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An *lrp*-Like Gene of *Bacillus subtilis* Involved in Branched-Chain Amino Acid Transport

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The *azlB* locus of *Bacillus subtilis* was defined previously by a mutation conferring resistance to a leucine analog, 4-azaleucine (J. B. Ward, Jr., and S. A. Zahler, J. Bacteriol. 116:727–735, 1973). In this report, *azlB* is shown to be the first gene of an operon apparently involved in branched-chain amino acid transport. The product of the *azlB* gene is an Lrp-like protein that negatively regulates expression of the *azlBCDEF* operon. Resistance to 4-azaleucine in *azlB* mutants is due to overproduction of AzlC and AzlD, two novel hydrophobic proteins.

Leucine-responsive protein (Lrp) of *Escherichia coli* is a global regulator affecting transcription of a diverse array of genes, many of which are involved in transport, biosynthesis, and degradation of amino acids; expression of most Lrp targets is modulated by leucine availability (9, 35). Lrp also negatively regulates its own expression (45). Lrp and its homologs, including *E. coli* AsnC (29), form an AsnC-Lrp family of transcription regulators that at present comprises more than 20 proteins from different groups of the domains *Eubacteria* and *Archaea.*

One of the *E. coli* operons positively regulated by Lrp is the glutamate synthase operon, *gltBDF* (8, 16). In *Bacillus subtilis*, transcription of the *gltA* and *gltB* genes, encoding the subunits of glutamate synthase, is subject to positive regulation by the product of the *gltC* gene (6, 7) but may be under the control of other proteins as well. The GltC protein belongs to the LysR family of bacterial regulators of transcription (41). Recently, a fragment of the *B. subtilis* chromosome containing one more gene of this family, *gltR*, that may be involved in *gltAB* regulation was cloned (5).

While determining the sequence of the *gltR* (233°) region (5) of the *B. subtilis* chromosome, we came upon an open reading frame that could encode a homolog of Lrp. Given the involvement of Lrp in regulation of glutamate synthase synthesis in *E. coli*, we were motivated to undertake a detailed analysis of its *B. subtilis* homolog. The *B. subtilis lrp*-like gene proved to be identical to *azlB*, described as a locus conferring resistance to 4-azaleucine (46), and to be the first gene of an operon apparently involved in transport of branched-chain amino acids. Two operons needed for branched-chain amino acid transport in *E. coli* are regulated by Lrp (22). The *azlB* gene was shown not to be involved in glutamate synthase regulation.

MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains were grown at 37°C in TSS minimal medium (20) with 0.5% glucose and a 0.2% nitrogen source, in Spizizen minimal medium with 0.5% glucose (42), or in DS nutrient broth medium (20). The same media with addition of agar or tryptose blood agar base medium (Difco) was used for growth of bacteria on plates. Strains carrying *azl-lacZ* transcriptional fusions integrated at the *amyE* locus were isolated after transformation of strain SMY with derivatives of pJPM82 (Table 1; also, see below), with selection for resistance to erythromycin and screening for loss of α-amylase production, which indicated a double-crossover, homologous recombination event. The Amy phenotype was assayed with colonies grown overnight on tryptose blood agar base–0.2% starch plates (20). The azaleucine resistance (Azl^r) phenotype was determined on TSS-agar plates containing 100 to 200 μg of 4-D,L-azaleucine (Sigma Chemical Co.) per ml. L broth or L agar (32) was used for growth of *E. coli* strains. The following antibiotics were used when appropriate: chloramphenicol (2.5 to 5 μg/ml), neomycin (5 μg/ml), tetracycline (15 μg/ml), phleomycin (1.0 μg/ml), and a combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) for *B. subtilis* strains and ampicillin (50 to 100 μg/ml), kanamycin (25 μg/ml), chloramphenicol (10 μg/ml), and tetracycline (10 μg/ml) for *E. coli* strains.

DNA manipulations and transformation. Methods for plasmid isolation, agarose gel electrophoresis, use of restriction and DNA modification enzymes, DNA ligation, Southern blot analysis, and PCR were as described by Sambrook et al. (39). *B. subtilis* chromosomal DNA was isolated by a previously published procedure (20). Preparation of electroporation-competent cells and plasmid transformation of *E. coli* strains with a GenePulser apparatus (Bio-Rad Laboratories) were done as described elsewhere (14). Transformation of *B. subtilis* by chromosomal or plasmid DNA was performed by the method of Dubnau and Davidoff-Abelson (15). DNA probes were radiolabeled with [α-^{32}P]dCTP by using the Rediprime DNA labeling system (Amersham Life Science) according to the manufacturer's instructions.

