

Reciprocal Regulation of L-Arabinose and D-Xylose Metabolism in *Escherichia coli*

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ABSTRACT

Glucose is known to inhibit the transport and metabolism of many sugars in *Escherichia coli*. This mechanism leads to its preferential consumption. Far less is known about the preferential utilization of nonglucose sugars in *E. coli*. Two exceptions are L-arabinose and D-xylose. Previous studies have shown that L-arabinose inhibits D-xylose metabolism in *Escherichia coli*. This repression results from L-arabinose-bound AraC binding to the promoter of the D-xylose metabolic genes and inhibiting their expression. This mechanism, however, has not been explored in single cells. Both the L-arabinose and D-xylose utilization systems are known to exhibit a bimodal induction response to their cognate sugar, where mixed populations of cells either expressing the metabolic genes or not are observed at intermediate sugar concentrations. This suggests that L-arabinose can only inhibit D-xylose metabolism in L-arabinose-induced cells. To understand how cross talk between these systems affects their response, we investigated *E. coli* during growth on mixtures of L-arabinose and D-xylose at single-cell resolution. Our results showed that mixed, multimodal populations of L-arabinose- and D-xylose-induced cells occurred at intermediate sugar concentrations. We also found that D-xylose inhibited the expression of the L-arabinose metabolic genes and that this repression was due to XylR. These results demonstrate that a strict hierarchy does not exist between L-arabinose and D-xylose as previously thought. The results may also aid in the design of *E. coli* strains capable of simultaneous sugar consumption.

IMPORTANCE

Glucose, D-xylose, and L-arabinose are the most abundant sugars in plant biomass. Developing efficient fermentation processes that convert these sugars into chemicals and fuels will require strains capable of cointilizing these sugars. Glucose has long been known to repress the expression of the L-arabinose and D-xylose metabolic genes in *Escherichia coli*. Recent studies found that L-arabinose also represses the expression of the D-xylose metabolic genes. In the present study, we found that D-xylose also represses the expression of the L-arabinose metabolic genes, leading to mixed populations of cells capable of utilizing L-arabinose and D-xylose. These results further our understanding of mixed-sugar utilization and may aid in strain design.

When bacteria are grown on a mixture of sugars, they will often consume the sugars one at a time in a defined hierarchy. The classic example is growth of *Escherichia coli* on glucose and lactose, where the cells will first consume the glucose and then the lactose. This process of ordered sugar consumption is known as carbon catabolite repression and has been studied in many species of bacteria (1–3). These studies have principally focused on the mechanisms governing the preferential consumption of glucose, which in the case of *E. coli* is known to involve the regulation of specific genes and metabolic fluxes (2, 4).

Far less is known about the mechanisms governing the preferential consumption of sugars other than glucose. One notable exception is the growth of *E. coli* on mixtures of L-arabinose and D-xylose (5–8), hereafter referred to simply as arabinose and xylose. Kang and coworkers first demonstrated that the xylose metabolic genes are repressed by arabinose (6). More recently, Desai and Rao investigated the mechanism for this hierarchy (5). Both the arabinose and xylose utilization pathways involve similar regulatory mechanisms (Fig. 1). The genes for these pathways are expressed only when their cognate sugar is present. AraC positively regulates the transcription of the arabinose metabolic and transporter genes in response to arabinose (9). Likewise, XylR positively regulates the transcription of the xylose metabolic and transporter genes in response to xylose (10). Desai and Rao found that arabinose-bound AraC binds to the promoter for the xylose metabolic genes and inhibits their expression, likely by a compet-

itive mechanism (5). While this previous work established the mechanism governing the hierarchy between arabinose and xylose, it did not explain how individual cells behave.

Both the arabinose and xylose genes are known to exhibit a bimodal/heterogeneous induction response to their cognate sugar (11–16). Of particular note, a previous study by Afroz and coworkers investigated the expression of the arabinose and xylose metabolic genes in *E. coli* in response to their cognate sugar (11). Using transcriptional fusions to green fluorescent protein, they measured expression from the P_{arab} and P_{xylA} promoters at single-cell resolution by flow cytometry. They found that both pathways exhibited a bimodal induction response to their cognate sugar at intermediate concentrations, where the promoters were active in some cells but inactive in others. In the case of xylose, the response

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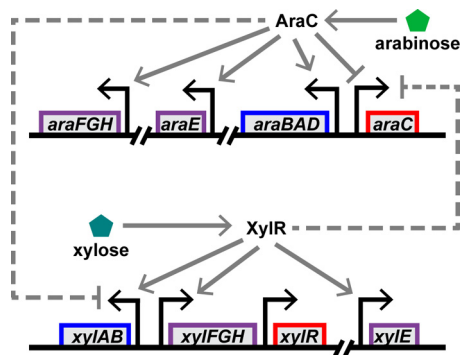


