JOURNAL OF BACTERIOLOGY, May 1998, p. 2574–2578 Vol. 180, No. 9
0021-9193/98/$04.00+0

Identification and Enzymatic Characterization of the Maltose-Inducible α-Glucosidase MalL (Sucrase-Isomaltase-Maltase) of *Bacillus subtilis*

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Received 29 December 1997/Accepted 3 March 1998

A gene coding for a putative α-glucosidase has been identified in the open reading frame *yvdL* (now termed *malL*), which was sequenced as part of the *Bacillus subtilis* genome project. The enzyme was overproduced in *Escherichia coli* and purified. Further analyses indicate that MalL is a specific oligo-1,4-1,6-α-glucosidase (sucrase-maltase-isomaltase). MalL expression in *B. subtilis* requires maltose induction and is subject to carbon catabolite repression by glucose and fructose. Insertional mutagenesis of *malL* resulted in a complete inactivation of the maltose-inducible α-glucosidase activity in crude protein extracts and a Mal⁻ phenotype.

Disaccharides such as maltose, sucrose, and trehalose can serve as sole carbon and energy sources for *Bacillus subtilis* (12, 17, 33). In most cases, the accumulation of sugars is coupled with phosphate bond energy (20). Sucrose and trehalose utilization is dependent on the uptake of the sugars by their specific permeases, which are phosphoenolpyruvate-dependent phosphotransferase systems (PTS) correlated with the phosphorylation of the sugar (9, 12, 26). The phosphorylated sugars are further hydrolyzed in the cytoplasm by a specific phosphosucrase (17, 31) or phospho-α-1,1-glucosidase (10, 12). The latter reaction results in glucose-6-phosphate and glucose in a ratio of 1:1 from 1 mol of trehalose-6-phosphate (10). In a further reaction, the resulting glucose can serve as a substrate for an ATP-dependent glucose kinase (29). Internal glucose presumably also is produced by hydrolysis of other disaccharides such as maltose. Previous studies indicated that maltose is taken up by a non-PTS system, because uncouplers negatively affected maltose transport. This finding led to the conclusion that *B. subtilis* does not possess an enzyme II for maltose and that maltose uptake is a proton motive-driven process (33).

Amino acid sequence analysis. Newly available sequence data from the *B. subtilis* genome sequencing project make possible the identification of several genes coding for potential α-glucosidases (15, 32). The predicted gene products deduced from the DNA sequence of one gene cluster (*yvdE* to *yvdM*) from 3,545.7 to 3,558.0 kb on the *B. subtilis* genomic map (Fig. 1) (32) showed amino acid similarities to proteins involved in maltose/maltodextrin utilization systems, which presumably belong to the ABC transporter family. Therefore, we have chosen this region for further investigation with regard to maltose utilization. The derived amino acid sequence from *yvdL* exhibits high similarities to several α-glucosidases (32), indicating that the protein belongs to the glucosidase protein family. Therefore, and because of the results presented in this work, we designated the gene, predicted to encode a protein composed of 561 amino acids with a calculated molecular mass of 66 kDa and a pI of 4.98, *malL*.

Expression of α-glucosidase in *B. subtilis*. If an α-glucosidase involved in maltose utilization exists in *B. subtilis*, one would expect it to be maltose inducible. Therefore, we investigated the dependence of *para*-nitrophenyl-α-D-glucopyranoside (PNPG) hydrolysis (PNPG is a synthetic substrate analogous for many α-glucosidases) on the presence or absence of maltose by MalL in crude cell extracts. MalL activity was determined as previously described for the phospho-α-1,1-glucosidase TreA (10, 12) with C minimal medium containing all the required components (18) and sugars as mentioned; cells were harvested at an optical density at 600 nm (OD_{600}) as indicated (Fig. 2). As expected, high α-glucosidase activity was detected only in cultures grown in C minimal medium containing 10 mM maltose, whereas only residual PNPG-hydrolyzing activity was present in cells grown without maltose (grown on 0.4% K-glutamate). The latter activity is about 15-fold lower than the maltose-induced level. These data lead to the conclusion that the α-glucosidase expression (MalL; see below) is maltose inducible.