Plasmid constructions. Plasmids used in this work are shown in Fig. 1 and 2A or are described in the text.

To clone the *azlB* region from a *B. subtilis* strain of the 168 lineage, the chromosomal DNA of strain 1A1 was completely digested with *Hin*dIII and size-fractionated by centrifugation in a 10 to 40% sucrose gradient. Two- to 6-kb fragments were ligated to *Hin*dIII-digested pBluescript II KS(−) (Stratagene, Inc.) and introduced into *E. coli* SURE by transformation. The resulting library, plated onto Hybond N membranes (Amersham Life Science), was screened with the radiolabeled 1.25-kb *Nsi*I_1-*Eco*RI_2 fragment of plasmid pBB408 (5), essentially as described by Sambrook et al. (39). The clone containing plasmid pMG001, harboring the 4.2-kb *Hin*$dIII_2$-*Hin*$dIII_3$ fragment (Fig. 1), was kept. By a similar procedure, the 4.6-kb *Pst*I_1-*Pst*I_2 fragment (pMG042; Fig. 1) was isolated by screening a library of 4- to 9-kb *Pst*I fragments.

To clone the chromosomal region containing the *azlB101* mutation, plasmid pBB589 (Fig. 2A), a derivative of pBB544 (described below), was introduced into the chromosome of strain BB34 (*azlB101*) by transformation via single-crossover, homologous recombination. The chromosomal DNA of the resulting strain was digested with *Hin*dIII, self-ligated, and introduced into *E. coli* JM107 by transformation. The isolated plasmid, pBB600, contained the 1.2-kb *Acc*I_1-*Hin*$dIII_3$ fragment of *B. subtilis* DNA (Fig. 1).

To construct plasmid pBB544, the *neo* gene from pBEST501 (27) was excised as a 1.3-kb *Pst*I-*Not*I fragment, treated with the large (Klenow) fragment of *E. coli* DNA polymerase I, and inserted at the filled-in *Afl*III site of pBluescript SK(−) (Stratagene, Inc.). The direction of *neo* transcription in pBB544 is away from the polylinker region; a plasmid with the opposite orientation of *neo* was called pBB543.

To construct most plasmids containing *azl-lacZ* transcriptional fusions (Fig.

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TABLE 1. Bacterial strains used

