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High-Affinity Arabinose Transport Mutants of *Escherichia coli*: Isolation and Gene Location

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The gene *araF*, the product of which is the L-arabinose-binding protein—a component of the high-affinity L-arabinose transport system, was located on the *Escherichia coli* linkage map at 45 min. We established this location using bacteriophage P2 eductates and bacteriophage P1 cotransduction frequencies with the adjacent genetic loci, *his* (histidine biosynthesis) and *mgl* (methylgalactoside transport). In addition, we isolated a number of mutants that phenotypically exhibited altered high-affinity L-arabinose transport capacities. At least two of these mutations were located in the *araF* gene, as binding protein purified from these strains exhibited altered in vitro arabinose-binding properties.

The pentose L-arabinose can serve as the sole carbon and energy source for *Escherichia coli*. There are two systems which transport L-arabinose into the cell, both of which are controlled by the arabinose regulatory protein, the product of the gene *araC* (6). The two transport systems can readily be distinguished by the biphasic nature of double reciprocal plots of uptake rate versus substrate concentration and by the properties of a number of mutants (10). Low-affinity L-arabinose transport was first described by Novotny and Englesberg (20) and is arabinose inducible, energy dependent, stable to osmotic shock, and observable in membrane vesicles and has a K_{en} of 1×10^{-4} M (10, 20). Mutations causing a defect in this system are located in the *araE* gene. High-affinity L-arabinose transport possesses a K_{en} of 1×10^{-6} M and contains as a component of the system the inducible, periplasmic L-arabinose-binding protein (ABP) (11). Mutants with altered ABP (25) or which produce no detectable ABP (CRM^- mutants) (3) lose the high-affinity transport system. ABP is the product of the *araF* gene and was tentatively located on the *E. coli* linkage map as being cotransducible with *his* (10).

The periplasmic binding proteins of gram-negative enteric organisms share many similar properties (2). They are essential for the high-affinity transport of many amino acids, carbohydrates, and ions (2) and, in addition, some carbohydrate-binding proteins also serve as essential receptors for chemotaxis (1, 14). The primary and tertiary structure of ABP has been solved in an attempt to determine its mechanism of action (12, 22). (This work is from a thesis submitted to the Department of Microbiology, Case Western Reserve University, in partial fulfillment of the requirements for the Ph.D. degree.)

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MATERIALS AND METHODS

Genetic methods. Bacterial strains used in this study are listed in Table 1. UP1654 and CW2022 are *E. coli* B/r; the P2 eductant QE35 and its parent are K-40 derivatives. All other strains are derivatives of *E. coli* K-12.

Mutants were obtained by ethyl methane sulfonate mutagenesis of strain CW2201 (18). Auxotrophs were isolated after two cycles of ampicillin amplification (18). P1bt bacteriophage transduction was by the method of Roth (24). Log-phase cells grown in LC broth (no glucose) were suspended in absorption media at a density of 4×10^8 cells/ml with donor phage at a multiplicity of infection of 0.05. This suspension was incubated at 37°C for 30 min. The cells were washed with E minimal medium (24), dispersed on appropriate selection media, and incubated at 37°C.

High-affinity L-arabinose transport mutants were isolated from an *E. coli* K-12 *araE* strain (CW2201). After mutagenesis, cells were grown on plates of eosin methylene blue or triphenyl tetrazolium chloride containing arabinose as a carbon source, and those colonies displaying an increased arabinose-negative phenotype compared with the parent were isolated. Mutations in *ara* operon structural genes were eliminated by testing all isolated strains for *araA* enzyme activity (5) and growth in the absence or presence of arabinose, indicative of mutations in *araD*. β-methylgalactoside transport mutants were identified by the inability to grow on 0.1 to 1 mM β-methylgalactoside and by the inability to transport 10^{-7} M D-galactose.

