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Allosteric Regulation of *Lactobacillus plantarum* Xylulose 5-Phosphate/Fructose 6-Phosphate Phosphoketolase (Xfp)

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ABSTRACT

Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp), which catalyzes the conversion of xylulose 5-phosphate (X5P) or fructose 6-phosphate (F6P) to acetyl phosphate, plays a key role in carbohydrate metabolism in a number of bacteria. Recently, we demonstrated that the fungal *Cryptococcus neoformans* Xfp2 exhibits both substrate cooperativity for all substrates (X5P, F6P, and P_i) and allosteric regulation in the forms of inhibition by phosphoenolpyruvate (PEP), oxaloacetic acid (OAA), and ATP and activation by AMP (K. Glenn, C. Ingram-Smith, and K. S. Smith. Eukaryot Cell 13:657–663, 2014). Allosteric regulation has not been reported previously for the characterized bacterial Xfps. Here, we report the discovery of substrate cooperativity and allosteric regulation among bacterial Xfps, specifically the *Lactobacillus plantarum* Xfp. *L. plantarum* Xfp is an allosteric enzyme inhibited by PEP, OAA, and glyoxylate but unaffected by the presence of ATP or AMP. Glyoxylate is an additional inhibitor to those previously reported for *C. neoformans* Xfp2. As with *C. neoformans* Xfp2, PEP and OAA share the same or possess overlapping sites on *L. plantarum* Xfp. Glyoxylate, which had the lowest half-maximal inhibitory concentration of the three inhibitors, binds at a separate site. This study demonstrates that substrate cooperativity and allosteric regulation may be common properties among bacterial and eukaryotic Xfp enzymes, yet important differences exist between the enzymes in these two domains.

IMPORTANCE

Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp) plays a key role in carbohydrate metabolism in a number of bacteria. Although we recently demonstrated that the fungal *Cryptococcus* Xfp is subject to substrate cooperativity and allosteric regulation, neither phenomenon has been reported for a bacterial Xfp. Here, we report that the *Lactobacillus plantarum* Xfp displays substrate cooperativity and is allosterically inhibited by phosphoenolpyruvate and oxaloacetate, as is the case for *Cryptococcus* Xfp. The bacterial enzyme is unaffected by the presence of AMP or ATP, which act as a potent activator and inhibitor of the fungal Xfp, respectively. Our results demonstrate that substrate cooperativity and allosteric regulation may be common properties among bacterial and eukaryotic Xfps, yet important differences exist between the enzymes in these two domains.

Xylulose 5-phosphate (X5P)/fructose 6-phosphate (F6P) phosphoketolase (Xfp), a member of the thiamine pyrophosphate (TPP)-dependent enzyme family, catalyzes the production of acetyl phosphate from the breakdown of xylulose 5-phosphate (equation 1; EC 4.1.2.9) or fructose 6-phosphate (equation 2; EC 4.1.2.22). In lactic acid bacteria and bifidobacteria, Xfp partners with either acetate kinase (Ack) to generate acetate and ATP (equation 3) or phosphotransacetylase (Pta) to generate acetyl coenzyme A (acetyl-CoA) and P_i (equation 4) (1, 2). More recently, Xfp open reading frames (ORFs) have been discovered in euascomycete and basidiomycete fungi as well (3). In fungi, Xfp is believed to partner with Ack, since all fungi that have an Ack ORF have at least one, and in some cases two, Xfp ORFs but lack Pta (3).

$$\text{X5P} + P_i \leftrightarrow \text{glyceraldehyde 3-phosphate} + \text{acetyl phosphate} + H_2O \quad (1)$$

$$\text{F6P} + P_i \leftrightarrow \text{erythrose 4-phosphate} + \text{acetyl phosphate} + H_2O \quad (2)$$

$$\text{Acetyl phosphate} + \text{ADP} \leftrightarrow \text{acetate} + \text{ATP} \quad (3)$$

$$\text{Acetyl-CoA} + P_i \leftrightarrow \text{acetyl phosphate} + \text{CoA} \quad (4)$$

Xfp has been biochemically and kinetically characterized from several bacterial species, including *Lactobacillus plantarum* (referred to by Yevenes and Frey as *L. plantarum* Xpk2) (2), *Bifidobacterium* spp. (1, 4), *Lactococcus lactis* (5), *Leuconostoc mesenteroides* (5), and *Pseudomonas aeruginosa* (5), and, more recently, one fungal species, *Cryptococcus neoformans* Xfp2 (6). The *Bifidobacterium* Xfp and the *L. plantarum*, *L. lactis*, *L. mesenteroides*, and *P. aeruginosa* Xfps displayed dual substrate specificity for both substrates X5P and F6P and followed Michaelis-Menten kinetics (1, 2, 4, 5). *C. neoformans* Xfp2 also displays dual substrate specificity but does not follow Michaelis-Menten kinetics (6). Instead, kinetic characterization of *C. neoformans* Xfp2 indicated the existence of both substrate cooperativity and allosteric regulation. *C. neoformans* Xfp2 was found to be inhibited by ATP, phosphoenolpyruvate (PEP), and oxaloacetic acid (OAA) and is activated by

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AMP (6). Substrate cooperativity and allosteric regulation have not been reported for any characterized bacterial Xfp (1, 2, 4, 5).