In carbohydrate utilization, the presence of different rapidly metabolized carbohydrates leads to a sequential expression of different sugar utilization systems. Glucose and fructose are sugars which are preferentially metabolized. This preferential metabolism leads to the repression of other sugar-metabolizing systems. In bacilli, this regulatory mechanism, designated carbon catabolite repression (CCR), contains the central component CcpA, which is essential for the mediation of CCR after interaction with HPr phosphorylated at Ser46 (6, 7). The phosphorylation of HPr Ser46 is catalyzed by an ATP-dependent HPr kinase (22). Introducing the *ptsH1* mutation, changing serine 46 to alanine, or the inactivation of CcpA results in the loss of CCR in many CCR systems (6, 7, 13). However, additional mechanisms of CCR have been proposed, including specific regulators, e.g., the contribution of the xylose or trehalose repressors and glucose-6-phosphate acting as an anti-inducer (4, 5), inducer exclusion (3, 27), the contribution of the glucose kinase (29, 30, 35), and potentially the regulation of enzymatic functions (10).

No α-glucosidase activity is detectable when wild-type cells are grown in 10 mM glucose or in a combination of 10 mM maltose and glucose (data not shown). The same results were

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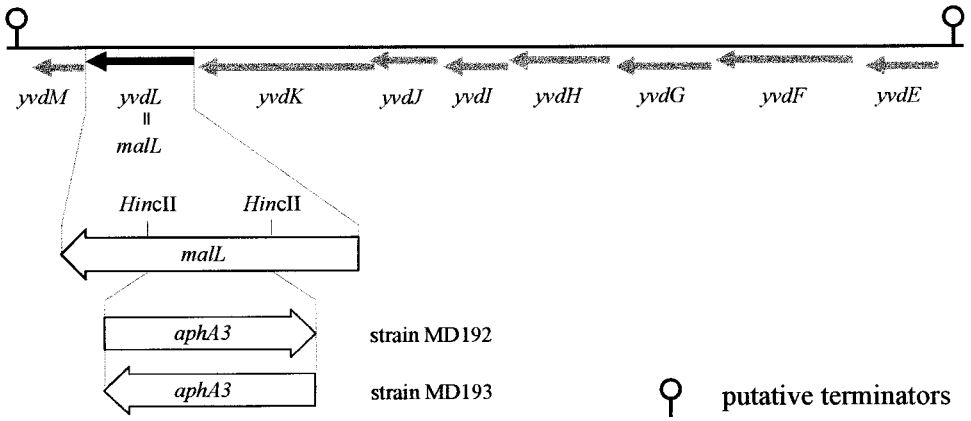

FIG. 1. The *yvdE* to *yvdM* region from 3,545 to 3,558 kb of *B. subtilis*. The *yvdL* gene (black arrow, now named *malL*) encoding the α-glucosidase is depicted below the DNA. Restriction sites used for *malL* inactivation by the *aphA3* cassette are listed. Potential transcription terminators as proposed in the SubtiList data bank (15, 32) are denoted at the ends of the DNA.

observed under the same growth conditions when glucose was substituted for 10 mM fructose (data not shown), suggesting that, besides being inducible by maltose, α-glucosidase is subject to glucose- and fructose-promoted CCR. Previous studies reported that a maltose-inducible α-glucosidase is under CCR (7). However, in strains harboring a *ccpA* and/or a *ptsH1* mutation, glucose-promoted CCR of the maltose-inducible α-glucosidase persisted (7). Therefore, additional mechanisms mediating the CCR of the maltose-inducible α-glucosidase which are independent of CcpA must be postulated.

We have also found that the expression of the maltose-inducible α-glucosidase is dependent on the growth phase in C minimal media containing 10 mM maltose. The highest MalL activity (139 nmol of PNPG hydrolyzed min^{-1} mg of crude protein $extract^{-1}$) occurred about 6 h after dilution of the cultures, corresponding to the mid-log phase of growth (Fig. 2). Upon reaching its maximal value, the specific activity of maltose-inducible PNPG hydrolysis remained constant, even when the culture entered stationary phase.