FIG 1 Regulation of the arabinose and xylose sugar utilization systems. Both systems are induced by their cognate sugar. Arabinose-bound AraC induces the expression of the arabinose metabolic genes (*araBAD*), high-affinity transporter genes (*araFGH*), and low-affinity transporter gene (*araE*) (9). In addition, arabinose-bound AraC represses its own expression. Xylose-bound XylR induces the expression of the xylose metabolic genes (*xylAB*), high-affinity transporter genes (*xylFGH*) and, presumably, a low-affinity transport gene (*xylE*) (10). Xylose-bound XylR does not appear to regulate the P_{xylR} promoter, though it may induce its expression due to transcription from the upstream P_{xylE} promoter. In addition, the two systems are subject to transcriptional cross talk. Arabinose-bound AraC represses the expression of the xylose genes (5) and, as demonstrated in this study, xylose-bound XylR represses the expression of the arabinose genes. Furthermore, the arabinose and xylose systems are both repressed by glucose (mechanism not shown).

was all or nothing: increasing the concentrations of sugar simply increased the number of induced cells where the P_{xylA} promoter was active. In the case of arabinose, the response was more complex. At low arabinose concentrations, the response was all or nothing. At higher concentrations, where the entire population was induced (P_{araB} promoter active), the response was graded: expression from the P_{araB} promoter increased with increasing arabinose concentrations.

The response of these two sugar utilization systems at single-cell resolution has not yet been investigated in mixtures of arabinose and xylose. An open question is: if induction is bimodal, then is this also true for catabolite repression? In other words, if only a fraction of cells is induced by arabinose, then is the remaining fraction capable of being induced by xylose? And, can distinct populations of arabinose- and xylose-induced cells coexist at some intermediate concentrations of the two sugars? To answer these questions, we investigated the single-cell response of *E. coli* during growth on arabinose and xylose by using fluorescent protein reporters. Our results show that mixed populations of arabinose- and xylose-induced cells occur at some intermediate sugar concentrations. We also found that xylose inhibits the expression of the arabinose genes and that this repression occurs through XylR. Collectively, these results demonstrate that a strict hierarchy does not exist between arabinose and xylose, as previously thought, but rather that repression is reciprocal between these two sugar utilization systems.

MATERIALS AND METHODS

Media and growth conditions. Luria-Bertani (LB) medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) was used for cell growth during strain and plasmid construction. Agar plates were prepared by adding 15 g/liter Bacto agar and antibiotics at the following concentrations: ampicillin at 100 μ g/ml, chloramphenicol at 20 μ g/ml, and kanamycin at 40 μ g/ml. Cells were grown in M9 glycerol medium (6.8 g/liter

Na_2HPO_4 , 3 g/liter KH_2PO_4 , 1 g/liter NH_4Cl , 0.5 g/liter NaCl, 2 mM MgSO_4 , 100 μ M CaCl_2 , 0.4% glycerol, and 0.001% thiamine hydrochloride) for the fluorescence measurement experiments. All growth experiments were performed at 37°C.

Bacterial strains and plasmid construction. All strains and plasmids are listed in Tables 1 and 2, respectively. The $\Delta xylAB$ mutant strain (mutated in the genomic region from nucleotide [nt] 3725940 to 3728788) was constructed using the method of Datsenko and Wanner (17). The *xylA'*-mCherry reporter plasmid pSK376 was constructed by PCR amplification of the mCherry gene and ribosome binding site from the plasmid pKW667 (18) using the primers SK353F (5'-GAC CGA ATT CTA AAA GGA GGA GAA AAT G) and SK353R (5'-ATG GCT AGC CTT TGA GTG AGC TGA TAC CGC TC) and the P_{xylA} promoter (genomic region from nt 3729038 to 3728713) from genomic DNA using the primers SK435F (5'-AGA GAG GTC GAC GAT TAC GAT TTT TGG TTT ATT TCT TGA TTT ATG ACC G) and SK435R (5'-AGA GAG GAA TTC ACG GAA TGC TAA CGG GTT TGA G). The two fragments were then cloned into the integrative plasmid pLC153 (18) by using the EcoRI/NheI and Sall/EcoRI restriction sites (restriction sites are underlined in the primer sequences). Similarly, the *araB'*-Venus reporter plasmid pSK459 was constructed by PCR amplification of the P_{araB} promoter (genomic region 70059 to 70386) using the primers SK380F (5'-AGA GAG GTC GAC ACT TTT CAT ACT CCC ACC ATT CAG AG) and SK380R (5'-AGA GAG GAA TTC CAT CCA AAA AAA CGG GTA TGG AGA AAC) and then cloning the fragment into the integrative plasmid pVenus (19) by using Sall and EcoRI restriction sites. The reporter plasmids pSK376 and pSK459 were integrated single copy into bacterial attachment sites for phages ϕ 80 and λ , respectively, using the conditional-replication, integration, and modular (CRIM) method (20). The integrated plasmids were moved to a clean background via P1 transduction (21, 22).