Strain	Genotype	Source or reference
E. coli		
JM83	*ara thi-1* Δ(*lac-proAB*) *strA* (ϕ80*lacZ*ΔM15)	47
JM107	*endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- Δ(*lac-proAB*) e14$^-$/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15	47
SURE	*endA1 gyrA96 thi* Δ*hsdRMS supE44 relA1 lac recB recJ sbcC201 uvrC umuC*::Tn*5*/F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*	Stratagene, Inc.
XL1-Blue	*endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*/F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*	Stratagene, Inc.
BU1255	*dam-3 dcm-6 gal lac ara thr leu*/F^+	Laboratory stock
B. subtilis		
168 lineage		
1A1	*trpC2*	BGSC[a]
BB34	*azlB101 pheA12*	5
LUW1	*trpC2* Δ*cypA*::*ble*	1A1 × pMG033
LUW5	*trpC2* Δ*azlB*::*ble*	1A1 × pΔlrp1
LUW7	*trpC2* Δ*orf91*::*ble*	1A1 × pMG041
SMY lineage		
SMY	Wild type	P. Schaeffer
BB272	Δ*azlB1* Ω(*orf91-azlB*)::*tet*[b]	SMY × pBB581
BB274	Δ*azlB2*	SMY × pBB587
BB284	Δ*azlCDE*::*neo*	SMY × pBB598
BB285	Δ*azlB2* Δ*azlCDE*::*neo*	BB274 × pBB599
BB286	′*orf282*::pBB404 (*cat* ′*orf182*′)	SMY × pBB404
BB831	Δ*amyE*::[*erm* Φ(*azlB*′-*lacZ*)]	SMY × pBB525
BB832	Δ*amyE*::[*erm* Φ(*azlBC*′-*lacZ*)]	SMY × pBB526
BB833	Δ*amyE*::[*erm* Φ(*azlBCD*′-*lacZ*)]	SMY × pBB527
BB834	Δ*azlB2* Δ*amyE*::[*erm* Φ(*azlB*′-*lacZ*)]	BB274 × DNA (BB831)
BB837	Δ*amyE*::[*erm* Φ(*azlB*′-*lacZ*)]	SMY × pBB529
BB838	Δ*azlB2* Δ*amyE*::[*erm* Φ(*azlB*′-*lacZ*)]	BB274 × DNA (BB837)
BB839	Δ*amyE*::[*erm* Φ(*azl*′*CD*′-*lacZ*)]	SMY × pBB528
BB840	Δ*azlB2* Δ*amyE*::[*erm* Φ(*azl*′*CD*′-*lacZ*)]	BB274 × DNA (BB839)
BB841	Δ*amyE*::[*erm* Φ(Δ*azlB2CD*′-*lacZ*)]	SMY × pBB530
BB842	Δ*azlB2* Δ*amyE*::[*erm* Φ(Δ*azlB2CD*′-*lacZ*)]	BB274 × DNA (BB841)
BB848	Δ*amyE*::[*erm* Φ(Δ*azlB3CD*′-*lacZ*)]	SMY × pBB531
BB849	Δ*azlB2* Δ*amyE*::[*erm* Φ(Δ*azlB3CD*′-*lacZ*)]	BB274 × DNA (BB848)
BB853	Δ*amyE*::[*erm* Φ(*azl*′*B*′-*lacZ*)]	SMY × pBB532
BB854	Δ*azlB2* Δ*amyE*::[*erm* Φ(*azl*′*B*′-*lacZ*)]	BB274 × DNA (BB853)
BB859	Δ*amyE*::[*erm* Φ(Δ*azlB2CDbrnQ*′-*lacZ*)]	SMY × pBB533
BB860	Δ*azlB2* Δ*amyE*::[*erm* Φ(Δ*azlB2CDbrnQ*′-*lacZ*)]	BB274 × DNA (BB859)
BB861	*orf105*::pBB535[*cat* Φ(*orf105*′-′*lacZ*)]	SMY × pBB535
BB862	Δ*azlB2 orf105*::pBB535[*cat* Φ(*orf105*′-′*lacZ*)]	BB274 × pBB535
BB865	Δ*amyE*::[*erm* Φ(*azl*′*BC*′-*lacZ*)]	SMY × pBB536

[a] BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus.
[b] The *tet* gene is inserted between the *orf91* and *azlB* genes (Fig. 1).

2A), appropriate DNA fragments from different derivatives of pBB544 (Fig. 2A) were subcloned between the *Bam*HI and *Eco*RI sites of pJPM82. The latter plasmid was made by J. Mueller (33) by first blunt-ending the *Eco*RI site of pJPM15 (34) to create pJPM80 and then replacing the 2.2-kb *Hin*dIII-*Sac*I fragment with the 2.1-kb *Hin*dIII-*Sac*I fragment of pJPM3 (34). This placed the *lacZ* reporter gene in pJPM82 under the control of the *spoIIAA* ribosomal binding site (17) with unique *Hin*dIII, *Bam*HI, and *Eco*RI sites upstream. Plasmid pBB528 (Fig. 2A) was made by deleting the 1.2-kb *Hin*dIII fragment of pBB527 (Fig. 2A). An *orf105-lacZ* translational fusion (pBB535; Fig. 2A) was constructed by cloning the 1.4-kb $HindIII_4$-*Bgl*II fragment, cleaved from pBB426 (5) with *Eco*RI (a vector site) and *Bgl*II, between the *Eco*RI and *Bam*HI sites of pJPM96. The latter plasmid was made by J. Mueller (33) by replacing the *Eco*RI-*Cla*I fragment of pJF751 (18) with the 0.8-kb *Eco*RI-*Cla*I fragment of pSGMU31 (17). The structure of the fusion junction in pBB535 was confirmed by sequencing.

Construction of *azlB* null mutants. To make an *azlB* deletion-insertion mutant, the 1.0-kb *Eco*RI (filled in)-*Hin*cII fragment of pBLE1 (21), containing the *ble* gene, was ligated to pMG001 (Fig. 1) cleaved with *Afl*II (filled in). The resulting plasmid, pΔlrp1 (Fig. 1), which has the *ble* gene reading in the direction opposite to that of *azlB*, was used to transform strain 1A1 to phleomycin resistance, determined by the *ble* gene. The substitution of the Δ*azlB*::*ble* allele for the wild-type *azlB* gene due to a double-crossover, homologous recombination event in one of the transformants, LUW5, was confirmed by Southern blot analysis (data not shown).