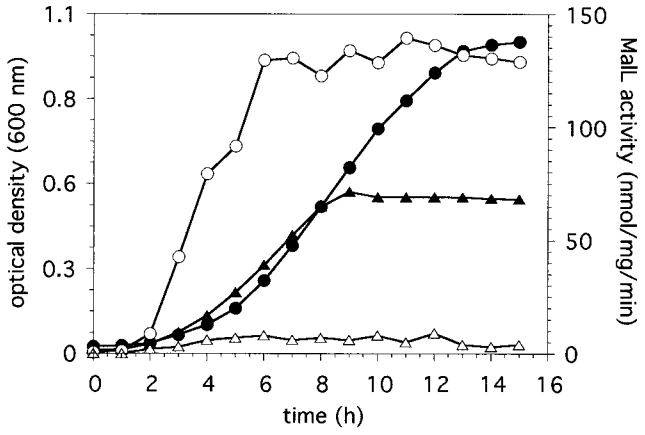

FIG. 2. Growth phase-dependent MalL activity. *B. subtilis* strains were grown in minimal media containing 10 mM maltose. Aliquots were harvested at the indicated times, and MalL activity was determined with PNPG as a substrate and expressed in nanomoles of product formed $minute^{-1}$ milligram of crude protein $extract^{-1}$ for the wild-type (○) and the $MalL^-$ strain MD193 (△). The corresponding OD_{600}s of the cultures are indicated for the wild-type (●) and the $MalL^-$ strain MD193 (▲). The growth and MalL activities of strains MD192 and MD193 were identical.

Cloning of the α-glucosidase-encoding gene. The *malL* gene was cloned by amplification via PCR (19) with a set of appropriate primers. *B. subtilis* chromosomal DNA was used as a template with the oligonucleotides 5′-CGATGTGAAAGGA GAAGGATCCATGAGTG and 5′-GATATTCTGCAGTAT CTGTTATCACTCCG, introducing a *Bam*HI site 5′ and a *Pst*I site 3′ to *malL*. The resulting 1,731-bp DNA fragment was ligated to the appropriate cloning sites of plasmid pQE-9 (21) after digestion with *Bam*HI and *Pst*I. In the resulting plasmid, pMalL, *malL* transcription is under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and the gene for the α-glucosidase is fused N-terminally in frame to the His-tag coding region of the plasmid. The encoded protein has a 12-residue N-terminal extension including the affinity tag (underlined): Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-**Met** . . .. Plasmid pMalL was transformed into *Escherichia coli* RB791 (2) and selected on Luria broth (25) supplemented with ampicillin (100 μg/ml). The addition of 2 mM IPTG to liquid cultures yielded an intense protein band in crude cell extracts migrating at the expected molecular mass of 66 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels (16) which were stained with Coomassie blue R250. Thus, the construct pMalL results in the overexpression of MalL in *E. coli.*

Inactivation of MalL. To show that MalL is indeed involved in maltose utilization in *B. subtilis* and that *malL* encodes the observed maltose-inducible α-glucosidase, we have constructed two *malL* mutations by insertional mutagenesis (Fig. 1 and Table 1) of the cloned gene in plasmid pMalL. In the first step, *malL* was inactivated on the plasmid, leading to plasmids pMalLK1 and pMalLK2, which were obtained by replacing an internal 549-bp *Hin*cII fragment of pMalL by a 1,494-bp *Sma*I/*Stu*I DNA fragment of plasmid pDG792 (11) carrying the *aphA3* gene (Fig. 1). Recombinants were selected in *E. coli* on Luria broth plates supplemented with ampicillin (100 μg/ml) and kanamycin (30 μg/ml). The orientation of the *aphA3* cassette was determined by digestion with *Ava*I, whose cleavage site is asymmetrically located in *aphA3*. The resulting plasmid, pMalK1, carries *aphA3* in the same orientation as *malL*, whereas in pMalLK2 *aphA3* is oriented in the opposite direction. Strains MD192 and MD193 were constructed by transformation (14) of a 2,676-bp *Bam*HI/*Pst*I DNA fragment obtained from plasmids pMalLK1 and pMalLK2, respectively, in *B. subtilis*, followed by selection on kanamycin (30 μg/ml) for