In this paper, we describe the characterization of *L. plantarum* Xfp, in which kinetic parameters were determined using the Hill equation, and the influence of potential allosteric effectors on *L. plantarum* Xfp activity was examined. *L. plantarum* Xfp was found to be an allosteric enzyme inhibited by PEP and OAA but unaffected by the presence of AMP or ATP. Additionally, glyoxylate was discovered to be an inhibitor of both *C. neoformans* Xfp2 and *L. plantarum* Xfp. Our results suggest that substrate cooperativity and allosteric regulation are common properties among bacterial and eukaryotic Xfp enzymes but are tailored to fit the metabolic pathways of the microbe.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich, VWR, Fisher Scientific, or Gold Biotechnology. The recombinant plasmid pET28b-*xpk2* in *Escherichia coli* BL21(DE3) was kindly provided by Perry Frey (University of Wisconsin—Madison) for the production of recombinant *L. plantarum* Xfp (2).

Production and purification of recombinant *L. plantarum* Xfp. BL21(DE3) containing the recombinant plasmid pET28b-*xpk2* was grown in Luria-Bertani (LB) medium with 25 μg/ml kanamycin at 37°C to an absorbance of ~0.8 at 600 nm. Recombinant *L. plantarum* Xfp production was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were allowed to grow overnight at room temperature and harvested by centrifugation.

Cells were suspended in buffer A (25 mM Tris, 150 mM sodium chloride, 20 mM imidazole, 1 mM dithiothreitol [DTT], and 10% glycerol [pH 7.4]) and lysed by two passages through a French pressure cell at approximately 130 MPa. Cell lysate was clarified by ultracentrifugation at 100,000 × *g* for 1.5 h. The supernatant was applied to a 5-ml His-Trap HP column (GE Healthcare) and subjected to column chromatography using an AKTA fast protein liquid chromatographer (GE Healthcare). After washing with at least seven column volumes of buffer A to remove any unbound protein, the column was subjected to a linear gradient of 20 to 500 mM imidazole to remove all column-bound protein. Fractions determined to contain *L. plantarum* Xfp by SDS-PAGE and by activity assays were pooled and dialyzed overnight against buffer containing 25 mM Tris, 1 mM DTT, and 10% glycerol (pH 7.0). Protein concentration was determined using a modified Bradford assay (7) with bovine serum albumin as the standard, and purified protein was stored at −80°C.

Production and purification of recombinant *C. neoformans* Xfp2. The recombinant plasmid pET21b-*XFP2* synthesized by GenScript was transformed into the *E. coli* expression strain RosettaBlue (Novagen). The recombinant strain was grown in LB medium supplemented with 1% dextrose to reduce basal-level transcription from the T7 RNA polymerase gene under the control of the L8-UV5 promoter in the DE3 prophage prior to the addition of IPTG, 50 μg/ml ampicillin, and 34 μg/ml chloramphenicol. At an absorbance of ~0.8 at 600 nm, IPTG was added to a final concentration of 1 mM to induce production of the enzyme. Expression was allowed to proceed overnight at room temperature, and cells were harvested by centrifugation. Recombinant *C. neoformans* Xfp2 was purified as previously described (6).

Xfp assay. Enzymatic activity was measured using the hydroxamate assay to detect the production of acetyl phosphate (1, 2, 6, 8). A standard 200-μl reaction mixture contained 0.5 mM thiamine pyrophosphate (TPP), 1 mM DTT, 5 mM magnesium chloride, and 50 mM morpholineethanesulfonic acid (at pH 6.0 for *L. plantarum* Xfp assays and pH 5.5 for *C. neoformans* Xfp2 assays) with varied concentrations of the substrates F6P and P_i, in the form of sodium phosphate (pH 6.0 for *L. plantarum* Xfp and pH 5.5 for *C. neoformans* Xfp2). Reactions were initiated by the addition of 38 to 46 μg enzyme and allowed to proceed for 30 min at 37°C for *L. plantarum* Xfp and 40°C for *C. neoformans* Xfp2. After 30 min, 100 μl of 2 M hydroxylamine hydrochloride (pH 7.0) was added, and reaction mixtures were allowed to incubate at room temperature for 10 min to fully convert all acetyl phosphate to acetyl hydroxamate. Reactions were terminated by the addition of 600 μl of a 50:50 mixture of 2.5% ferric chloride in 2N hydrochloric acid and 10% trichloroacetic acid to generate the ferric-hydroxamate complex. The color change due to product formation was measured by a change in absorbance at 540 nm. All data sets correspond to reactions performed in triplicate.