Transport assays. Cells were grown in minimal salts [0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, 0.003% $MnCl_2$], 0.2% glycerol, 0.2% Casamino Acids, and thiamine (1 mg/ml) at 37°C and induced in early- to mid-log phase by the addition of 0.4% L-arabinose. After three generations of growth, cells were centrifuged and washed three times with

TABLE 1. *Bacterial strains*

Strain	Genotype	Relevant phenotype	Origin or reference
UP1654	B/r F⁻ *araE201*,	Ara⁻, low-affinity-Ara-transport	Hogg and Englesberg (11)
CW2022	B/r *araF404*	ABP⁻	Brown and Hogg (3)
CW2200	K-12 Hfr *thi* wild type)	Ara⁺, thiamine⁻	Schleif (25)
RSF3	K-12 *araA3*	Ara⁻, thiamine⁻	Schleif (25)
CW2201	K-12 *araE201 thi*	Wild type, low-affinity-Ara-transport⁻	From CW2200 by cotransduction with thymine, using P1bt grown on UP1654
CW2202-2215	K-12 *araE201 araF412-425*	Low-affinity-Ara-transport⁻ High-affinity-Ara-transport⁻	Ethyl methane sulfonate mutagenesis of CW2201
CW2216	K-12 *his* CW2205	Low-affinity-Ara-transport⁻ High-affinity-Ara-transport⁻	Ethyl methane sulfonate mutagenesis of CW2205
S183-71-17	K-12 *leu his ΔlacZYA mglDC*	High-affinity-Gal-transport⁻	B. Rotman
S183-72-2	K-12 *leu his ΔlacZYA mglDA*	High-affinity-Gal-transport⁻	B. Rotman
AW5014	K-12 *thr leu met mglB*	GBP⁻	J. Adler
AW5015	K-12 *thr leu met mglB*	GBP⁻	J. Adler
K-40	K-40 (wild type)	Wild type	M. Sunshine (27)
QE35	K-40 Δ(*his-rfb-gnd*)	High-affinity-Gal-transport⁻ High-affinity-Ara-transport⁻	Derived from K-40 by P2 eduction, Sunshine and Kelly (27)

minimal glycerol media. Cells were suspended in minimal glycerol media plus chloramphenicol (50 μg/ml) to an optical density of 0.125 at 600 nm. Transport assays were performed at 25°C and started by the addition of 1.0 ml of cells to 0.25 ml of the appropriate concentration of L-[^{14}C]arabinose (60 mCi/mmol; Amersham Corp.) or D-[^{3}H]galactose (1 mCi/0.3 mg; New England Nuclear Corp.). Final cell density was approximately 1×10^9 cells/ml (optical density at 600 nm of 0.1). Uptake was terminated by membrane filtration (0.45 μm, Millipore Corp.) of 1 ml of reaction mixture. The membrane filter was rinsed with 1 ml of room temperature minimal glycerol medium, dried, placed in scintillation fluid {0.4% PPO [2,5-dephenyloxazole]; 0.01% POPOP [1,4-bis-(5-phenyloxazolyl)benzene] in toluene}, and counted in a Packard Prias or Packard Tri-Carb liquid scintillation spectrometer. L-arabinose transport mutants were characterized by 30-s assays and concentrations of 10^{-5} and 10^{-6} M L-arabinose and 10^{-7} M D-galactose. The purity of the D-[^{3}H]galactose and L-[^{14}C]arabinose was ascertained by paper or thin-layer chromatography in water-saturated 1-butanol.

ABP production. ABP production in isolated colonies was ascertained by the CRM (ABP cross-reacting material) plate method (9). ABP production was also determined in disrupted L-arabinose-induced cells by equilibrium dialysis in the presence of 5×10^{-5} M L-[^{14}C]arabinose (50 mM $KxPO_4$ [pH 7.8]–0.1 mM dithiothreitol) and by reactivity to anti-ABP antiserum in double-immunodiffusion assays.