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Reference or source[a]
Strains		
Bacillus subtilis		
168	*trpC2* (wild type)	BGSC, 1A1
MD192	*trpC2 malL*::*aphA3*	pMalLK2 tf> 168
MD193	*trpC2 malL*::*aphA3*	pMalLK1 tf> 168
Escherichia coli RB791	F′ (*lacI*^{q} L8) *hsdR*$^+$ *hsdM*	2
Plasmids		
pDG792	pMTL23 derivative containing the *aphA3* antibiotic cassette	11
pMalL	pQE-9 derivative containing *malL* fused in frame to the His-tag coding region under *tac* promoter control	This work
pMalLK1	pMalL derivative carrying *malL*::*aphA3*	This work
pMalLK2	pMalL derivative carrying *malL*::*aphA3*	This work
pQE-9	Expression vector for His-tag fusions under *T5* promoter control	21

[a] tf> indicates transformation of DNA mentioned. BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

recombinants, which had arisen by a double crossover. The correct insertion in the chromosome of the integrants has been verified by PCR (see above) (19) with chromosomal DNA of *B. subtilis* wild type and the resulting strains MD192 and MD193 as templates. Both *B. subtilis* mutants were also analyzed for maltose-inducible α-glucosidase activity. No significant enzymatic activity could be observed even in the maltose-induced state (Fig. 2).

We also analyzed the phenotype of the *B. subtilis* α-glucosidase mutants. On solid C minimal maltose medium, *malL* inactivation leads to a Mal$^-$ phenotype (data not shown), although some residual growth is still observable. This finding agrees with the behavior of cultures in liquid C minimal maltose medium (shown in Fig. 2 for *B. subtilis* wild type and mutant MD193). Wild-type cells showed a typical growth curve reaching an OD_{600} of more than 1.0. In contrast, the cultures of the *malL* mutations start with identical doubling times, but growth stopped when the culture reached an OD_{600} of 0.5. However, *malL* mutant strains and wild-type strains exhibited no difference in growth when glucose (10 mM) was substituted for maltose (data not shown). One explanation for the incomplete Mal$^-$ phenotype of the *malL* strains could be the impurity of the maltose (possibly containing contaminating glucose) we used in this assay. Another interpretation for this phenotype may be the existence of an additional maltose utilization system. Nevertheless, the results presented clearly identify MalL as the only maltose-inducible α-glucosidase which is also subject to CCR.

Purification of the α-glucosidase. *E. coli* RB791/pMalL was grown in 100 ml of Luria broth at 37°C. Expression of *malL* was induced by the addition of 2 mM IPTG when the culture reached an OD_{600} of 0.5. Growth was allowed to proceed for another 5 h, and the cells were harvested by centrifugation at 5,000 × *g*. The resulting cell pellet was washed once in lysis buffer (25 mM imidazole [pH 7.0], 10 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 2 mM 1,4-dithiothreitol), resuspended in the same buffer, and frozen at −70°C. Frozen cell pellets were thawed on ice and sonicated six times for 30 s each at 40 W with 30-s intervals with a Labsonic U sonicator (B. Braun, Melsungen, Germany). After centrifugation for 30 min at 40,000 × *g* in a Sorvall SS34 rotor, overproduced soluble MalL was present in the supernatant. The crude extract was passed over a 1-ml Ni^{2+}-loaded HiTrap chelating column (Pharmacia, Freiburg, Germany) which had been equilibrated with lysis buffer with a Pharmacia Äkta purifier apparatus. The column was washed with 5 column volumes of lysis buffer until the absorption at 280 nm showed a stable baseline, and protein was eluted with a 10-ml linear gradient between lysis buffer and lysis buffer containing 500 mM imidazole at a flow rate of 1 ml/min (Fig. 3). Fractions (2 ml for column washing and 0.5 ml for gradient elution) were collected. The fusion protein typically eluted at about 250 mM imidazole. During protein purification, we analyzed the total and specific MalL activities from each purification step and the enrichment of MalL protein by SDS-PAGE (Fig. 3). The enrichment of MalL activity was calculated to be 45-fold. A 100-ml culture yielded about 2.4 mg of pure protein. Purified MalL was reasonably stable when stored in elution buffer at 4°C for at least 4 weeks.

