fig. 2 and 3B). The deduced polypeptide lacks 300 amino acids of MalR, including the residues that constitute the helix-turn-helix DNA binding motif. Therefore, the introduced deletion (*malR*Δ) should lead to a completely nonfunctional protein without interrupting translation of the mRNA.

To integrate the internally truncated *malR*Δ gene into the genome of *S. xylosus*, we could not use the wild-type strain, because no selectable resistance marker had been cloned in *malR* and we did not expect an easily detectable phenotype for the *malR* deletion. But with the *malA* mutant TX150, it seemed feasible to devise a selection. In the course of creating specific disruptions of several genetic loci with resistance genes in *S. xylosus*, we observed mutant colonies to arise at a rather high frequency (3). Molecular analysis of some of these mutants indicated that gene conversion by double crossover occurred. It was conceivable, therefore, that the truncated *malR* gene could spontaneously integrate into the chromosome of TX150 and replace *ermB* (Fig. 3A). This would then lead to at least one detectable phenotype, erythromycin sensitivity, and perhaps to a second, the ability to utilize maltose.

Plasmid pDR1 was introduced into TX150, and the transformants were patched on maltose fermentation test plates. All colonies were Mal^+, indicating that *malA* was expressed and complemented the MalA defect in TX150. One transformant was grown overnight in medium containing chloramphenicol. Dilutions were plated on nonselective agar plates, and 300 colonies were checked for erythromycin resistance, maltose utilization, and chloramphenicol resistance. Two erythromycin-sensitive, Mal^+ clones were detected, but both were chloramphenicol resistant, indicating that the plasmid was still present. To cure the cells from pDR1, they were grown without selection for about 15 generations and plated onto nonselective medium. The antibiotic resistance of 100 colonies for each isolate was tested. About 40% of the colonies were erythromycin and chloramphenicol sensitive. The genomic organization at the *malRA* locus of several clones was analyzed by Southern blotting and PCR and found to be as depicted in Fig.

TABLE 4. Characterization of the *S. xylosus malR* deletion mutant TX152 and inducibility of the maltose utilization system in *S. xylosus* C2a[a]

S. xylosus strain	α-Glucosidase activity (nmol of nitrophenol produced/min/mg of protein)[b]		Maltose transport (nmol of maltose transported/min/mg of protein)[c]	
	−Maltose	+Maltose	−Maltose	+Maltose
C2a	13	14	16	130
TX150 (*malR′* Δ*malA*)	NA	NA	9	30
TX152 (*malR*Δ)	20	19	8	36

[a] Cultures were grown in B medium containing 0.5% maltose or without addition of a carbohydrate to an optical density at 578 nm of 2.

[b] α-Glucosidase assays were performed with *p*-NP-glucoside (10 mM) as the substrate. Values represent averages of two measurements, each with bacterial extracts of two cultures. Standard deviations were in the range of ±10%. NA, not applicable.

[c] Determined by using whole cells and 100 μM [^{14}C]maltose (13 mCi/mmol). Mean values were taken from three assays, each of two cultures. Standard deviations were in the range of ±20%.

3B. Somewhat surprisingly, these clones developed a less intense red color on the maltose test plates than the wild type did. One colony, TX152, was then chosen for further analysis.

Phenotype of the *malR* null mutant. Maltase activity was determined in cells grown in B medium without additional carbohydrate and supplemented with maltose. As summarized in Table 4, no significant induction of maltase activity could be detected in the wild-type strain after addition of maltose to the growth medium. In the *malR*Δ mutant TX152, α-glucosidase activity rises about 1.5-fold under all conditions. It seems that there is no maltose-specific regulation of *malA* expression even in the wild-type strain. Consequently, there is virtually no effect of *malR* deletion on *malA* expression. The slight increase of MalA activity in TX152 may be a consequence of shortening the mRNA. It also demonstrates that the introduced mutation in *malR* was indeed nonpolar for *malA* expression.