Two more deletions within the *azlB* gene were created by cutting out the 0.29-kb *Afl*II fragment of pBB577 (Fig. 1) and religating the remaining fragment after filling in its ends. Plasmid pBB578 (Δ*azlB1*), in which one A nucleotide was missing at the *Afl*II-*Afl*II junction, was first isolated (Fig. 1). The *tet* gene excised from pBEST307 (26) as a 1.9-kb *Hin*dIII-*Eco*RI fragment and blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase I was inserted at the *Nru*I site of pBB578 (0.4 kb upstream of the *azlB* coding region), creating pBB581 and pBB582 (in which *tet* is transcribed in the same direction as *azlB* and in the opposite direction, respectively) (Fig. 1). pBB581 was introduced into strain SMY by transformation, with selection for tetracycline resistance and screening for neomycin sensitivity (a vector marker), indicating a double-crossover, homologous recombination event. The replacement of the wild-type chromosomal *azlB* gene by the Δ*azlB1* allele in strain BB272 was confirmed by sizing the PCR fragments generated from the *azlB* locus (data not shown).

The Δ*azlB1* deletion shifts the reading frame of the distal part of *azlB* and causes premature termination of translation. In pBB587, similar to pBB578 but with a correct *Afl*II junction (Fig. 1), the deletion does not alter the reading frame of *azlB* (and creates the *Pac*I site at its junction). pBB587 was introduced by a single-crossover, homologous recombination event into the chromosome of strain SMY, with selection for neomycin resistance (a vector marker). About

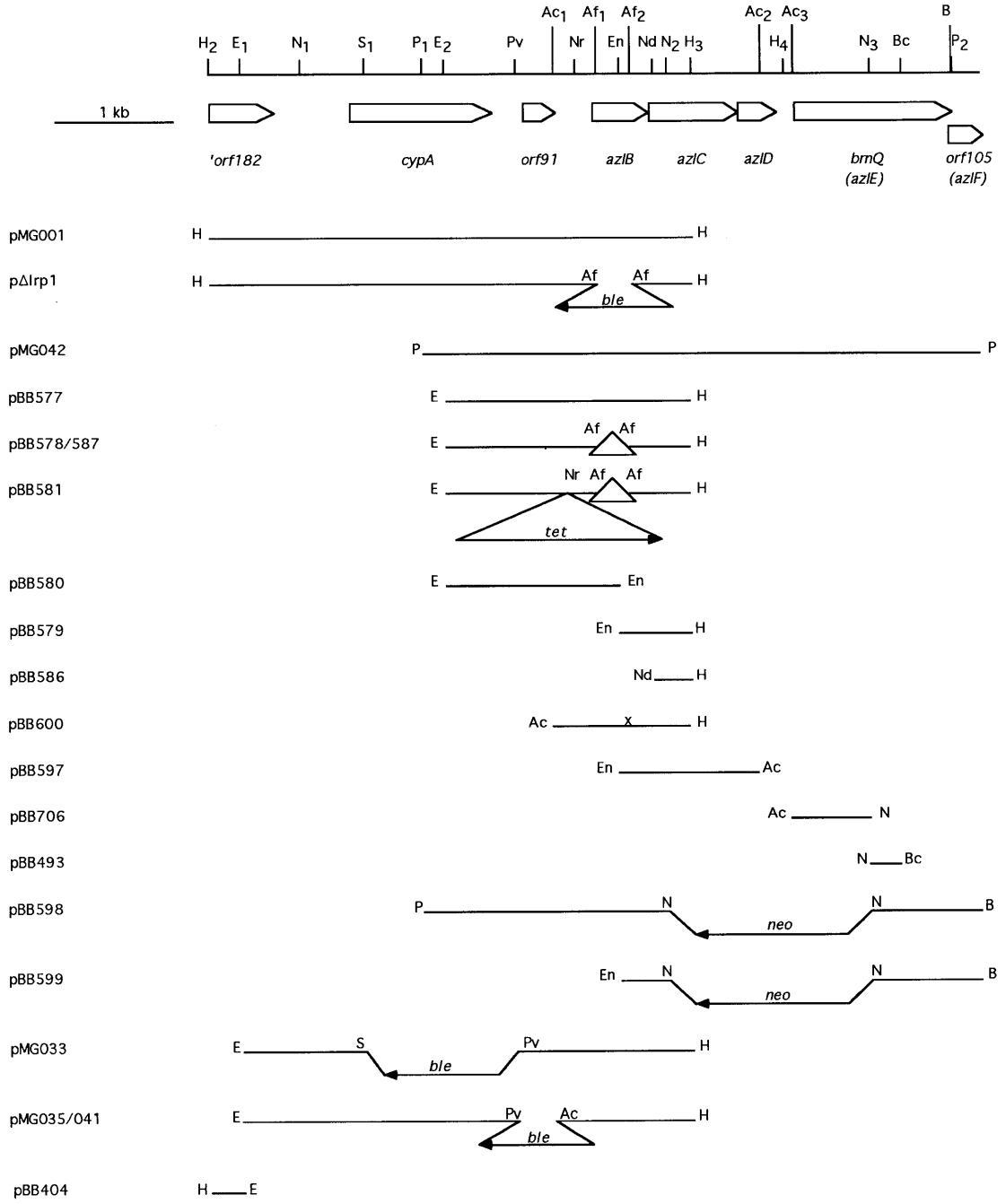
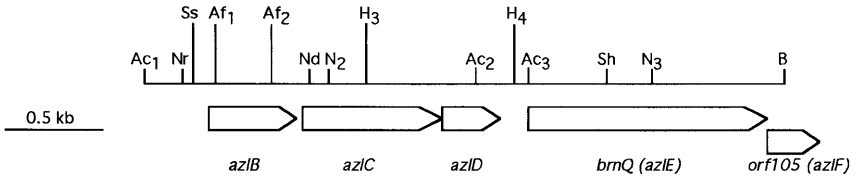