Physical properties of MalL activity. To compare the stability of MalL with that of other α-glucosidases, we examined its

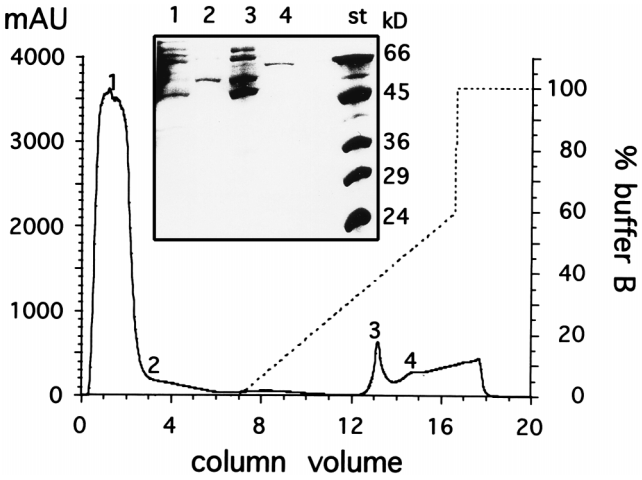

FIG. 3. Elution profile in Ni^{2+} HiTrap chelating column and purification of MalL. Protein absorption at 280 nm (solid line) and the imidazole gradient as the percentage of buffer B (dashed line) are presented as a function of the column volume (in milliliters). Analysis of selected fractions (indicated by numbers on the elution profile) on SDS-PAGE is shown in the insert. Pure MalL is in fraction 4. Molecular mass standards with the indicated sizes in kilodaltons (kD) are shown in lane st. mAU, milli-absorption units.

TABLE 2. MalL substrate specificity and kinetic parameters

Carbohydrate	Systematic name of carbohydrate	V_{max} (μmol min^{-1} mg of protein^{-1})	K_m (mM)
Sucrose	1-*O*-α-D-glucopyranosyl-β-D-fructofuranoside	357	10.2
Isomaltose	6-*O*-α-D-glucopyranosyl-D-glucose	162	0.455
Maltose	4-*O*-α-D-glucopyranosyl-D-glucose	65	0.135
PNPG	*p*-Nitrophenyl-α-D-glucopyranoside	3.3	0.210

activity under the influence of different parameters. A standard MalL activity test was used (containing 3 μg of purified protein per ml at 25°C) to determine the optimum pH. The pH of the reaction buffer was varied with either HCl or NaOH or supplemented with different concentrations of NaCl and KCl. With PNPG as the substrate, the pH profile was essentially symmetrical with optimal activity at pH 7.0. At pHs 5.75 and 8.0, the activity was reduced to 50%. MalL activity was inhibited by salt and exhibited 50% inhibition at 500 mM NaCl and 40% inhibition at 1 M KCl. These findings differ from the previously described effects of salt on the homologous phospho-α-1,1-glucosidase TreA, whose enzymatic activity is stimulated up to 10-fold at the appropriate salt concentrations (10).