***L. plantarum* Xfp kinetic analysis.** To determine the *L. plantarum* Xfp apparent kinetic parameters $K_{m\ app}$ and $k_{cat\ app}$, one substrate was varied while the other substrate was held constant at a saturating concentration (60 mM for F6P and 8 mM for P_i). Since the commercial availability of X5P has been discontinued, all kinetic parameters were determined using F6P. Data were plotted using KaleidaGraph software (Synergy), and kinetic parameters were found by applying the Hill equation (equation 5) (9, 10) to the data set, where V_0 is initial velocity, [*S*] is substrate concentration, *V* is maximum velocity, $K_{0.5}$ is substrate concentration at half-maximal velocity, and *h* is the Hill constant.

$$V_0 = V + [S]^h/(K_{0.5}{}^h + [S]^h) \quad (5)$$

Determination of inhibitor IC_{50} values (individually and in combination). The half-maximal inhibitory concentration (IC_{50}) was determined for each *L. plantarum* Xfp inhibitor and an additional *C. neoformans* Xfp2 inhibitor not previously described by measuring the decrease in enzyme activity at $K_{0.5}$ substrate concentrations (11 mM F6P and 1 mM P_i for *L. plantarum* Xfp and 16 mM F6P and 13 mM P_i for *C. neoformans* Xfp2) in the presence of increasing inhibitor concentration. The IC_{50} was found by fitting the data with a log (inhibitor) versus response curve in Graphpad Prism 5 software. In order to determine if inhibitors share the same or overlapping allosteric binding sites, the effect on IC_{50} of one inhibitor in the presence of another inhibitor was measured.

RESULTS

Effect of pH on *L. plantarum* Xfp activity. Recombinant *L. plantarum* Xfp was produced and purified using nickel affinity chromatography and kinetically characterized. *C. neoformans* Xfp2 activity is significantly reduced with increasing pH, and maximal activity occurs between pH 4.5 and 6.0 (6). As with *C. neoformans* Xfp2, *L. plantarum* Xfp activity decreases with increasing pH. However, the decrease in activity occurs at pH 6.5 versus at pH 6.0 for *C. neoformans* Xfp2 (Fig. 1), suggesting that *L. plantarum* Xfp is slightly more tolerant to elevated pH than *C. neoformans* Xfp2. Maximum *L. plantarum* Xfp activity occurs around pH 6.0, which was used in all kinetic assays.

Kinetic characterization of *L. plantarum* Xfp. We have recently shown that *C. neoformans* Xfp2 displays substrate cooperativity and is subject to allosteric regulation (6), neither of which has been reported for any of the bacterial Xfp enzymes (1, 4, 5, 11), including the *L. plantarum* Xfp (2). To establish whether substrate cooperativity exists for *L. plantarum* Xfp, apparent kinetic parameters (Table 1) were determined in the acetyl phosphate-forming direction for the substrates F6P and P_i by fitting experimental data to the Hill equation (equation 5), in which a Hill constant (*h*) greater than 1.0 represents positive cooperativity and a Hill constant less than 1.0 represents negative cooperativity (12, 13). *L. plantarum* Xfp displays negative cooperativity for P_i, as indicated by a Hill constant of 0.68 ± 0.02 (Table 1), similar to the Hill constant of 0.59 ± 0.03 for the *C. neoformans* enzyme (6). The $K_{0.5}$ value of 1.0 ± 0.1 mM for P_i ($K_{0.5}$ is a kinetic parameter for enzymes that display substrate cooperativity and is similar to K_m in defining the substrate concentration required to obtain half-maximum activity) is similar to the K_m previously determined for the *L. plantarum* enzyme (2).

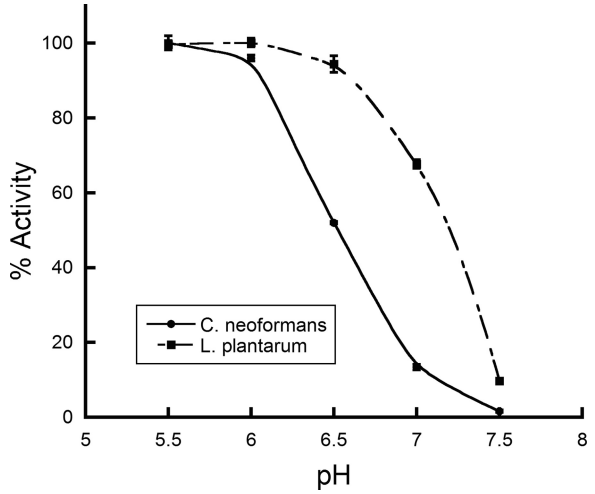

FIG 1 Effect of pH on *L. plantarum* Xfp and *C. neoformans* Xfp2 activity. Enzyme reactions were performed using F6P and P_i $K_{0.5}$ concentrations for *L. plantarum* Xfp and *C. neoformans* Xfp2. Activity begins to decrease at pH values above 6.0 for *C. neoformans* Xfp2 and above 6.5 for *L. plantarum* Xfp.

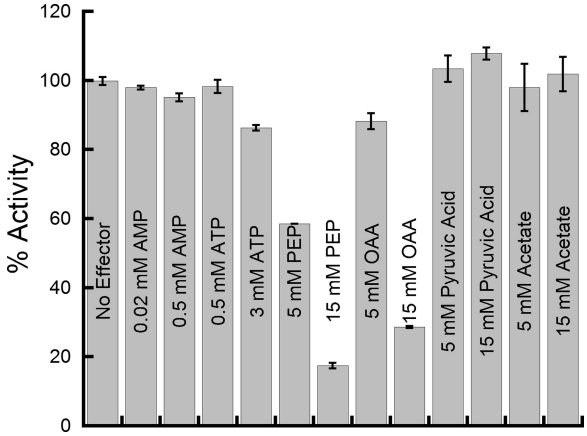

FIG 2 Effect of various ligands on *L. plantarum* Xfp activity. Reactions were performed using *L. plantarum* Xfp F6P and P_i $K_{0.5}$ substrate concentrations. Two concentrations of each coenzyme or metabolic intermediate were tested. Reactions were performed in triplicate. Activity is reported as the percentage of activity with no ligand present.

