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Regulation of the Raffinose Permease of *Escherichia coli* by the Glucose-Specific Enzyme IIA of the Phosphoenolpyruvate:Sugar Phosphotransferase System

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In enteric bacteria, chromosomally encoded permeases specific for lactose, maltose, and melibiose are allosterically regulated by the glucose-specific enzyme IIA of the phosphotransferase system. We here demonstrate that the plasmid-encoded raffinose permease of enteric bacteria is similarly subject to this type of inhibition.

Previous studies have identified an allosteric mechanism by which the enteric bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) regulates the uptake of non-PTS carbohydrates (15–20). This mechanism involves the direct binding of a central PTS regulatory protein, the glucose-specific enzyme IIA (IIA^{Glc}), to the target protein. Recent studies have identified residues which when mutated prevent allosteric binding of IIA^{Glc} to the target permeases (7, 9, 10, 22). Additionally, the three-dimensional structure of the *Escherichia coli* glycerol kinase-IIA^{Glc} complex has been solved to a resolution of 2.6 Å (0.26 nm), revealing residues involved in this protein-protein interaction (8).

Transport systems shown to be subject to allosteric regulation include chromosomally encoded permeases specific for lactose (LacY), maltose (MalK), and melibiose (MelB) as well as glycerol kinase and adenylate cyclase. We here demonstrate that in *E. coli* cells bearing the plasmid-encoded raffinose catabolic system (4), sensitivity of [^{3}H]raffinose uptake to inhibition by the glucose analog, methyl α-glucoside (αMG), is enhanced by partial thermal inactivation of enzyme I and abolished by mutational loss of IIA^{Glc}. The results suggest that the raffinose permease (RafB), which is similar in sequence to LacY, is subject to PTS-mediated regulation. Demonstration of the sensitivity of RafB to PTS-mediated regulation allows refinement of the previously postulated consensus sequence for IIA^{Glc} binding (7, 9).

The *E. coli* strains used in the present study are listed in Table 1 with their genotypes and sugar fermentation properties. The wild-type strain LJ50 (*cpd-453*) is Crooke's strain, which lacks cyclic AMP (cAMP) phosphodiesterase (6, 11). The temperature-sensitive enzyme I mutant LJ176 (*ptsI313*) was constructed in the LJ50 genetic background as described previously (6). A *crr* mutation was introduced in the genetic background of *ptsI313* to yield LJ2703 (*ptsI313 crr-213*). This last strain was isolated at 42°C on minimal glycerol (0.4%) plus αMG (0.2%) plates essentially as described previously (19).

To these three strains, the plasmid, pRSD2 (inducible for *raf* operon expression) or the plasmid pRSD2-1 (constitutive for *raf* operon expression) was introduced by conjugation (14), first from strain PS9 (kindly provided by Kurt Schmid), then to strain HB101 (3), and finally to each of the three aforementioned recipients.

The fermentation properties of these strains at three temperatures in the presence and absence of αMG were as expected with respect to known PTS and non-PTS (but PTS-regulated) carbon sources. Representative data are presented in Table 1 and are compared with those for raffinose fermentation.

LJ50 fermented both PTS sugars (mannitol) and non-PTS sugars (maltose) regardless of the growth temperature or the presence of αMG. The same was true for raffinose fermentation, provided that one of the plasmids bearing the raffinose catabolic system was present. Strain LJ176, which exhibits a temperature-sensitive phenotype for enzyme I, fermented mannitol effectively when grown at 30°C, poorly at 37°C, and not at all at 42°C. This phenotype was independent of the presence or absence of αMG, of the plasmid pRSD2 or pRSD2-1, and of the *crr* mutation present in strain LJ2703 (Table 1 and unpublished results) as expected. Maltose fermentation in the absence of αMG occurred in strain LJ176 at 30 or 37 but not at 42°C. In the presence of αMG, maltose was fermented at 30 but not at 37 or 42°C. The pRSD2-1 plasmid increased maltose fermentation at borderline temperatures, presumably because of competition for IIA^{Glc} binding to RafB (see the discussion below and references 16 and 17). The *crr* mutation in the isogenic strain LJ2703 abolished the inhibitory effect of elevated temperature or of αMG on maltose fermentation as expected (19, 20).