As mentioned above, TX152 cells appeared to utilize maltose less efficiently than the wild type does. Since MalA activity was even higher in TX152, we wondered whether transport of maltose was affected by the *malR* deletion. Cultures of the wild type, of TX150, and of TX152 were grown in B medium without and with maltose, and maltose uptake was measured. Strain TX150 was included because it harbors a C-terminal deletion of *malR*. In contrast to *malA* expression, maltose transport in the wild-type strain could be stimulated eightfold by adding maltose to the growth medium. In the mutant strains TX150 and TX152, two effects were observed. First, the basal, uninduced rate of maltose uptake dropped about twofold. Second, transport activity after induction with maltose did not reach the wild-type level, showing a fourfold reduction. Thus, reduced maltose uptake is the reason for the pale red appearance of TX152 colonies on maltose indicator plates.

DISCUSSION

In *S. xylosus*, cleavage of maltose and maltodextrins is mediated by the enzyme MalA, encoded by the *malRA* locus. It hydrolyzes maltose more efficiently than longer maltosaccharides (Table 2), and it is also able to cleave sucrose (Table 1). Glucose release was observed with maltodextrins up to a chain length of seven glucose units, whereas amylose did not seem to be a substrate for MalA. The end product of maltosaccharide cleavage by MalA is glucose, as determined by thin-layer chromatography (data not shown). Since enzymatic reactions were carried out in crude extracts, the results may have been overshadowed by other maltosaccharide-degrading activities. However, we found no indication for such enzymes. Even with fivefold more cell extract of the *malA* null mutant than in the standard assays and 24 h of incubation, no glucose was produced from maltosaccharides. In thin-layer chromatography analysis, no change in the amount or mobility of the added substrates was observed (data not shown). It cannot be ruled out that we have missed very low abundance enzymes, but it seems that the enzymatic activity toward maltosaccharides detected in *S. xylosus* extracts really reflects intrinsic properties of MalA.

Substrate specificity, especially the ability to cleave *p*-NP-glucoside, and the release of glucose as the end product of hydrolysis clearly distinguish MalA from amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase, enzymes found to be involved in maltosaccharide catabolism in *E. coli* and *S. pneumoniae*. Instead, the *S. xylosus* MalA enzyme resembles the α-glucosidases or maltases found in yeasts. MalA also shows considerable sequence similarity to these yeast enzymes. There have been reports of bacterial α-glucosidases with properties similar to those of MalA (13), but their role in maltose utilization has not been established.

Of the maltodextrins, *S. xylosus* utilizes only maltose and maltotriose, as determined on sugar fermentation test plates, although MalA can cleave maltodextrins up to a chain length of seven glucose units in vitro. This finding suggests that longer maltodextrins cannot enter the cytoplasm of *S. xylosus*. It could well be that MalA also plays a role in breakdown of storage polysaccharides.

The *malA* gene was detected by its ability to confer sucrase activity to *E. coli*. Its inactivation in the chromosome led to reduced sucrase activity in *S. xylosus*. Nearly 20% of the sucrase activity found in crude extracts could be attributed to MalA (Table 1). On the other hand, the *malA* null mutant showed no detectable phenotype on sucrose utilization test plates. This apparent contradiction may be explained by the use of sucrose in the sucrose hydrolase assay. *S. xylosus* possesses a sucrose-specific permease of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (44), and sucrose enters the cell as sucrose-6-phosphate, which is subsequently cleaved by the *scrB* sucrase (4). Thus, hydrolysis of sucrose-6-phosphate is physiologically important. Since this sugar phosphate is not commercially available and difficult to synthesize, we were not able to test whether MalA can also cleave phosphorylated sucrose. It is conceivable that we overestimated the activity of MalA compared with ScrB activity by using sucrose rather than the sucrose-6-phosphate in the hydrolase assays. In any case, the phenotypes of the *scrB* and *malA* mutants on sugar fermentation test plates and growth experiments using different carbon sources demonstrate that MalA plays a minor role, if any, in sucrose catabolism in *S. xylosus.*