Unlike that for *C. neoformans* Xfp2, the *L. plantarum* Xfp Hill constant of approximately 1.0 does not indicate the existence of substrate cooperativity in regard to F6P binding. F6P progress curves for *L. plantarum* Xfp were found to fit both the Michaelis-Menten and Hill equations equally well, with *R* values of around 0.99. Using the Michaelis-Menten equation, $K_{m\ app}$ for F6P was determined to be 10.8 ± 0.8 mM, while a $K_{0.5}$ for F6P of 11.0 ± 1.4 mM was calculated using the Hill equation for the same data set (Table 1). Both F6P $K_{0.5}$ and $K_{m\ app}$ are on the same order of magnitude as the $K_{m\ app}$ for F6P previously determined by Yevenes and Frey (2).

***L. plantarum* Xfp is inhibited by PEP and OAA but unaffected by AMP and ATP.** The same ligands examined as possible allosteric effectors of *C. neoformans* Xfp2 (6) were tested to determine their effect on *L. plantarum* Xfp activity. As with *C. neoformans* Xfp2, *L. plantarum* Xfp is inhibited by phosphoenolpyruvate (PEP) and oxaloacetic acid (OAA) (Fig. 2) and slightly inhibited by citrate (data not shown). *L. plantarum* Xfp activity was unaffected by the presence of AMP or ATP, the primary allosteric activator and inhibitor, respectively, of *C. neoformans* Xfp2. Additional ligands tested, such as acetate and pyruvic acid, that had no effect on *C. neoformans* Xfp2 activity also had no effect on *L. plantarum* Xfp activity.

TABLE 1 Apparent kinetic parameters for *L. plantarum* Xfp and *C. neoformans* Xfp2

Enzyme and substrate	$K_{0.5}$ (mM)	$k_{cat\ app}$ (s^{-1})	$k_{cat\ app}/K_{0.5}$ (s^{-1} mM^{-1})	*H*
L. plantarum Xfp				
F6P	11.0 ± 1.4	1.05 ± 0.05	0.10 ± 0.01	0.99 ± 0.04
P_i	1.0 ± 0.1	1.12 ± 0.03	1.11 ± 0.09	0.68 ± 0.02
C. neoformans Xfp2[a]				
F6P	15.9 ± 1.3	3.47 ± 0.10	0.22 ± 0.01	1.41 ± 0.11
P_i	13.3 ± 1.5	4.22 ± 0.13	0.32 ± 0.03	0.59 ± 0.03

[a] Previously reported kinetic parameters (6).

Glyoxylate inhibits both *L. plantarum* Xfp and *C. neoformans* Xfp2 activity. Since PEP and OAA serve as common allosteric effectors for bacterial *L. plantarum* Xfp and eukaryotic *C. neoformans* Xfp2, various PEP analogs were tested to determine the specificity of this allosteric site for PEP and the primary chemical moiety that contributes to the allosteric inhibitory effect. Nonphosphorylated PEP analogs previously have been utilized to determine the chemical moiety that contributes to allostery in muscle pyruvate kinase (14), and each of these PEP analogs were used to test *L. plantarum* Xfp inhibition (Fig. 3A). Interestingly, glyoxylate was found to inhibit Xfp in addition to PEP and OAA. *L. plantarum* Xfp was almost fully inhibited by 8 mM glyoxylate, with only 7.2% ± 1.0% activity remaining, but was only partially inhibited by 8 mM PEP (45.7% ± 3.4% activity remaining). Pyruvate showed no inhibition, while the PEP analogs D-lactate, L-lactate, methyl pyruvate, hydroxyacetone, and glycolate displayed intermediate levels of inhibition. These same PEP analogs were used to test their inhibitory effect against *C. neoformans* Xfp2 as well, and the results were similar to those found for the *L. plantarum* Xfp. Glyoxylate also inhibits *C. neoformans* Xfp2 activity, with 8 mM reducing activity to 15.3% ± 1.0%. Similar to *L. plantarum* Xfp, pyruvate had no effect on *C. neoformans* Xfp2 activity, while all other PEP analogs show intermediate inhibition between that of pyruvate and glyoxylate (Fig. 3B).

Determination of allosteric effector IC_{50}s. The half-maximal inhibitory concentration (IC_{50}) was determined for each *L. plantarum* Xfp inhibitor (Table 2) using $K_{0.5}$ substrate concentrations. The IC_{50} for glyoxylate was lower than those for PEP and OAA by approximately 3-fold and 5-fold, respectively. In order to determine if any of these inhibitors share the same site, the IC_{50} of one inhibitor was determined in the presence of a second inhibitor held constant at its IC_{50}. If two inhibitors share the same site or if their sites overlap, then approximately half the sites should be occupied by the inhibitor held constant, thereby lowering the amount of the varied inhibitor required to reduce activity by an additional 50%.