FIG. 1. Restriction map of the *azlB* region and plasmids carrying different parts of this region. At the top, the restriction map of part of the 12.7-kb chromosomal segment cloned in previous work (5) is shown. The restriction sites are abbreviated as follows: A, *Afl*II; Ac, *Acc*I; B, *Bgl*II; Bc, *Bcl*I; E, *Eco*RI; En, *Eco*NI; H, *Hin*dIII; N, *Nsi*I; Nd, *Nde*I; Nr, *Nru*I; Pv, *Pvu*II; P, *Pst*I; and S, *Sac*I. Only relevant *Acc*I, *Bcl*I, *Pvu*II, *Nde*I, and *Nru*I sites are shown. Plasmids pMG001, pΔIrp1, and pMG042 are derivatives of pBluescript II KS(−) (Stratagene, Inc.); plasmids pMG033 and pMG035 are derivatives of pBLE1 (21); plasmid pMG041 is a derivative of pHV32 (36); plasmids pBB404, pBB493, pBB598, pBB599, and pBB706 are derivatives of pJPM1 (34); and other plasmids are derivatives of pBB544 (this work). Construction of most plasmids is described in Materials and Methods; other plasmids were constructed by deleting or subcloning fragments of the *azlB* region. *E. coli* BU1255 was used to isolate plasmid DNA sensitive to *Bcl*I. x, location of the *azlB101* mutation within pBB600.

25% of these transformants, merodiploid for the *azlB* region, acquired the Azl^r phenotype, reflecting that this phenotype is conferred by an *azlB* deletion and that this region underwent gene conversion (the Azl^r phenotype is recessive; see Results). After growth of one of the Neo^r Azl^r strains in the absence of neomycin, a Neo^s Azl^r derivative in which the integrated pBB587 was apparently excised from the chromosome and lost was isolated. Replacement of the wild-type *azlB* gene by the Δ*azlB2* allele in strain BB274 and excision of pBB587 were confirmed by sizing the PCR fragments generated from the *azlB* locus (data not shown).

Inactivation of the *azlCD* and *brnQ* genes. Plasmids pBB586 and pBB493 (Fig. 1), carrying internal fragments of the *azlC* and *brnQ* genes, respectively, were integrated into the chromosome of strain SMY or BB274 (Δ*azlB2*) via a single-crossover recombination event. These integration events disrupted the open reading frames of *azlC* or *brnQ*.



A)

		ß-galactosidase activity in azlB+		ΔazlB2	
		DSM	TSS	DSM	TSS
pBB589/525	Ac — Af	14	1.0	364	328
pBB592/529	Nr — Af	17	0.9	353	349
pBB723/532	Ss — Af	24	37	37	87
pBB725/536	Ss — Nd	ND	5.1	NA	
pBB590/526	Ac — Nd	ND	1.1	NA	
pBB588/527	Ac — Ac	5.8	ND	NA	
pBB596/530	Ac — Af Af — Ac	14	ND	214	ND
pBB593/531	Ac — Nr Af — Ac	4.2	ND	5.9	ND
pBB528	H — Ac	3.4	1.7	3.3	1.8
pBB727/533	Ac — Af Af — Sh	2.6	0.2	68	58
pBB535	H — B	≤0.6	≤0.1	3.0	9.5