The *araC'*-Venus reporter plasmid pXW8 was constructed by PCR amplification of the P_{araC} promoter (genomic region from nt 70089 to 70386) by using the primers XW20F (5'-ACA CAC GTC GAC AGA GAG TTG CGA TAA AAA GCG) and XW20R (5'-ACA CAC GAA TTC ACT TTT CAT ACT CCC ACC ATT C) and then cloning the fragment into the integrative plasmid pVenus by using Sall and EcoRI restriction sites (underlined). The *araE'*-Venus reporter plasmid pXW10 was constructed by PCR amplification of the P_{araE} promoter (genomic region from nt 2980205 to 2980657) using the primers XW22F (5'-AGA GAG GTC GAC AAT TCT CGA CCG CAT TCC AG) and XW22R (5'-AGA GAG GAA TTC TTT TTC CTG CCA GCA GAG AG) and then cloning the fragment into the integrative plasmid pVenus using Sall and EcoRI restriction sites. The *araF'*-Venus reporter plasmid pXW11 was constructed by PCR amplification of the P_{araF} promoter (genomic region from nt 1984153 to 1984752) using the primers XW23F (5'-AGA GAG GTC GAC TTC ATT GAT GAA ATC AAT GTA ACT GC) and XW23R (5'-AGA GAG TCT

TABLE 1 *E. coli* strains used in this study

Strain	Relevant characteristics	Source or reference
MG1655	λ <i>rph-1</i> (wild type)	CGSC
CR413	$\Delta xylR::\text{FRT}$	5
SK517	$\Delta xylAB::\text{FRT}$	
SK459	<i>attL::[kan P_{araB}-Venus oriR6K]</i>	
SK463	<i>attϕ80::[cm P_{xylA}-mCherry oriR6K]</i>	
SK504	<i>attL::[kan P_{araB}-Venus oriR6K]</i> <i>attϕ80::[cm P_{xylA}-mCherry oriR6K]</i>	
SK518	$\Delta xylR::\text{FRT}$ <i>attL::[kan P_{araB}-Venus oriR6K]</i>	
SK519	$\Delta xylAB::\text{FRT}$ <i>attL::[kan P_{araB}-Venus oriR6K]</i>	
XW81	<i>attL::[kan P_{araC}-Venus oriR6K]</i>	
XW83	<i>attL::[kan P_{araE}-Venus oriR6K]</i>	
XW84	<i>attL::[kan P_{araF}-Venus oriR6K]</i>	
BL21(DE3)	F ⁻ <i>ompT gal dcm hsdS</i> (r _B ⁻ m _B ⁻) λ (DE3)	GE HealthCare

TABLE 2 Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pVenus	<i>kan attλ Venus oriR6K</i>	19
pLC153	<i>cm attφ80 oriR6K</i>	18
pInt-ts	<i>bla int oriR6K</i> (helper plasmid for <i>attλ</i> integration)	20
pAH123	<i>bla int oriR6K</i> (helper plasmid for <i>attφ80</i> integration)	20
pKW669	<i>cm P_{LtetO-1}-mCherry colE1</i>	18
pmCherry	<i>cm attφ80 mCherry oriR6K</i>	
pSK376	<i>cm attφ80 P_{xyIA}-mCherry oriR6K</i>	
pSK459	<i>kan attλ P_{araB}-Venus oriR6K</i>	
pXW8	<i>kan attλ P_{araC}-Venus oriR6K</i>	
pXW10	<i>kan attλ P_{araE}-Venus oriR6K</i>	
pXW11	<i>kan attλ P_{araF}-Venus oriR6K</i>	
pET-28(a)	<i>kan lacI P_{T7}-His₆ T7 pBR322</i>	Novagen
pSK451	<i>kan lacI P_{T7}-His₆-xyIR T7 pBR322</i>	

AGA GGT TCT CTC CAG CTT TAG TGT C) and then cloning the fragment into the integrative plasmid pVenus using Sall and XbaI restriction sites. The reporter plasmids were integrated single copy into bacterial attachment sites for λ by using the CRIM method and then moved to a clean background via P1 transduction.

To construct the XylR expression vector with an N-terminal 6 \times His tag, the *xyIR* gene was PCR amplified using the primers SK413F (5'-CAG CGC TAG CTT TAC TAA ACG TCA CCG CAT C) and SK411R (5'-GCT CGA ATT CTT ATT ACT ACA ACA TGA CCT CGC TAT TTA C) and then cloned into the pET-28(a) vector (Novagen) by using the NheI and EcoRI restriction sites (underlined).

Fluorescence assays. Cells were grown overnight in M9 glycerol minimal medium supplemented with 0.2% glucose to an optical density at 600 nm (OD_{600}) of ~ 1 . The overnight culture was diluted 1:10⁶ and grown in M9 glycerol medium supplemented with various amounts of arabinose and xylose for 20 h to an OD_{600} of ~ 0.002 . This method has been shown to allow cells be in exponential phase for most of the incubation period (11). The cells were then analyzed using a BD LSR Fortessa flow cytometer. Fluorescence values for approximately 20,000 cells were collected using the fluorescein isothiocyanate channel (excitation at 488 nm, emission at 530/30 nm) for Venus and the phycoerythrin-Texas Red channel (excitation at 561 nm, emission at 610/20 nm) for mCherry-tagged fluorescent proteins. The population was gated using side scatter (SSC) and forward scatter (FSC) channels to distinguish cells from other debris. Data from the flow cytometry experiments were analyzed using the FCS Express program, version 4 (De Novo Software). The data were exported to Microsoft Excel 2010 and further processed in Origin Pro 9.0 to obtain density and histogram plots. The populations were segregated using the quadrant gate in the FCS Express version 4 software. Wild-type strain MG1655 was used as a negative control to set up the gate. All experiments were performed on three separate days; however, we have provided the histogram for only a single experiment, as little deviation was observed among the replicates.