In the presence of 10.5 mM OAA, the PEP IC_{50} decreased by more than half of the original value, demonstrating that the *L.*

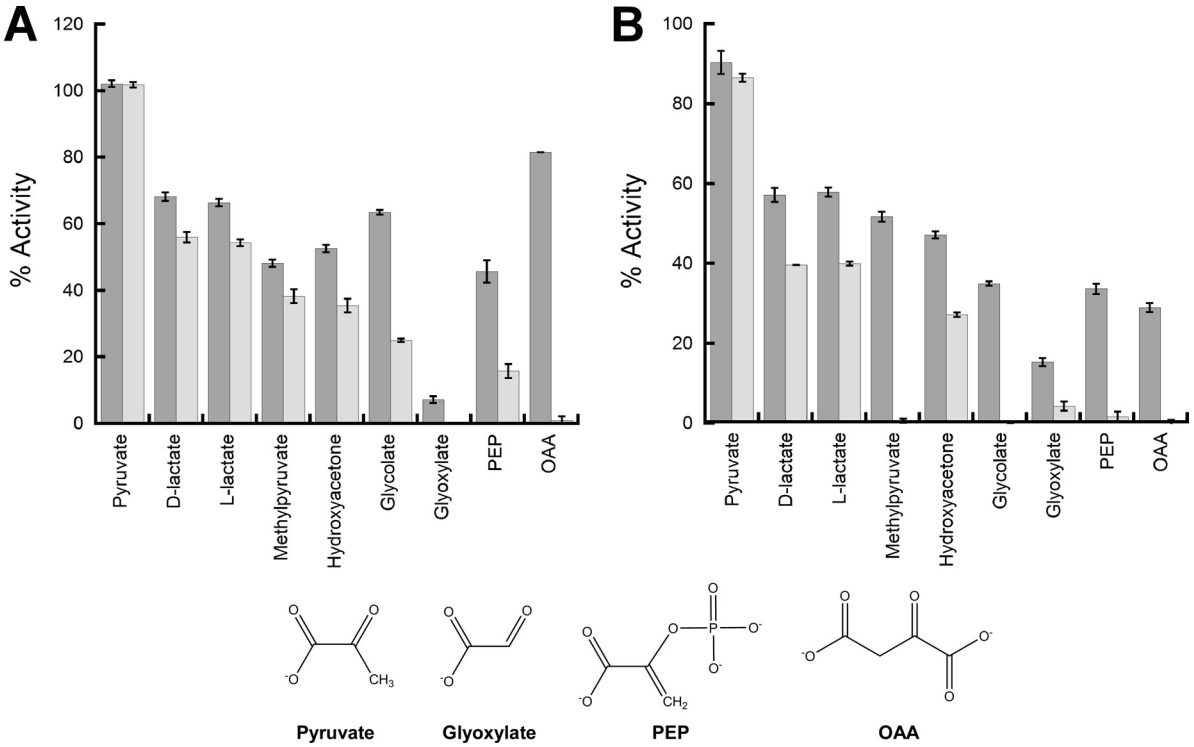

FIG 3 Effect of nonphosphorylated PEP analogs on *L. plantarum* Xfp and *C. neoformans* Xfp2 activities. Reactions were performed using *L. plantarum* Xfp (A) and *C. neoformans* Xfp2 (B) F6P and P_i $K_{0.5}$ substrate concentrations. Concentrations of 8 mM (dark gray) and 16 mM (light gray) for each nonphosphorylated PEP analog were tested in triplicate. Activity is reported as a percentage of activity with no ligand present.

plantarum PEP and OAA binding sites are the same or overlapping, as indicated previously for *C. neoformans* Xfp2 (6). The reason the IC_{50} of PEP is reduced by more than half in the presence of OAA most likely is due to the unique inhibitory effect of OAA (Fig. 4A) not seen in glyoxylate (Fig. 4B) or PEP (Fig. 4C) inhibition profiles. High concentrations of OAA were required to initiate *L. plantarum* Xfp inhibition, followed by a sharp decrease in activity in the presence of additional OAA (Fig. 4A). This inhibitory concentration threshold suggests that PEP and OAA interact with the binding site differently or that the PEP and OAA binding sites are overlapping instead of identical. The IC_{50} of PEP does not change significantly in the presence of 2 mM glyoxylate, while the IC_{50} of OAA decreases by approximately 26%. Therefore, it appears that the PEP/OAA site is separate from the glyoxylate site, since neither PEP nor OAA IC_{50} display close to a 50% decrease in the presence of 2 mM glyoxylate.

TABLE 2 Half-maximal inhibitory concentrations (IC_{50}s)

Enzyme	Inhibitor		IC_{50} (mM)
	Varied	Constant	
L. plantarum Xfp	Glyoxylate		1.93 ± 0.05
	PEP		6.70 ± 0.12
		OAA (10.5 mM)	1.14 ± 0.12
		Glyoxylate (2 mM)	6.04 ± 0.11
	OAA		10.5 ± 0.07
		Glyoxylate (2 mM)	7.78 ± 0.20
C. neoformans Xfp2	Glyoxylate		4.93 ± 0.04
		PEP (8 mM)	3.13 ± 0.18
	PEP		5.31 ± 0.13
		Glyoxylate (5 mM)	4.01 ± 0.07

Since glyoxylate was not previously recognized as an Xfp inhibitor (6), the IC_{50} of glyoxylate was determined for *C. neoformans* Xfp2 (Table 2). The *C. neoformans* Xfp2 PEP IC_{50} of 5.31 ± 0.13 mM, slightly lower than the 8 mM IC_{50} previously reported, (6) decreased to 4.01 ± 0.07 mM in the presence of 5 mM glyoxylate. Since the IC_{50} of PEP only decreases by about 24% in the presence of 5 mM glyoxylate, it is likely that the binding of PEP and glyoxylate occur at separate sites on *C. neoformans* Xfp2 as well.