From subcloning experiments, we could deduce that *malA* does not possess its own promoter (Table 3) and must, therefore, be cotranscribed with *malR*. Inspection of the DNA sequence upstream of *malR* (Fig. 2) revealed an almost perfect *E. coli* Eσ^{70} promoter consensus sequence (10) with appropriate spacing (17 bp) between the −35 and −10 regions and only one deviation from consensus in the −10 box. A region of dyad symmetry centered around −50 of the putative *malR* promoter suggested a possible binding site for MalR as part of an autoregulatory circuit. Somewhat surprisingly, expression of *malA* showed no maltose-specific modulation, and maltodextrins also had no influence. In addition, deleting the *malR* gene did not result in a significant change in *malA* expression. If one

takes MalA activity as a measure of *malRA* expression, the data also indicate that *malR* is not subject to maltose-specific regulation and that MalR does not autoregulate transcription of its own gene. The palindrome in the putative *malR* promoter region resembles the catabolite-responsive element implicated in catabolite repression in a number of gram-positive bacteria (12). This similarity could indicate that *malRA* expression is under global catabolite control.

In contrast to the constitutive expression of *malRA*, maltose transport is induced by the addition of maltose to the culture medium (Table 4). Since MalR belongs to the LacI-GalR family of regulators, the majority of which act as transcriptional repressors, we anticipated finding elevated maltose transport in the absence of inducer as a result of *malR* deletion in TX152. Instead, maltose uptake rates were reduced no matter how the cells were grown (Table 4). The same result was obtained in TX151, the strain carrying *malR* deleted at its 3′ end. Removal of 106 amino acids at the C terminus of MalR led to a complete loss of function, perhaps by abolishing substrate-binding and oligomerization abilities or both. Hence, MalR is indeed involved in controlling the maltose uptake system. It has a stimulatory rather than a repressive effect, but inducibility is not completely lost upon *malR* inactivation.

Activation of gene expression by members of the LacI-GalR group would have a precedent. FruR, the fructose operon repressor of *E. coli* (26), and CcpA, the catabolite control protein from *B. subtilis* (9), act as repressors as well as activators of transcription. To act as a sugar-specific regulator, MalR should respond specifically to maltose by binding to DNA and activating gene expression. Effector-dependent DNA binding has been shown for another protein of the LacI-GalR family, the PurR repressor of *E. coli* (37). In this case, hypoxanthine and guanine act as corepressors. Thus, effector-induced DNA binding would also be consistent with properties found within the LacI-GalR group.

More surprising than the positive regulation mediated by MalR was the apparent inducibility of maltose transport in the *malR* mutant TX152. The induction rate is reduced only about twofold relative to the wild-type value in this strain (Table 4). To explain this result, we consider two possibilities. First, there may be more than one inducible maltose transport system in *S. xylosus*, only one being controlled by MalR. This situation would be reminiscent of the high- and low-affinity galactose transporters regulated by GalR and GalS, respectively (48). Second, another protein could cooperate with MalR in the regulation of one uptake system.

To clarify these questions, target genes should be isolated from the genome of *S. xylosus*. Likely candidates would be the gene(s) encoding the maltose transport system(s), which do not seem to be located next to *malRA* (data not shown). By cloning the *malRA* genes, a first step has been taken in the analysis of the maltose system in *S. xylosus.*