Protein purification. The 6 \times His-XylR expression vector was transformed into overexpression strain BL21(DE3) expressing T7 RNA polymerase and grown overnight in LB supplemented with 40 μ g/ml kanamycin. The overnight culture was diluted 1:33 in fresh LB and grown to an OD_{600} of 0.6 before being induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside. Cells were then grown for an additional 4 h at 37°C and harvested by centrifugation at 5,000 \times g for 15 min. The cell pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole; pH 8) containing 1 mg/ml lysozyme and incubated on ice for 30 min. The cells were then sonicated (8 cycles of 10-s pulses at 2-min intervals) on ice. The cell lysate was separated from cells debris by centrif-

ugation at 9,000 \times g for 30 min. The lysate was then added to a half volume of Ni-nitrilotriacetic acid-agarose (Qiagen) and gently mixed on a rotary shaker at 4°C for 1 h. The mixture was then centrifuged at 1,000 \times g for 5 min, and then the supernatant was discarded. The resin was washed twice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole; pH 8). Finally, the protein was eluted with 2 volumes (0.5 ml) of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole; pH 8). The eluate was analyzed by SDS-PAGE.

Electrophoretic mobility shift assay. DNA fragments for the P_{xyIA} (genomic region from nt 3729038 to 3728713), P_{araC}/P_{araB} (genomic region from nt 70059 to 70386), and P_{flgB} (genomic region from nt 1129902 to 1130228) promoters were ³²P-labeled in a phosphorylation reaction mixture containing 5 pmol DNA, 2 μ l T4 polynucleotide kinase 10 \times buffer, 1 μ l T4 polynucleotide kinase (New England BioLabs), and 2 μ l of [γ -³²P]ATP in a final volume of 20 μ l. The mixture was incubated at 37°C for 20 min. The reaction was then quenched by adding 2 μ l of 0.5 M EDTA. The binding reaction was performed by mixing 0.5 pmol ³²P-labeled DNA, 0.5 μ g salmon sperm DNA (Invitrogen), 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 100 μ g/ml bovine serum albumin (BSA), 10% glycerol, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 10 mM xylose (only when indicated), and 100 ng purified XylR protein (only when indicated) in a total volume of 50 μ l. The mixture was incubated at room temperature for 30 min. The electrophoresis was carried out on a 5% native polyacrylamide gel in TBE buffer (10.8 g/liter Tris, 5.5 g/liter boric acid, 0.37 g/liter EDTA disodium salt) at room temperature. The gel was vacuum dried on a filter paper and placed on a phosphor screen overnight. The phosphor screen was scanned using a Storm 840 PhosphorImager.

RESULTS

As arabinose is known to inhibit the expression of the xylose metabolic genes, we sought to determine how this cross talk would affect the single-cell response of *E. coli* grown in a mixture of arabinose and xylose. In particular, would we observe mixed, multimodal populations of arabinose- and xylose-induced cells or would we observe some cells induced for arabinose but not xylose and other cells induced for xylose but not arabinose? To simultaneously measure the expression of the arabinose and xylose metabolic pathways in single cells, we constructed transcriptional fusions of the P_{araB} and P_{xyIA} promoters to the fluorescent proteins Venus (23) and mCherry (24). These constructs were then integrated single copy into the chromosome by using the phage λ and ϕ 80 attachment sites (20). This design enabled us to simultaneously measure the expression from the P_{araB} and P_{xyIA} promoters in single cells by using flow cytometry. Following the protocol of Afroz and coworkers (11), the cells were grown for 20 h in M9 minimal medium supplemented with 0.4% glycerol and various amounts of arabinose and xylose, starting with a very low cell density to ensure that the cells were harvested during exponential phase.

We first investigated the response to a single sugar in order to validate our strains. As shown in Fig. 2, the cells exhibited a bimodal induction response to both sugars: coexisting populations of induced (promoter active) and uninduced (promoter inactive) cells were observed at intermediate sugar concentrations. As a control, we also measured fluorescence in a strain lacking both fluorescent proteins. The measured control level of fluorescence was similar to that observed for the uninduced population, indicating that both promoters were inactive in the absence of their cognate sugar (see Fig. S1 in the supplemental material). We also found that the xylose pathway exhibited an all-or-nothing response, whereas the arabinose pathway exhibited a more complex response: all or nothing at low concentrations and a graded response at higher ones (>1 μ M). These results are entirely