Allosteric inhibitors influence F6P binding. Progress curves of substrate concentration versus activity were generated for *L. plantarum* Xfp substrates F6P and P_i in the presence of increasing PEP concentrations (Fig. 5). The presence of PEP had the same effect on *L. plantarum* Xfp kinetic parameters as it did for *C. neoformans* Xfp2 (6). In regard to P_i, the presence of PEP had little effect on $K_{0.5}$, which remained between 1.0 ± 0.03 mM and 1.3 ± 0.30 mM at 0 mM PEP and 16 mM PEP, respectively, or Hill constant, which ranged between 0.66 ± 0.01 and 0.49 ± 0.02. However, a gradual reduction in V_{max} from 1.06 ± 0.01 μmol of product formed per 30-min reaction at 0 mM PEP to 0.80 ± 0.04 μmol at 16 mM PEP was observed. The $K_{0.5}$ of F6P increased from 9.8 ± 0.2 mM in the absence of inhibitor to 37.8 ± 0.9 mM in the presence of 16 mM F6P, and the Hill constant also increased from 1.31 ± 0.01 to 1.79 ± 0.01. The presence of PEP had little effect on F6P maximal activity, with V_{max} ranging between 0.93 ± 0.02 and 0.87 ± 0.01 μmol of product formed per 30-min reaction. Since PEP and OAA bind at the same or overlapping sites on *L. planta-*

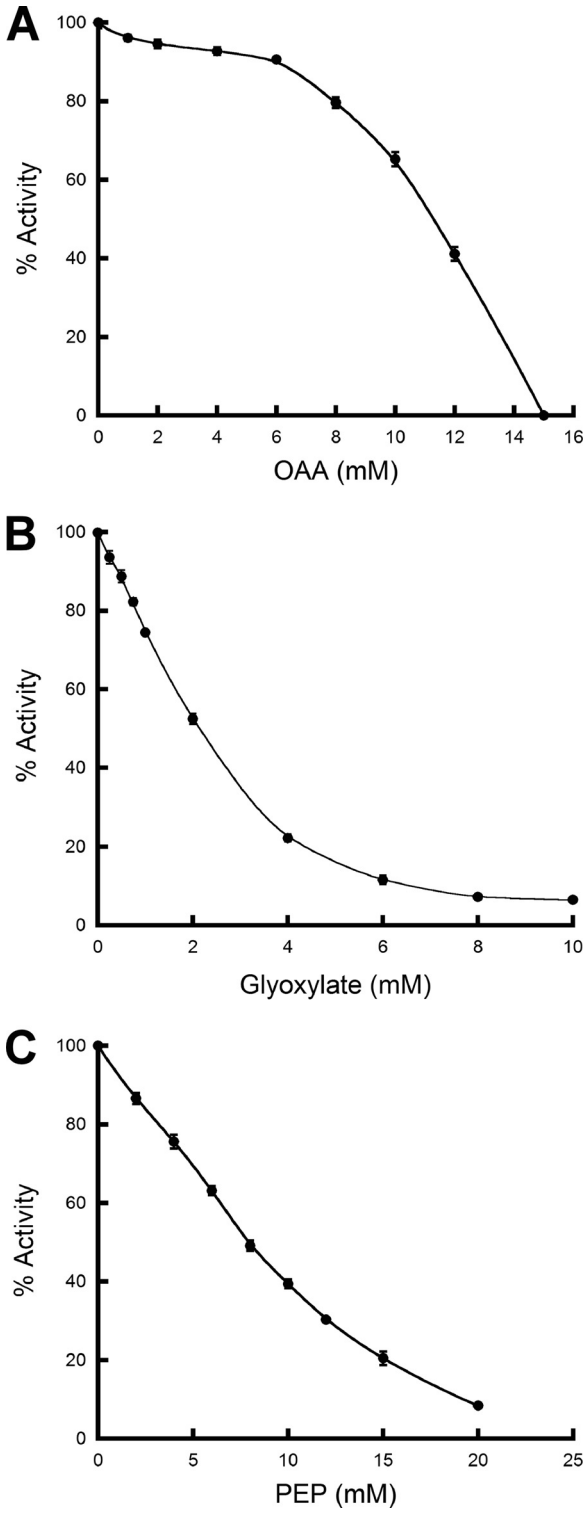

FIG 4 Inhibition of *L. plantarum* Xfp by OAA, PEP, and glyoxylate. Reactions were performed in triplicate using *L. plantarum* Xfp F6P and P_i $K_{0.5}$ substrate concentrations. Activity was measured in the presence of increasing concentrations of OAA (A), glyoxylate (B), or PEP (C) and are reported as a percentage of maximal activity with no ligand present.

rum Xfp, PEP was utilized to represent the effect of both PEP and OAA inhibition on substrate binding. The influence that PEP inhibition has on the F6P $K_{0.5}$ and Hill constant suggests that, similar to *C. neoformans* Xfp2 (6), the binding of PEP and OAA directly influences the binding of F6P to the *L. plantarum* Xfp active site. Glyoxylate binding did not significantly influence F6P $K_{0.5}$ but had a greater influence on V_{max} (data not shown).