ABP L-arabinose-binding assays. ABP was purified from each of the mutants (21), and purity was verified by electrophoresis on polyacrylamide gels under denaturing (15) and nondenaturing (4) conditions. The ABP was partially denatured in 2 M guanidine-hydrochloride to remove arabinose which is bound after purification (23) and dialyzed extensively against 50 mM $KxPO_4$–0.1 mM dithiothreitol, pH 7.8. Equilibrium binding assays were carried out using 10 μM ABP (in EDTA-treated dialysis tubing) in 100 ml of assay buffer (50 mM $KxPO_4$–0.1 mM dithiothreitol, pH 7.8) containing the appropriate concentration of L-[^{14}C]-arabinose (60 mCi/mmol, Amersham Corp.). The binding assays were allowed to reach equilibrium (18 h) while rotating at 6°C. Protein concentration was determined with samples from the dialysis bags and the ABP extinction coefficient, $E_{280} = 0.94$ liter mg^{-1} cm^{-1} (19), or by the method of Lowry et al. (16). Samples (0.2 to 0.3 ml) were mixed with scintillation fluid (33% Triton X-100 in toluene, 0.067 g of POPOP per liter, and 2.67 g of PPO per liter) and counted for 10 min in a Packard Prias or Packard Tri-Carb liquid scintillation counter. All assays were conducted in duplicate and repeated at least twice. The slopes of all plots were determined by linear regression analysis.

Peptides of ABP were prepared with lyophilized protein. For the preparation of tryptic peptides, ABP was dissolved in freshly prepared 0.2 M NH_4HCO_3 (pH 8.0), and 1% (wt/wt) trypsin (L-tosylamido 2-phenol ethyl chloromethyl ketone treated; Worthington Biochemical Corp.) was added. After incubation at 37°C for 10 h, an additional 1% trypsin was added, and the sample was incubated at 37°C for an additional 10 h before lyophilization.

ABP peptides were analyzed by high-pressure liquid chromatography (HPLC) by the method of Mahoney and Hermodson (17). The HPLC system consisted of a SynChropak RP-P C-8 HPLC column (4.1 by 250 mm; SynChrom, Inc.) connected to a Beckman model 334 gradient liquid chromatograph, model 421 controller, model C-R1A integrator, and a variable wavelength Hitachi spectrophotometer, model 100-10. Tryptic peptides were run at a flow rate of 1.5 ml/min on a linear gradient of 0 to 50% acetonitrile (Burdick and Jackson Laboratories) in 0.1% trifluoroacetic acid.

The trifluoroacetic acid was redistilled after refluxing over CrO_3 for 2 h.

Sequence analysis of HPLC-purified peptides was performed on a Beckman model 890C sequencer, using a peptide precipitation program (8) modified to include Polybrene (3 to 5 mg per analysis; Pierce Chemical Co.) (13). PTH-amino acids were identified by HPLC, using the method of Somack (26), or by gas chromatography, using a Hewlett-Packard model 5830A gas chromatograph as described previously (7).

RESULTS AND DISCUSSION

The gene responsible for ABP, *araF*, had previously been reported as being cotransducible with *his*, using the bacteriophage P1 (10). P2 eductants were used in an attempt to better locate *araF*. P2 eductants are bacteria that were lysogenic for the bacteriophage P2, which upon phage excision have also lost a portion of the host chromosome. Sunshine and Kelly have reported that education occurs precisely at the P2 attachment site (43.5 map units) and deletions extend various lengths through the *his* locus (27). The eduction in QE35 would appear to extend through *mgl* since QE35 did not grow on 1 mM methylgalactoside as sole source of carbon and did not transport D-galactose at 10^{-7} M (Table 2). In the control experiment, the K-40 parent grew on 1 mM methylgalactoside and effectively transported D-galactose at 10^{-7} M. QE35 did not produce ABP as detected by equilibrium arabinose-binding or double-immunodiffusion analysis, whereas the K-40 parent did. Strain differences in arabinose and galactose uptake rates (Table 2) (K-12 versus B/r versus K-40) were considerable, and therefore we used growth, binding assays, and immunodetection methods to confirm the presence or absence of the binding protein. On the basis of the eduction origin reported by Sunshine and Kelly (27) (*attP2H*, 43.5 min) and the extent of the deletion in QE35 (through *mgl*, this report) and the absence of immunodetectable ABP in this strain it appears that *araF* is located between *attP2H* and *mgl*.

Thirty-second assays at two arabinose concentrations, 10^{-5} M and 10^{-6} M, were used to screen ethyl-methane-sulfonate-mutagenized cells for high-affinity L-arabinose transport, and putative mutants are noted in Table 2.