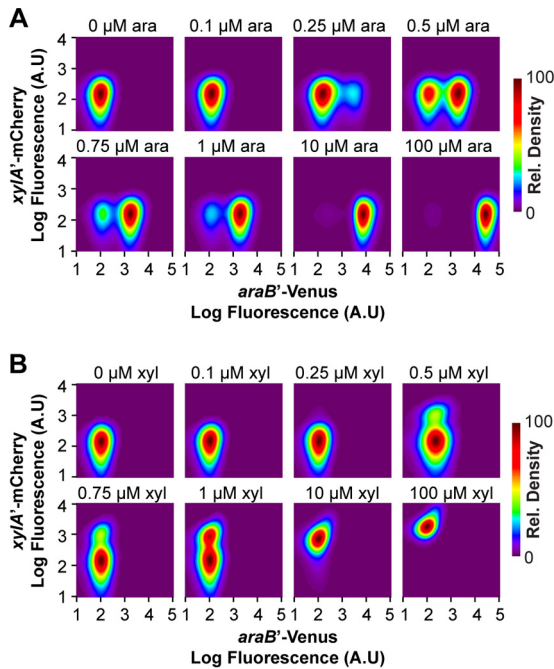


FIG 2 Both the arabinose and xylose systems exhibit a bimodal response to their cognate sugar. The response was measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xyIA} promoter to the mCherry fluorescent protein. The density plots show the distributions of promoter activities in individual cells as determined based on Venus and mCherry fluorescence. Data were smoothed and normalized to a peak value of 100 to facilitate interpretation. The cells were grown in M9 minimal medium containing 0.4% glycerol and various concentrations of arabinose (A) or xylose (B) as indicated. Table S1 in the supplemental material lists the fractions of cells in the uninduced and induced states.

consistent with those of Afroz and coworkers, though we did see a somewhat graded response for xylose at high concentrations ($>10 \mu\text{M}$).

We next investigated the response to mixtures of sugars. Here we investigated two concentrations of arabinose, $0.5 \mu\text{M}$ in association with a mixed population (Fig. 3A) and $1 \mu\text{M}$ in association with a nearly induced population (Fig. 3B), and a range of xylose concentrations. At low concentrations ($<0.5 \mu\text{M}$), xylose did not have an effect, consistent with the single-sugar response (Fig. 2B). At intermediate xylose concentrations (0.5 to $1 \mu\text{M}$) and $0.5 \mu\text{M}$ arabinose, we observed four distinct populations: one where both the P_{araB} and P_{xyIA} promoters were inactive, one where only the P_{araB} promoter was active, one where only the P_{xyIA} promoter was active, and one where both promoters were active (Fig. 3A). The same behavior was also observed with $1 \mu\text{M}$ arabinose (Fig. 3B), though the results were less pronounced because the population where only the P_{araB} promoter is active predominated, presumably due to repression of the P_{xyIA} promoter. These results indicated that the cells exhibit a mixed, multimodal response to arabinose and xylose, with some cells capable of consuming both sugars and others just one. At high xylose concentrations ($>1 \mu\text{M}$), the P_{xyIA} promoter was active in all cells. Interestingly, the fraction of cells where the P_{araB} promoter was active decreased. These results demonstrated that xylose can inhibit the expression of the arabinose genes. This inhibition became more apparent when expression from the P_{araB} promoter was averaged over the

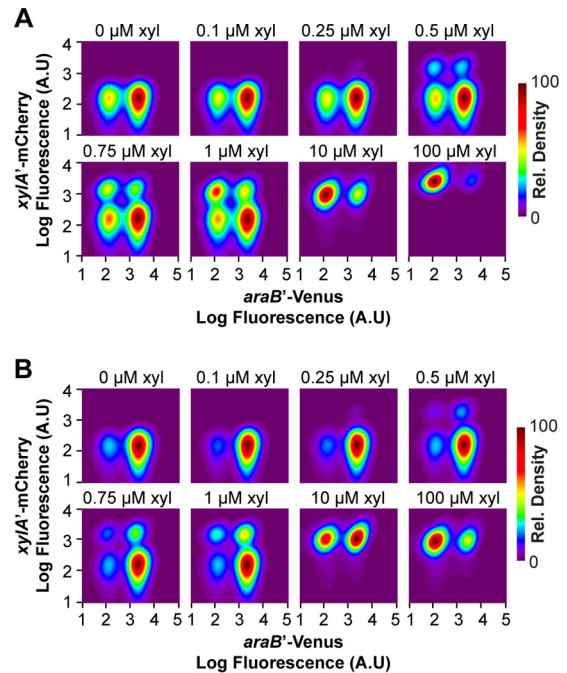


FIG 3 The arabinose and xylose systems exhibit a multimodal response to two sugars. The response was measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xyIA} promoter to the mCherry fluorescent protein. The density plots show the distributions of promoter activities in individual cells as determined based on Venus and mCherry fluorescence. Data were smoothed and normalized to a peak value of 100 to facilitate interpretation. The cells were grown in M9 minimal medium containing 0.4% glycerol, $0.5 \mu\text{M}$ (A) or $1 \mu\text{M}$ (B) arabinose, and various concentrations of xylose, as indicated. Table S1 in the supplemental material lists the fractions of cells in the uninduced and induced states.

entire population (Fig. 4A). We note, however, that the inhibition by xylose was much weaker than inhibition by arabinose (Fig. 4B). In addition, high concentrations of xylose did not completely inhibit the utilization of arabinose, presumably due to the weak inhibition (data not shown).