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Brückner, R.** 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. Gene **122:**187–192.
3. **Brückner, R.** Unpublished data.
4. **Brückner, R., E. Wagner, and F. Götz.** 1993. Characterization of a sucrase gene from *Staphylococcus xylosus*. J. Bacteriol. **175:**851–857.
5. **Decker, K., R. Peist, J. Reidl, M. Kossmann, B. Brand, and W. Boos.** 1993. Maltose and maltotriose can be formed endogenously in *Escherichia coli* from glucose and glucose-1-phosphate independently of enzymes of the maltose system. J. Bacteriol. **175:**5655–5665.
6. **Ehrmann, M., and W. Boos.** 1987. Identification of endogenous inducers of the *mal* system in *Escherichia coli*. J. Bacteriol. **169:**3539–3545.
7. **Götz, F., and B. Schumacher.** 1987. Improvements of protoplast transformation in *Staphylococcus carnosus*. FEMS Microbiol. Lett. **40:**285–288.
8. **Götz, F., J. Zabielski, L. Philipson, and M. Lindberg.** 1983. DNA homology between the arsenate resistance plasmid pSX267 from *Staphylococcus xylosus* and the penicillinase plasmid pI258 from *Staphylococcus aureus*. Plasmid **9:**126–137.
9. **Grundy, F. J., D. A. Waters, S. H. Allen, and T. M. Henkin.** 1993. Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. J. Bacteriol. **175:**7348–7355.
10. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
11. **Hong, S. H., and J. Marmur.** 1986. Primary structure of the maltase gene of the Mal6 locus of *Saccharomyces carlsbergensis*. Gene **41:**75–84.
12. **Hueck, C. J., W. Hillen, and M. H. Saier, Jr.** 1994. Analysis of a *cis*-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. **145:**503–518.
13. **Kelly, C. T., M. E. Heffernan, and W. M. Fogarty.** 1980. A novel α-glucosidase produced by *Bacillus amylolyticus*. Biotechnol. Lett. **2:**351–356.
14. **Kopetzki, E., P. Buckel, and G. Schuhmacher.** 1989. Cloning and characterization of baker's yeast α-glucosidase: over-expression in a yeast strain devoid of vacuolar proteinases. Yeast **5:**11–24.
15. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
16. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–89.
17. **Morse, M. L., and M. L. Alire.** 1958. An agar medium indicating acid production. J. Bacteriol. **76:**270–271.
18. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. Methods Enzymol. **155:**335–350.
19. **Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelmann.** 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2:**109–129.
20. **Peschke, U., V. Beuck, H. Bujard, R. Gentz, and S. F. J. Le Grice.** 1985. Efficient utilization of *Escherichia coli* transcriptional signals in *Bacillus subtilis*. J. Mol. Biol. **186:**547–555.
21. **Pugsley, A. P., and C. Dubreuil.** 1988. Molecular characterization of *malQ*, the structural gene for the *Escherichia coli* enzyme amylomaltase. Mol. Microbiol. **2:**473–479.
22. **Puyet, A., and M. Espinosa.** 1993. Structure of the maltodextrin-uptake locus of *Streptococcus pneumoniae*. Correlation to the *Escherichia coli* maltose regulon. J. Mol. Biol. **230:**800–811.
23. **Puyet, A., A. M. Ibánez, and M. Espinosa.** 1993. Characterization of the *Streptococcus pneumoniae* maltosaccharide regulator MalR, a member of the LacI-GalR family of repressors displaying distinctive genetic features. J. Biol. Chem. **268:**25402–25408.
24. **Raha, M., I. Kawagishi, V. Müller, M. Kihara, and R. M. MacNab.** 1992. *Escherichia coli* produces a cytoplasmic α-amylase, AmyA. J. Bacteriol. **174:**6644–6652.
25. **Raibaud, O., and E. Richet.** 1987. Maltotriose is the inducer of the maltose regulon. J. Bacteriol. **169:**3059–3061.
26. **Ramseier, T. M., D. Nègré, J. C. Cortay, M. Scarabel, A. J. Cozzone, and M. H. Saier, Jr.** 1993. *In vitro* binding of the pleiotropic transcriptional regulatory protein, FruR, to the *fru*, *pps*, *ace*, *pts* and *icd* operons of *Escherichia coli* and *Salmonella typhimurium*. J. Mol. Biol. **234:**28–44.
27. **Reidl, J., and W. Boos.** 1991. The *malX malY* operon of *Escherichia coli* encodes a novel enzyme II of the phosphotransferase system recognizing glucose and maltose and an enzyme abolishing the endogenous induction of the maltose system. J. Bacteriol. **173:**4862–4876.
28. **Reidl, J., K. Römisch, M. Ehrmann, and W. Boos.** 1989. MalI, a novel protein involved in regulation of the maltose system of *Escherichia coli*, is highly homologous to the repressor proteins GalR, CytR, and LacI. J. Bacteriol. **171:**4888–4899.
29. **Reyes, M., N. A. Treptow, and H. A. Shuman.** 1986. Transport of *p*-nitrophenyl-α-maltoside by the maltose transport system of *Escherichia coli* and its subsequent hydrolysis by a cytoplasmic α-maltosidase. J. Bacteriol. **165:**918–922.
30. **Richet, E., and O. Raibaud.** 1989. MalT, the regulatory protein of the *Escherichia coli* maltose system, is an ATP-dependent transcriptional activator. EMBO J. **8:**981–987.
31. **Robeson, J. P., R. G. Barletta, and R. Curtiss III.** 1983. Expression of a *Streptococcus mutans* glucosyltransferase gene in *Escherichia coli*. J. Bacteriol. **153:**211–221.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a

laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Schinzel, R., and D. Palm.** 1990. *Escherichia coli* maltodextrin phosphorylase: contribution of active site residues glutamate-637 and tyrosine-538 to the phosphorolytic cleavage of alpha-glucans. Biochemistry **29:**9956–9962.
35. **Schleifer, K. H., and W. E. Kloos.** 1975. Isolation and characterization of staphylococci from human skin. I. Amended description of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and descriptions of three new species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus* and *Staphylococcus xylosus*. Int. J. Syst. Bacteriol. **25:**50–61.
36. **Schneider, E., S. Freundlieb, S. Tapio, and W. Boos.** 1992. Molecular characterization of the MalT-dependent periplasmic α-amylase of *Escherichia coli* encoded by *malS*. J. Biol. Chem. **267:**5148–5154.
37. **Schuhmacher, M. A., K. Y. Choi, H. Zalkin, and R. G. Brennan.** 1994. Crystal structure of LacI member PurR, bound to DNA: minor groove binding by α-helices. Science **266:**763–770.
38. **Schwarz, M.** 1987. The maltose regulon, p. 1482–1502. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
39. **Shine, J., and L. Dalgarno.** 1974. The 3′ terminal sequence of *E. coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
40. **Stassi, D. L., J. J. Dunn, and S. A. Lacks.** 1982. Nucleotide sequence of DNA controlling expression of genes for maltosaccharide utilization in *Streptococcus pneumoniae*. Gene **20:**359–366.
41. **Stassi, D. L., P. Lopez, M. Espinosa, and S. A. Lacks.** 1981. Cloning of chromosomal genes in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **78:**7028–7032.
42. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43:**77–90.
43. **Tapio, S., F. Yeh, H. A. Shuman, and W. Boos.** 1991. The *malZ* gene of *Escherichia coli*, a member of the maltose regulon, encodes a maltodextrin glucosidase. J. Biol. Chem. **266:**19450–19458.
44. **Wagner, E., F. Götz, and R. Brückner.** 1993. Cloning and characterization of the *scrA* gene encoding the sucrose-specific enzyme II of the phosphotransferase system from *Staphylococcus xylosus*. Mol. Gen. Genet. **241:**33–41.
45. **Watanabe, K., K. Chishiro, K. Kitamura, and Y. Suzuki.** 1991. Proline residues responsible for thermostability occur with high frequency in the loop regions of an extremely thermostable oligo-1,6-glucosidase from *Bacillus thermoglucosidasius* KP1006. J. Biol. Chem. **266:**24287–24294.
46. **Watanabe, K., K. Kitamura, H. Iha, and Y. Suzuki.** 1990. Primary structure of the oligo-1,6-glucosidase of *Bacillus cereus* ATCC7064 deduced from the nucleotide sequence of the cloned gene. Eur. J. Biochem. **192:**609–620.
47. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
48. **Weickert, M. J., and S. Adhya.** 1993. The galactose regulon of *Escherichia coli*. Mol. Microbiol. **10:**245–251.
49. **Yanish-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–109.