Using P1 cotransduction frequencies, *araF* appears to be located between *his* and *mgl*. *araF* transfer was determined with the ABP CRM^- mutant CW2022. A *his* auxotroph (CW2216) of mutant CW2205 proved suitable as an *mgl* phenotypic marker when it was determined that CW2205 produced ABP CRM, though still defective in high-affinity L-arabinose transport, and was found to be mutant for *mgl* by growth assays and galactose uptake.

The results of the present study, using a three-point genetic cross involving *his*, *araF*, and *mgl*, located *araF* at 45 units on the *E. coli* linkage map between the *his* (44.2) and *mgl* (45.4) loci (Table 3). This location is based on a 21% cotransduction frequency of *araF* with *his*, a 15% cotransduction frequency of *mgl* with *his*, and a 67% linkage of *araF* and *mgl*. If *araF* were

TABLE 2. *L-Arabinose and D-galactose transport*

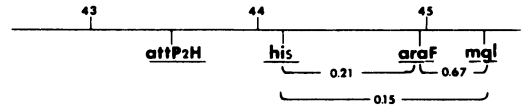
Strain	Genotype	Transport sp act[a] of:		
		L-Arabinose		D-Galactose (L-arabinose induced)
		10^{-5} M	10^{-6} M	10^{-7} M
CW2201	*araE201 thi*	172	34.8	13.2
CW2022	*araE201 araF*(CRM^-) *thi*		4.3	0.1
CW2215	CW2205 *his*	12.3	0.2	
AW5014	*mglB*		1.6	0.1
AW5015	*mglB*		6.0	0.3
S183-71-17	*mglDC*		3.0	0.2
S183-72-2	*mglDA*		4.8	0.1
K-40	Wild type (control)		0.9	9.8
QE35	P2 eductant of K-40		0.4	0.2
CW2202 to CW2215	Putative high-affinity Ara-transport mutants	0–12.6	0–1.4	

[a] Nanomoles per 30 s per milliliter (optical density of 0.10).

TABLE 3. *Cotransduction and genetic mapping of araF*[a]

No. of transductants with:		
his$^+$ (selected marker)	*araF* (as crm^-)	*mgl*$^+$
140	30	21
	30	20

[a] Cross P1bt bacteriophage generated on strain CW2022 × CW2216. In CW2022 (*his*$^+$ *araF mgl*$^+$) the *araF* mutation resulted in an ABP CRM^- phenotype; in CW2216 (*his araF mgl*) the *araF* mutation resulted in an ABP CRM^+ phenotype. Transductants were screened for ABP CRM production (10) and *mgl* expression (growth on 0.1 to 1 mM β-methylgalactoside). Of 30 ABP crm^- transductants, 20 were mgl^+. Map distances, calculated according to Wu (28), were *his-mgl*, 1.08 units; *his-araF*, 0.92 units; *araF-mgl*, 0.29 units. Cotransduction frequencies are indicated on the map under appropriate loci.



located on the other side of *mgl*, its cotransduction frequency with *his* would be expected to be below 15%. We therefore assume the proposed gene order is correct.

Equilibrium dialysis L-arabinose-binding assays demonstrated that most of the putative L-arabinose transport mutants isolated have wild-type binding ability with a dissociation constant (K_d) of approximately 2×10^{-7} M and a ν_0 (moles of ligand bound per mol of ABP) of 0.5 to 0.7. Mutants CW2208 and CW2210 had altered binding parameters, displaying K_d's of 4×10^{-7} M and 2×10^{-7} M and ν_0's of 0.3 and 0.02, respectively (Fig. 1). Initial analysis of ABP tryptic peptides from these mutants indicated an alteration in CW2208 ABP (Fig. 2), although CW2210 ABP was refractory to this type of analysis. The peptide designated by an asterisk in the wild type (Fig. 2a) was absent in the mutant (Fig. 2b), which acquired two additional peptides designated by asterisks in Fig. 2b. The peptide in the wild-type ABP, which is absent in the mutant CW2208, was isolated by HPLC methods, and analysis of the amino acid sequence indi-