Raffinose fermentation was qualitatively similar to that of maltose, provided that the plasmid present was pRSD2. Thus, at 42°C, strain LJ176 could not ferment raffinose and at 37°C, a temperature at which raffinose was fermented, αMG inhibited raffinose fermentation. The same strain bearing the constitutive plasmid pRSD2-1 fermented raffinose at all temperatures. This behavior is in agreement with previously reported results with LacY. *E. coli* strains that synthesize LacY constitutively or at high levels are similarly resistant to PTS-mediated regulation (21). The results are therefore in full agreement with expectations assuming that RafB is subject to allosteric regulation by IIA^{Glc} of the PTS.

Figure 1 shows time courses for [^{3}H]raffinose uptake. Without a plasmid bearing the raffinose catabolic system, no uptake was observed under the specific conditions used in the experiment. After growth of LJ176/pRSD2-1 in Luria-Bertani medium in the presence of 0.2% glucose and 5 mM cAMP at 30°C, uptake of [^{3}H]raffinose was subject to inhibition by

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TABLE 1. Sugar fermentation after growth of *E. coli* strains at 30, 37, and 42°C in the absence and presence of αMG

Strain	Genotype	Fermentation response[a] with:													
		Mannitol[b]			Maltose			Maltose + αMG			Raffinose			Raffinose + αMG		
		30°[c]	37°	42°	30°	37°	42°	30°	37°	42°	30°	37°	42°	30°	37°	42°
LJ50	*cpd-453*	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
LJ50/pRSD2	*cpd-453/raf*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LJ50/pRSD2-1	*cpd-453/rafR*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LJ176	*cpd-453 ptsI313*	+	±	−	+	+	−	+	−	−	−	−	−	−	−	−
LJ176/pRSD2	*cpd-453 ptsI313/raf*$^+$	+	±	−	+	+	−	+	−	−	+	+	−	+	±	−
LJ176/pRSD2-1	*cpd-453 ptsI313/rafR*	+	±	−	+	+	−	+	±	−	+	+	+	+	+	+
LJ2703	*cpd-453 ptsI313 crr-213*	+	±	−	+	+	+	+	+	+	−	−	−	−	−	−
LJ2703/pRSD2	*cpd-453 ptsI313 crr-213/raf*$^+$	+	±	−	+	+	+	+	+	+	+	+	+	+	+	+
LJ2703/pRSD2-1	*cpd-453 ptsI313 crr-213/rafR*	+	±	−	+	+	+	+	+	+	+	+	+	+	+	+

[a] Fermentation was observed on MacConkey agar plates containing 1% carbohydrate and 0.2% αMG. Responses were as follows: +, red colonies (strong fermentation); ±, pink colonies (intermediate fermentation); −, white colonies (no fermentation).
[b] Fermentation responses observed with the PTS substrates glucitol, fructose, and *N*-acetylglucosamine were the same as those for mannitol.
[c] Temperatures are given in degrees Celsius.

αMG. Activity of LacY, which transports raffinose at an appreciable rate (1), was elevated relative to the control in which cAMP was omitted. Consequently, excess thiomethyl-β-D-galactopyranoside was included in the transport assay mixture in order to ensure that all of the radioactive raffinose was transported via RafB. As a positive control, these cells were shown to exhibit LacY activity which was subject to PTS-mediated regulation as reported previously (6, 20). As summarized in Table 2, this inhibition was abolished by the *crr* mutation. Additionally, when strain LJ176 bearing pRDS2-1 was examined, complete inactivation of enzyme I due to growth at 42°C resulted in a loss of sensitivity to inhibition (Table 2).