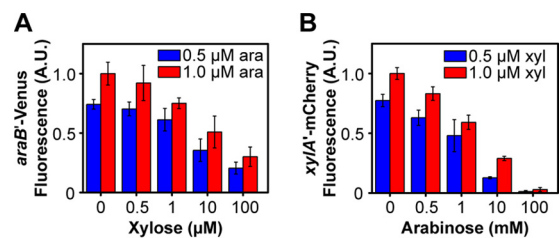


FIG 4 The arabinose and xylose systems are subject to transcriptional cross talk. (A) Xylose represses expression of the arabinose metabolic genes. (B) Arabinose represses expression of the xylose metabolic genes. The responses were measured in a single strain containing single-copy, chromosomal transcriptional fusions of the P_{araB} promoter to the Venus fluorescent protein and the P_{xyIA} promoter to the mCherry fluorescent protein. The cells were grown in M9 minimal medium containing 0.4% glycerol and the specified concentrations of arabinose and xylose. Note that repression by arabinose was stronger than repression by xylose. Fluorescence values were averaged from single-cell, flow cytometry data. Error bars denote the standard deviations from three experiments performed on separate days. Histograms for these data are provided in Fig. S2 in the supplemental material.

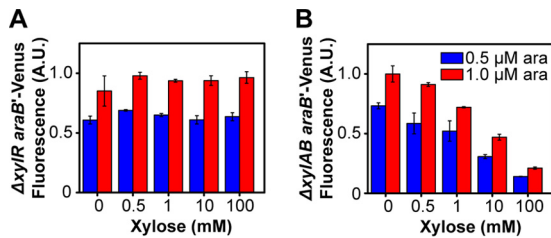


FIG 5 Xylose does not repress arabinose gene expression in the absence of XylR ($\Delta xylR$ mutant strain) (A) but does in the absence of xylose metabolism ($\Delta xylAB$ mutant strain) (B). The responses were measured in the specified strain containing a single-copy, chromosomal transcriptional fusion of the P_{araB} promoter to the Venus fluorescent protein. The cells were grown in M9 minimal medium containing 0.4% glycerol and the specified concentrations of arabinose and xylose. Fluorescence values were averaged from single-cell, flow cytometry data. Error bars denote the standard deviations from three experiments performed on separate days. Histograms for these data are provided in Fig. S3 in the supplemental material.

Desai and Rao (5) previously found that arabinose represses the xylose genes through AraC, where arabinose-bound AraC inhibits activation of the P_{xylA} promoter by xylose-bound XylR (5). To determine if the reciprocal mechanism occurs with xylose, namely, that xylose-bound XylR binds and inhibits activation of the P_{araB} promoter by arabinose-bound AraC, we measured the response of the P_{araB} promoter in a $\Delta xylR$ mutant strain. No repression by xylose was observed (Fig. 5A). The results suggested that repression is XylR dependent, as opposed to xylose somehow inhibiting AraC. One caveat with this experiment is that the xylose utilization genes were not expressed in a $\Delta xylR$ mutant strain, suggesting that xylose may not be able to enter the cells due to the xylose transporters not being expressed. Numerous studies, however, have shown that the arabinose transporters are promiscuous and capable of taking up xylose (5, 25, 26). We also measured the response of the P_{araB} promoter in a $\Delta xylAB$ mutant strain to determine whether xylose was inhibiting the arabinose genes or some downstream metabolite. As shown in Fig. 5B, repression of the arabinose genes by xylose still occurred in the absence of xylose metabolism but the presence of xylose transport.

Based on the results above, XylR likely binds and competitively inhibits the arabinose promoters. To test this mechanism, we performed an electrophoretic mobility shift assay with purified XylR and a DNA fragment containing the divergently arranged P_{araC} and P_{araB} promoters. We also tested binding to the P_{xylA} promoter as a positive control and binding to the P_{flgB} promoter from the flagellar regulon as a negative control, because the latter was unlikely to be bound by XylR. As shown in Fig. 6A, XylR binds both the P_{xylA} and P_{araC}/P_{araB} promoters in a xylose-dependent manner. In particular, a shift was observed only in the presence of XylR and xylose. Not surprisingly, no shift was observed with the P_{flgB} promoter. These results demonstrate that xylose-bound XylR binds the divergent P_{araC}/P_{araB} promoter.

We next used the FIMO program (27) to search for likely XylR binding sites within the P_{araC}/P_{araB} promoter by using the consensus motif derived from known XylR binding sites within the P_{xylA} and P_{xylF} promoters (28). One putative XylR binding site was identified ($P < 0.005$) that overlapped the P_{araC} promoter (Fig. 6B). These results suggested that XylR does not directly repress the P_{araB} promoter but rather indirectly represses it by inhibiting expression of AraC. To test this mechanism, we measured expres-