DISCUSSION

Bacteria with nonstandard (i.e., not "*E. coli*-like") central metabolic pathways play major roles in the human microbiome. These include large numbers of *Lactobacillu*s and *Bifidobacterium* species, which metabolize sugars by glycolytic pathways that are quite different from the conventional Emden-Meyerhoff-Parnas pathway. One of those pathways, the pentose phosphoketolase pathway, requires Xfp, an enzyme that has been neglected until quite recently.

We recently determined that a fungal Xfp, *C. neoformans* Xfp2, displayed both substrate cooperativity (positive cooperativity for F6P and negative cooperativity for P_i) and allosteric regulation in the form of inhibition through the binding of ATP, PEP, and OAA and activation by the binding of AMP (6). Here, we report the discovery of substrate cooperativity and allosteric regulation for bacterial *L. plantarum* Xfp. This report describes the first indication that substrate cooperativity and allosteric regulation also exists among at least some bacterial Xfp enzymes. Kinetic parameters for *L. plantarum* Xfp originally were determined by Yevenes and Frey by fitting substrate progress curves with the Michaelis-Menten equation to determine the apparent K_m for F6P and P_i, which were found to be 24 ± 4 mM and 2.9 ± 0.5 mM, respectively (2). We produced the recombinant *L. plantarum* Xfp and demonstrated that this enzyme displays negative cooperativity in regard to P_i binding with a Hill constant less than one but little cooperativity in regard to F6P binding with a Hill constant roughly equal to one.

In addition to substrate cooperativity, *L. plantarum* Xfp, like *C. neoformans* Xfp2, is allosterically regulated. It is inhibited by PEP and OAA, but unlike *C. neoformans* Xfp2, the presence of ATP or AMP had little to no effect on activity. Initially, *L. plantarum* Xfp kinetic parameters did not suggest the presence of substrate cooperativity for F6P binding alone; however, the presence of the inhibitor PEP induces positive cooperativity, as demonstrated by the sigmoidal progress curves and increase in F6P Hill constant shown in Fig. 5. Unlike PEP, glyoxylate inhibits enzyme activity without greatly influencing F6P binding, since both F6P K_m and the Hill constant were not greatly altered. In regard to *C. neoformans* Xfp2 and *L. plantarum* Xfp regulation by excess PEP and OAA, the presence of these intermediates indicate the energy needs of the cell have been met. The cell can switch from glycolysis, the breakdown of glucose, to gluconeogenesis to synthesize and ultimately store glucose until it is needed; therefore, Xfp may be inhibited by these intermediates in order to limit additional energy production through the Xfp/Ack pathway (6).

In addition to Xfp, *L. plantarum* can produce acetyl phosphate from pyruvate using pyruvate oxidase (Pox) and from acetyl-CoA using phosphotransacetylase (Pta). It has been shown that at least some Acks in heterofermentative bacteria are allosterically regulated (15). Perhaps Xfp regulation by the presence of ATP and AMP is less necessary in *L. plantarum*, which has additional sources for acetyl phosphate production and may also possess an

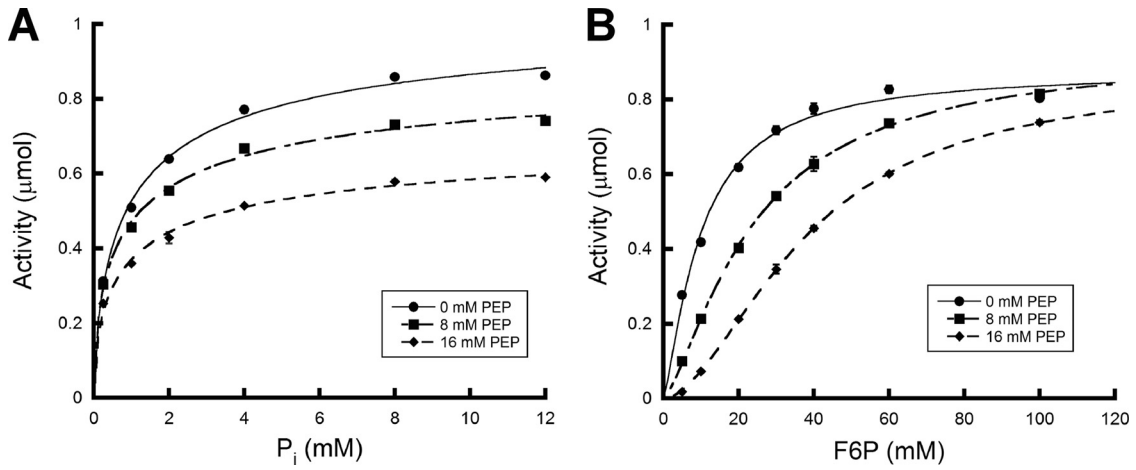

FIG 5 Effect of PEP on *L. plantarum* Xfp substrate progress curves. Progress curves were generated in the presence of 0, 8, and 16 mM PEP for the substrates P_i (a) and F6P (b). Reactions were performed in triplicate, and activities were reported as μmol of product formed.

allosterically regulated Ack, although this has not been experimentally proven. Xfp regulation by ATP and AMP may have evolved in *C. neoformans*, where there is no evidence of an allosterically regulated Ack (C. Ingram-Smith, A. Guggisberg, S. Henry, J. Welch, K. Laws, A. Mattison, A. Bizhanova, and K. Smith, unpublished data) or the presence of additional acetyl phosphate producing enzymes, such as Pox and Pta. Therefore, the control of ATP production by Ack in *C. neoformans* may rest solely on the production of acetyl phosphate by Xfp.