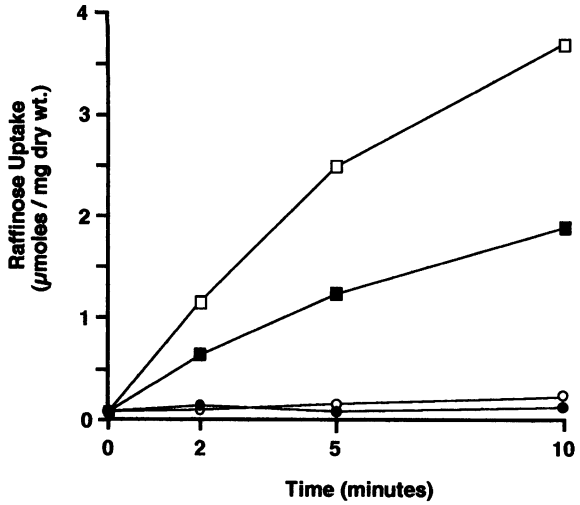

FIG. 1. Time courses for [^{3}H]raffinose uptake. *E. coli* LJ176 and LJ176/pRSD2-1 were grown at 30°C to mid-exponential phase in Luria-Bertani broth. Glucose (0.1%) and 5 mM cAMP were added 1.5 h before harvesting. Cells were washed three times in 100 mM potassium phosphate buffer (pH 7.5). Transport was initiated by the addition of [^{3}H]raffinose (NEN) (0.5 mM final concentration; 1.2 μCi/μmol) to cells incubated for 5 min at 30°C in potassium phosphate buffer containing 10 mM $MgCl_2$ with or without 1 mM αMG (final volume, 1 ml; A_{600} = 5 optical density U). The uptake buffer also contained 10 mM thiomethyl-β-galactopyranoside to reduce background activity due to low-affinity uptake of raffinose through LacY (1). Aliquots (100 μl) were filtered rapidly through nitrocellulose filters (Millipore HA; pore size, 0.45 μm) and immediately washed two times with 5 ml of potassium phosphate buffer. Radioactivity on the dry filters was determined by liquid scintillation counting. ○, LJ176 without αMG; ●, LJ176 with αMG; □, LJ176/pRSD2-1 without αMG; ■, LJ176/pRSD2-1 with αMG.

An αMG titration curve for inhibition of [^{3}H]raffinose uptake is shown in Fig. 2. Partial heat inactivation of enzyme I shifted the titration curve on this activity versus the αMG concentration plot to the left, demonstrating that raffinose uptake became increasingly sensitive to inhibition by low concentrations of αMG as the enzyme I activity decreased. This behavior is a characteristic of PTS-mediated regulation (6). Regardless of growth temperature, conditions of growth, or αMG concentration, inhibition was never seen when the *crr* mutation was present (data not shown).

The results presented above are fully consistent with the conclusion that the plasmid-encoded RafB is subject to direct allosteric regulation by IIA^{Glc} in *E. coli*. This RafB is a member of the major facilitator superfamily and clusters tightly together with the sequenced genes for LacY and sucrose permease (CscB) of *E. coli* and *Klebsiella pneumoniae* (2, 12). The region of *E. coli* LacY implicated in IIA^{Glc} binding is the central loop of this protein, between transmembrane α-helices 6 and 7 (5, 22). The sequence of the relevant portion of this loop in RafB was therefore compared with the homologous sequence in LacY as well as with the region in the MalK that has been implicated in IIA^{Glc} binding (7, 9). The sequences are aligned in Fig. 3. The underlined residues in LacY and MalK when mutated as indicated abolished IIA^{Glc}-mediated regulation of these two permeases (7, 9, 22). It can be seen that

TABLE 2. Effects of temperature and αMG on [^{3}H]raffinose uptake in *E. coli*[a]

Strain	Growth	[^{3}H]raffinose uptake (μmol/min/mg [dry wt])	
		−αMG	+αMG
LJ176/pRSD2-1	30°C	0.63 ± 0.03	0.29 ± 0.01
LJ176/pRSD2-1	42°C	0.52 ± 0.04	0.49 ± 0.07
LJ2703/pRSD2-1	30°C	0.66 ± 0.03	0.64 ± 0.06
LJ2703/pRSD2-1	42°C	0.47 ± 0.03	0.50 ± 0.04

[a] Growth conditions and [^{3}H]raffinose uptake were as described in the legend to Fig. 1.