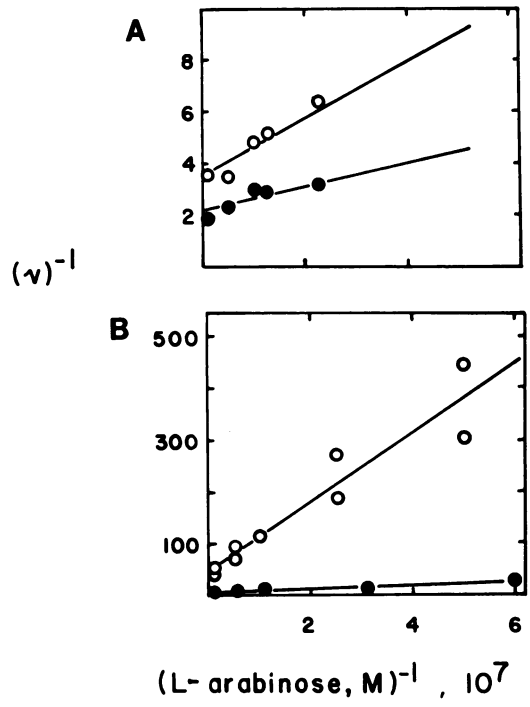


FIG. 1. *L-arabinose equilibrium binding kinetics of wild-type ABP (●) isolated from RSF3 (araA3 thi) and mutant ABP (○). ABP was at a concentration of 10 μM. Specific activity of L-[^{14}C]arabinose was 1.1 × 10^{14} cpm/mol; ν is moles of ligand bound per mole of ABP; data are the results of three assays done in duplicate. Lines were drawn by linear regression analysis of data. (A) Mutant CW2208; (B) mutant CW2210.*

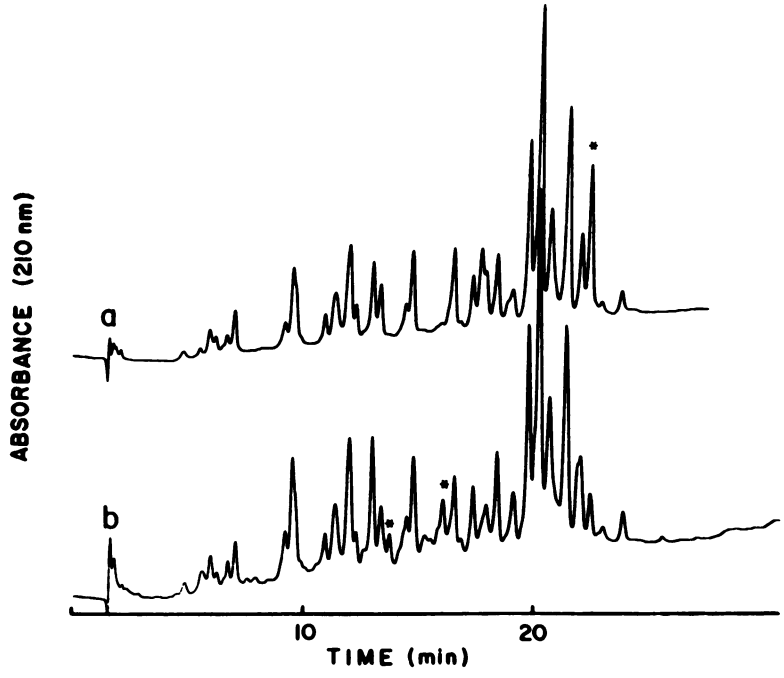


FIG. 2. *HPLC of ABP tryptic peptides (35 μg) from (a) wild-type and (b) mutant CW2208 on a C-8 column. Linear gradient was 0 to 50% $CNCH_3$ in 0.1% trifluoroacetic acid; flow rate was 1.5 ml/min.*

cated it to be the tryptic fragment located between residues 198 and 214 of the complete molecule. In the three-dimensional structure of the molecule, this peptide is located in the hinge region, which contains the binding site for L-arabinose. Attempts to isolate the altered peptide(s) from CW2208 ABP have been unsuccessful.

The arabinose transport mutants isolated in this study will be used for structure-function studies of the ABP. Since not all of the mutations are likely to occur in *araF*, these and additional mutants will be used in future studies to positively identify the other components required for high-affinity arabinose transport.

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