Interestingly, acetyl phosphate has been shown in several bacteria (e.g., *E. coli*, *Salmonella enterica*, *Xenorhabdus nematophilus*, *Borrelia burgdorferi*, and *Campylobacter*) to donate its phosphoryl group to certain response regulators, with physiologically important consequences (for a review, see reference 16). Two groups have recently shown that acetyl phosphate is the acetyl donor for most of the protein acetylation that occurs in *E. coli* (17, 18). Acetyl phosphate-dependent phosphorylation or acetylation has not yet been reported in bacteria that possess Xfp, but it is reasonable to imagine that these posttranslational modifications would occur, especially considering acetyl phosphate is a key central metabolite.

Nonphosphorylated PEP analogs were tested to determine the primary chemical moiety that contributes to PEP allosteric inhibition for both *L. plantarum* Xfp and *C. neoformans* Xfp2. Interestingly, pyruvate displayed no inhibition, while glyoxylate, which differs from pyruvate by the absence of a single methyl group, inhibits *L. plantarum* Xfp more than PEP or OAA at the same concentration. Our results suggest that PEP and OAA bind to the same or overlapping sites in both *L. plantarum* Xfp and *C. neoformans* Xfp2, but glyoxylate binds at a distinct site.

Since glyoxylate is an allosteric inhibitor presumably with its own allosteric site on both *L. plantarum* Xfp and *C. neoformans* Xfp2, there is likely a metabolic connection between the presence of excess glyoxylate and the inhibition of Xfp, consequently limiting the production of acetyl phosphate from X5P and F6P. The glyoxylate cycle functions as a bypass of the decarboxylation steps of the tricarboxylic acid cycle, allowing for the use of simple two-carbon compounds, such as acetate and ethanol, to generate malate from the combined action of the enzymes isocitrate lyase (Icl) and malate synthase (Mls) (19–21). The glyoxylate cycle is utilized when glucose is limiting (19), but the production of excess glyoxylate indicates that other 2-carbon compounds, such as acetate, are prevalent. Since we hypothesize that *C. neoformans* Xfp2 partners with Ack to generate acetate and ATP, the presence of excess glyoxylate indicates that acetate is in abundance, so glyoxylate inhibits Xfp, thereby inhibiting the production of acetyl phosphate and consequently the production of acetate by Ack.

We have failed to identify genes encoding the enzymes Icl and Mls within the *L. plantarum* genome, suggesting it lacks a glyoxylate cycle. However, *L. plantarum* cell extract is capable of metabolizing glyoxylate (22). An ORF within the *L. plantarum* genome designated 2-hydroxyacid dehydrogenase (accession number YP_004888759) has 61% identity to a gene designated a glyoxylate reductase (equation 6) in *Lactobacillus otakiensis*, and it also has been shown in *Rhizobium etli* that an enzyme previously labeled as 2-hydroxyacid dehydrogenase displays glyoxylate reductase activity (23).

$$\text{Glyoxylate} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{glycolate} + \text{NAD(P)}^+ \quad (6)$$

In plants, glyoxylate reductase is believed to function as a way of removing excess reducing equivalents like NADPH (24), and glyoxylate reductase in *L. plantarum* could serve a similar purpose. A possible connection between *L. plantarum* Xfp inhibition by excess glyoxylate is that the presence of glyoxylate indicates the presence of excess NADPH. The pentose phosphate pathway (PPP) serves as a major source of the NADPH produced in the cell. Thus, inhibiting Xfp, which utilizes PPP end products, may hinder the PPP from producing additional end products, thereby also reducing the amount of NADPH produced upstream. The regulation of Xfp by glyoxylate in *L. plantarum* may serve as a means of balancing the production and utilization of NADPH by the PPP and glyoxylate reductase, respectively, to aid in cellular redox balance.

Concluding remarks. Substrate cooperativity and allosteric regulation no longer can be considered a purely eukaryotic Xfp phenomenon with the discovery of its existence in at least some bacterial Xfps, specifically *L. plantarum* Xfp. However, there are differences between the degree of substrate cooperativity and the allosteric effectors that inhibit or activate eukaryotic Xfp and bac-

terial Xfp from *C. neoformans* and *L. plantarum*, respectively. Both *C. neoformans* Xfp2 and *L. plantarum* Xfp share PEP, OAA, and glyoxylate as allosteric inhibitors, but *C. neoformans* Xfp2 also is inhibited by ATP and activated by AMP, while an activator of *L. plantarum* Xfp has yet to be discovered. Additionally, regulation by glyoxylate appears to result from different phenomena in *C. neoformans*, which primarily produces glyoxylate through the glyoxylate cycle, versus *L. plantarum*, which appears to lack a glyoxylate cycle. Allosteric regulation of both the bacterial and eukaryotic Xfps suggests that tight control over the pathway is important for responding to the different environmental stresses that ultimately influence cellular metabolism.

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