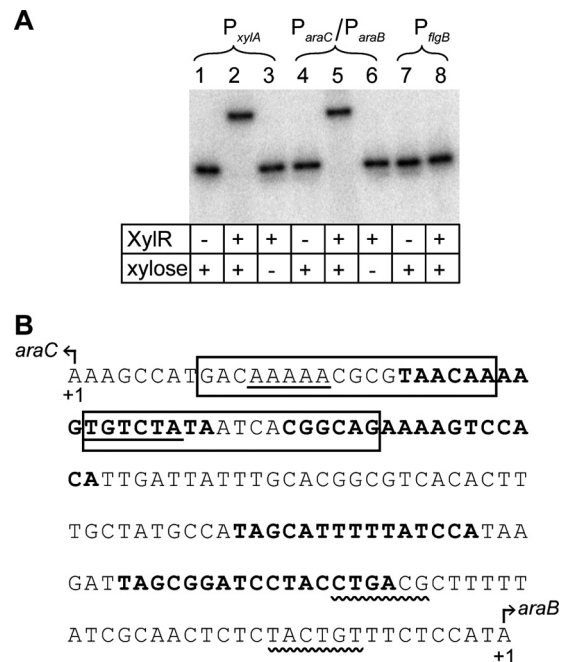


FIG 6 (A) XylR binds the P_{araC}/P_{araB} promoter in a xylose-dependent manner, as determined in an electrophoretic mobility shift assay. The P_{xylA} promoter was included as a positive control, and the P_{flgB} promoter was included as a negative control. (B) Nucleotide sequence of the P_{araC}/P_{araB} promoter region. The $-10/-35$ regions of P_{araC} and P_{araB} promoters are shown with straight and wavy underlines, respectively. The AraC binding sites are shown in boldface. These annotations were taken from RegulonDB (28). The putative XylR binding sites, as determined through sequence analysis, are boxed.

sion from the P_{araC} , P_{araE} , and P_{araF} promoters by using single-copy transcription fusions to the Venus fluorescent protein at different concentrations of xylose. With all three promoters, xylose was found to weakly inhibit their expression (Fig. 7). Because we also did not identify any clear XylR binding sites within the P_{araE} and P_{araF} promoters, these results suggest that xylose-bound XylR binds to the P_{araC} promoter and inhibits expression of AraC, which in turn reduces expression from the P_{araB} , P_{araE} , and P_{araF} promoters.

DISCUSSION

The original motivation for this study was to understand how individual *E. coli* cells behave when grown in mixtures of arabinose and xylose. Both sugar utilization systems exhibit bimodal responses (11–16), and our initial goal was to understand how catabolite repression affected the response of individual cells. Our data show that the response to the two sugars is multimodal and that the shape of this distribution is determined by the reciprocal regulation of these two sugar utilization systems. Multiple studies have shown that arabinose inhibits the utilization of xylose in *E. coli* by repressing the expression of the xylose metabolic genes (5, 6). The key finding in the present study is that xylose can also inhibit, albeit weakly, the expression of the arabinose metabolic genes. These results demonstrate that the hierarchy between arabinose and xylose is not fixed, as previously believed, but rather determined by the respective sugar concentrations. An immediate question is why.

The repression of xylose utilization by arabinose ostensibly

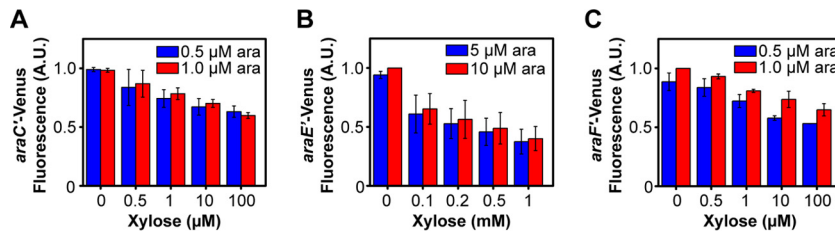


FIG 7 Xylose represses the expression of arabinose transporters and the transcriptional activator AraC. The response was measured in a single strain containing a single-copy, chromosomal transcriptional fusion of the P_{araC} (A), P_{araE} (B), or P_{araF} (C) promoter to the Venus fluorescent protein. Fluorescence values were averaged from single-cell, flow cytometry data. Error bars denote the standard deviations from three experiments performed on separate days. Histograms for these data are provided in Fig. S4 in the supplemental material. Note that higher arabinose concentrations were required in order to induce expression from the P_{araE} promoter.

makes sense, because *E. coli* grows at a faster rate on arabinose than it does on xylose due to differences in the route of transport employed. In particular, both the arabinose and xylose systems separately employ two similar pairs of transporters for sugar uptake, a low-affinity proton symporter and a high-affinity ATP-dependent transporter (Fig. 1). Arabinose is primarily transported through the more-energy-efficient AraE symporter, whereas xylose, for reasons unknown, is primarily transported through the more-energy-costly ATP-dependent XylFGH transporter, even at high sugar concentrations (25, 29). The resulting differences in growth rates fit the general pattern observed with hierarchical sugar consumption and presumably reflect the efficient allocation of metabolic resources by the cell (8). If xylose is in excess, however, then it presumably makes sense for the cells not to ignore it and to allocate their metabolic machinery in proportion to the availability of this alternate growth substrate. Indeed, both arabinose and xylose are principally derived from hemicellulose hydrolysates, where xylose is the more predominant sugar (30). Alternatively, mutual repression of these two sugar utilization systems could reflect a division-of-labor strategy, where some cells grow on arabinose and others grow on xylose. As our data show (Fig. 3), these mixed populations are observed during growth at intermediate sugar concentrations. Furthermore, we observed fewer cells induced for growth on both sugars than would be expected if the pathways operated independently of one another (see Table S1 in the supplemental material). Likely, individual cells can employ either strategy, because we observed that some cells adapted to growth on just one sugar and others adapted to growth on both. For either strategy, be it proportional allocation or division of labor, one would expect repression by arabinose to be stronger, because it is the better growth substrate. In other words, when the concentrations are equal, we would expect more cells to be adapted to growth on arabinose than on xylose. This in fact is what we observed.