Substrate specificity and kinetic parameters of MalL. The phenotype of the *malL* mutants and the analysis of the amino acid sequence of MalL suggested that the gene encodes an α-glucosidase. However, the enzyme specificity cannot be determined definitively from the sequence analysis. Therefore, the specific activity of purified MalL was monitored by determining the release of free glucose after disaccharide hydrolysis by coupling the enzymatic activity with hexokinase and glucose-6-phosphate dehydrogenase following the method described by Seno and Charter (28). For this purpose, we used the glucose determination kit HK10 from Sigma (Deisenhofen, Germany) with modifications as described by the manufacturer. The reaction mixture contained 50 mM Tris-HCl, (pH 7.0), 25 mM $MgCl_2$, 0.5 mM NAD, 1 mM ATP, and 0.8 U of glucose-6-phosphate dehydrogenase and was incubated at 25°C. Glucose-6-phosphate dehydrogenase activity was assayed with NAD as the cofactor by monitoring the change in OD_{340}. Kinetic parameters were determined with 3 μg of purified MalL in a 1-ml reaction volume. Using substrates at 8 mM allowed us to conclude initially that in addition to PNPG, MalL hydrolyzes sucrose, maltose, and isomaltose, with specific activities of 4.47, 1.7, and 2.02 μmol of glucose per min per mg of protein, respectively. Under these assay conditions, no hydrolysis of trehalose, *ortho*-nitrophenyl galactopyranoside (ONPG), or melibiose and only weak lactose hydrolysis (0.04 μmol/min/mg) were observed. Analysis of the K_m and V_{max} of MalL for different substrates showed that MalL has 22- to 76-fold-higher affinities for isomaltose and maltose than for sucrose (Table 2). However, the V_{max} for sucrose is faster than that for cleavage of isomaltose and maltose. The K_m for PNPG is similar to that for maltose, but the V_{max} for cleavage of PNPG is up to 100-fold lower than that observed for various disaccharides. From these data, it can be concluded that MalL efficiently hydrolyzes α-glycosidic 1,4- and 1,6-disaccharides but not α-1,1- or β-glycosidic bonds. The enzyme must also discriminate between galactosides and glucosides, because no hydrolysis of melibiose (α-1,6-galactopyranosyl-α-D-glucose) was detected. Therefore, we categorize this enzyme as an oligo-1,4-1,6-α-glucosidase. By virtue of its ability to hydrolyze sucrose and PNPG, this enzyme may be assigned the general designation oligo-α-glucosidase.

The substrate specificity of MalL, its ability to hydrolyze PNPG, and its release of glucose as an end product of disaccharide cleavage clearly distinguish MalL from the amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase enzymes involved in maltosaccharide catabolism in *E. coli* and *Streptococcus pneumoniae* and the phospho-α-glucosidases described for *B. subtilis*, *E. coli*, and *Fusarium mortiferum* (12, 23, 34). MalL of *B. subtilis* bears a greater resemblance to the α-glucosidase or maltase MalA of *Staphylococcus xylosus* (8) or to those found in yeast; this is also reflected in the considerable sequence similarity between these proteins (data not shown). However, *S. xylosus* MalA is a specific α-1,4-glucosidase which does not cleave isomaltose (8).

For utilization of sucrose at concentrations of 1 mM or less, *B. subtilis* possesses a sucrose-specific permease (SacP) which belongs to the phosphoenolpyruvate-dependent PTS as well as a phosphosucrase (1, 9). At higher sucrose concentrations, sucrose is cleaved extracellularly (31) and the resulting monosaccharides are taken up by the glucose- and fructose-specific PTS-dependent permeases (24). Therefore, a functional role for MalL in sucrose metabolism seems doubtful, at least when externally hydrolyzed sucrose is used as a carbon source. However, MalL might play a role in the breakdown of internal storage polysaccharides containing α-1,4 and α-1,6 bonds.

It appears most likely that MalL has a direct function in maltose and/or isomaltose metabolism. The location of *malL* in a cluster of genes with high homologies to maltose utilization systems suggests that these genes encode an ABC transporter for maltose. As previously suggested, maltose uptake in *B. subtilis* is an energy-dependent mechanism. The most plausible explanation for the negative effect of uncouplers on maltose transport was the role of the proton motive force in this process (33). Tangney and coworkers also reported the contribution of a putative maltose phosphorylase (33). However, it is possible that two maltose utilization systems exist in *B. subtilis*; this would explain the partial Mal$^-$ phenotype in the *malL* mutants as discussed above. In future work we will focus on the putative maltose and/or isomaltose transport and utilization system encoded by the *yvdE* to *yvdM* region and the molecular mechanisms of the regulation of these genes via induction and glucose repression.

We thank U. Ehmann for her interest in this work and many helpful suggestions and K. Oliva for editing the manuscript. This work was carried out in the laboratories of W. Hillen, whose support is greatly appreciated.

Financial support was obtained from the Deutsche Forschungsgemeinschaft (Da248/2-2, Da248/5-2), SFB473, and the Fonds der Chemischen Industrie.

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