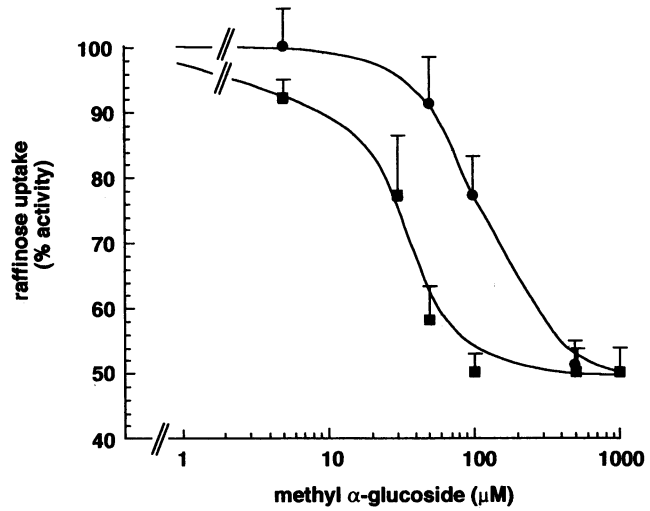

FIG. 2. Effect of thermal inactivation of enzyme I on the sensitivity of raffinose uptake to inhibition by αMG. Growth, preparation of cells, and [^{3}H]raffinose uptake experiments were as described in the legend to Fig. 1. LJ176/pRSD2-1 was grown at 30°C and assayed directly (●) or incubated at 42°C for 20 min to heat inactivate enzyme I (■) in the absence of αMG. Transport assays were conducted in the presence of various αMG concentrations as indicated. The data were derived from five independent sets of titration experiments. Error bars indicate the standard errors of the mean.

within the region implicated in IIA^{Glc} binding to LacY and MalK, these two permeases as well as RafB exhibit significant sequence similarity. Particularly striking is the V(L)-G-A-N-X-S-L(A) sequence motif shared by all of these permeases. This sequence represents a new consensus sequence for IIA^{Glc} binding.

Interestingly, it has been claimed by Bockmann et al. (2) that the central loop might not be involved in IIA^{Glc} binding. This suggestion was based on their interpretation of the multiple alignment of CscB, RafB, and LacY from *E. coli* and *K. pneumoniae*. When we reexamined this region, we found that only CscB lacks the consensus sequence, and this permease exhibits a deletion of three residues adjacent to it. LacY from *K. pneumoniae* (13) lacks the last two residues of the consensus sequence. Therefore, it seems unlikely that CscB is regulated by the PTS. This suggestion is supported by the fact that the *ptsI* mutant ECB30 is positive for sucrose fermentation but negative for the fermentation of melibiose, maltose, and glycerol (references 1a, 2, and 20 and unpublished results). Whether LacY from *K. pneumoniae* is regulated by this mechanism remains to be established.

The studies presented in this communication provide further definition of the requirements for IIA^{Glc} binding to target permeases. Mutagenic studies with RafB will be required to further substantiate the involvement of the central loop region of this permease in the binding of IIA^{Glc}.

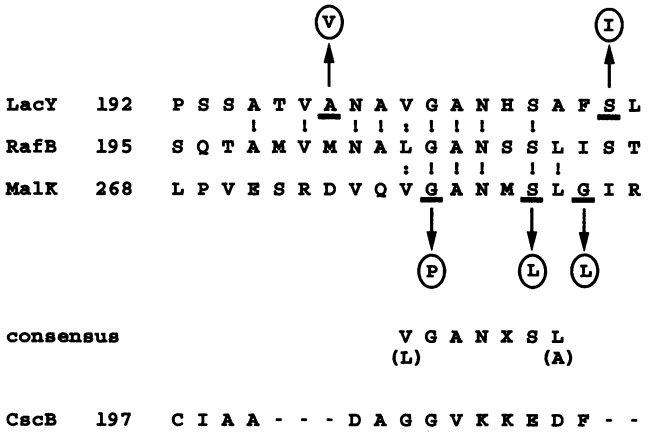

FIG. 3. Sequences of the putative IIA^{Glc} binding regions in LacY, RafB, and MalK, which are regulated by IIA^{Glc}, and comparison with that of the corresponding region of CscB, which seems not to be regulated by IIA^{Glc}. The number of the first residue shown in the protein is indicated in front of the sequence. !, identity; :, conservative substitution. Consensus, that portion of the proposed consensus sequence for IIA^{Glc} binding that is conserved in the three regulated permeases but not in CscB. A dash in the CscB sequence indicates a gap in the computer alignment of LacY, RafB, and CscB.

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