While both the arabinose and xylose metabolic genes exhibit a bimodal response to their cognate sugar, the nature of this response is different. As first documented by Afroz and coworkers, the xylose response is all or nothing at all sugar concentrations, whereas the arabinose response is all or nothing at low sugar concentrations and graded at high sugar concentrations. While the physiological significance of these differences is not known (at least in response to a single sugar), they potentially explain why repression by arabinose is significantly stronger than repression by xylose: the arabinose genes exhibit a greater range of expression levels in individual cells than the xylose genes. Presumably, the degree of repression by arabinose-bound AraC also exhibits a greater range than xylose-bound XylR.

While transcriptional cross talk provides one mechanism to explain catabolite repression among nonglucose sugars, other mechanisms have also been proposed. Aidelberg and coworkers recently investigated the selective consumption of six non-phosphotransferase system (PTS) sugars (8). They observed a hierarchy in the expression of the genes associated with α -lactose, L-arabinose, D-xylose, D-sorbitol, L-rhamnose, and D-ribose metabolism in *E. coli*. The hierarchy, with lactose at the top and ribose at the bottom, matches the ordering of the growth rates supported by these sugars. In other words, a sugar is preferred to another if it supports faster growth. These results demonstrate that catabolite repression is widespread among non-PTS sugars. In addition to discovering this hierarchy, Aidelberg et al. also found that the cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP) differentially activates the promoters for these metabolic genes, where the relative degree of activation follows the same hierarchy. This observation is significant, because previous studies have demonstrated that cAMP synthesis is inversely proportional to the growth rate of the cell (31–33). Thus, a cell growing on lactose will produce less cAMP than one growing on any one of the other five sugars. Based on these findings, those authors proposed an alternate model for catabolite repression among non-PTS sugars that was based on sequential activation as opposed to one based on competitive inhibition. According to this model, the metabolic genes for the less-preferred sugar are not expressed, because cAMP concentrations are too low to induce their expression due to the faster growth supported by the preferred sugar. While this model is appealing, it does not explain the selective utilization of arabinose and xylose. For one, the present study demonstrates that the hierarchy between these two sugars is not fixed but rather is determined by their relative concentrations. Furthermore, Desai and Rao demonstrated that arabinose still inhibits xylose gene expression in a mutant ($\Delta araBAD$ strain) unable to metabolize arabinose (5). Moreover, we found in the present study that xylose inhibited arabinose gene expression in a mutant ($\Delta xylAB$ strain) unable to metabolize xylose (Fig. 5B). These results clearly demonstrate that catabolite repression is not due to differential activation by CRP-cAMP but instead transcriptional cross talk; how else can xylose (or arabinose) repress arabinose (or xylose) gene expression when xylose (or arabinose) is not being metabolized? Our results, however, do not invalidate their general model, as only two sugars were considered in the present study. In addition, the relative degree of activation of the arabinose and xylose genes by CRP-cAMP, as reported by Aidelberg and coworkers, is small and substantially less than many other sugars investigated in their work. It is unlikely to result in the

hierarchical expression of these two sets of metabolic genes. Whether the differences are sufficiently great to govern the hierarchical expression of other sugar genes is unknown.

In addition to identifying a new facet to the coregulation of arabinose and xylose metabolism, the present work may also aid efforts to produce chemical and fuels from plant biomass. Plant biomass is a renewable and low-carbon feedstock for many value-added compounds. Fermentation of the constituent sugars using engineered microorganisms provides one promising route for the conversion of plant biomass to chemicals and fuels (34, 35). After glucose, xylose and arabinose are the next-most-abundant sugars in plant-derived hydrolysates. For the fermentation process to be economic and efficient, the microorganisms need to be able to use all of these sugars and, ideally, at the same time. Not surprisingly, numerous design strategies have been proposed to enable simultaneous sugar utilization in *E. coli* as well as other bacteria and yeast. Most of these efforts have focused on the cointegration of glucose and another sugar (36). Less effort has been directed toward the cointegration of nonglucose sugar (7, 37–39). In the context of this work, the work of Groff and coworkers (37) is notable. They engineered an *E. coli* strain capable of simultaneously consuming arabinose and xylose by overexpressing XylR. Presumably, the increased concentration of XylR counterbalances inhibition by arabinose-bound AraC, thus enabling the expression of both sets of metabolic genes. In the course of designing this strain, they found that arabinose utilization was inhibited when they overexpressed XylR from too strong of a promoter. They hypothesized that high levels of XylR repress expression of the arabinose metabolic and transport genes. The present work validates their hypothesis and further shows that xylose can repress arabinose gene expression even under native conditions. It also shows that regulation of these two sugar utilization systems is not as simple as previously believed, and further engineering will be required to design optimal cointegrating strains. An open question concerns how the individual cells of the strain engineered by Groff and coworkers actually behave. Are they in fact simultaneously consuming the two sugars or are they separating into two balanced populations selectively consuming the two sugars? The present work demonstrates that both scenarios are possible. An additional question is whether one scenario would be preferred over the other in the context of industrial fermentations; in particular, are generalists preferred to specialists? Nature seems to prefer both. More work is required to resolve these issues.

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