B)

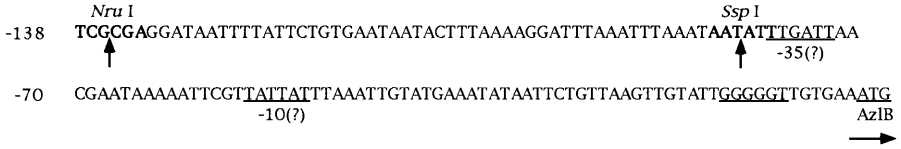


FIG. 2. (A) Construction and activities of various *azl-lacZ* fusions. Plasmid pJPM82 (see Materials and Methods) was used for construction of most fusions (pBB525 to pBB533 and pBB536), which were subsequently integrated by double-crossover recombination at the *amyE* locus of the *B. subtilis* chromosome. Plasmid pJPM96 (see Materials and Methods) was used for construction of an *orf105-lacZ* (pBB535) translational fusion; this fusion was integrated at the *azl-brnQ* region of the chromosome by single-crossover recombination. Plasmids pBB588 to pBB590, pBB592, pBB593, pBB596, pBB723, pBB725, and pBB727 are derivatives of pBB544 (this work) and were used as intermediates in construction of *lacZ* fusions. The restriction sites are abbreviated as for Fig. 1; additional relevant sites are *Sph*I (Sh) and *Ssp*I (Ss). Strains carrying the respective fusions (Table 1) were grown in TSS glucose-ammonia medium or DS nutrient broth medium (DSM). The presented activities of *lacZ* fusions were determined at the middle of exponential phase (optical density of ~0.5 at 600 nm), when those activities were close to maximal. β-Galactosidase activity is expressed in Miller units as described in Materials and Methods. All the numbers are averages of at least two experiments, and the mean errors did not exceed 20%. NA, not applicable (a full-length allele of *azlB* is present within a fusion); ND, not determined. (B) Sequence of the *azlB* regulatory region. A likely initiation codon, a possible ribosomal binding site, and the putative −10 and −35 promoter regions of the *azlB* gene (underlined) and the direction of translation (horizontal arrow) are indicated. The coordinates are given with respect to the *azlB* translation start point. The *Nru*I and *Ssp*I sites used to construct truncated *azlB-lacZ* fusions are shown, with the beginning of the *azlB* sequence within these fusions indicated (vertical arrows). The *Acc*I site used to construct a full-length *azlB-lacZ* fusion lies at positions −337 to −332.

Plasmids pBB579, pBB597, and pBB706 (Fig. 1) were integrated into the chromosome of strain SMY or BB274 (Δ*azlB2*) via a single-crossover recombination event. These integration events separated *azlC*, *azlD*, *brnQ*, and *orf105* (for pBB579), *azlD*, *brnQ*, and *orf105* (for pBB597), or only *brnQ* and *orf105* (for pBB706) from the *azlB* promoter.

Construction of an *azlCDE* null mutant. Plasmid pBB419 (5) was cut with *Nsi*I to delete the 1.7-kb $NsiI_2$-$NsiI_3$ fragment containing the *azlD* gene and parts of *azlC* and *brnQ* and ligated with the 1.3-kb *neo* gene excised from pBEST501 (27) with *Pst*I. Plasmid pBB598 has the *neo* gene transcribed in the direction opposite to that of *azlB* (Fig. 1). pBB599 is a version of pBB598 in which the 1.7-kb *Pst*I-*Eco*NI fragment was deleted (Fig. 1). These two plasmids were introduced into strain SMY or BB274 (Δ*azlB2*) by transformation, with selection for neomycin resistance and screening for chloramphenicol sensitivity, an indication of a double-crossover recombination event. Replacement of the wild-type chromosomal *azlCDE* genes by the Δ*azlCDE*::*neo* allele in strains BB284 and BB285 was confirmed by sizing the PCR fragments generated from this chromosomal region (data not shown).

Construction of a *cypA* null mutant. The 1.0-kb $EcoRI_1$-$SacI_1$ fragment (Fig. 1) was ligated to pBLE1 (21) that had been cut with the same enzymes to create pMG030, and then the 1.5-kb $PvuII$-$HindIII_3$ fragment (Fig. 1) was ligated to pMG030 cleaved with *Hin*cII and *Hin*dIII. The resulting plasmid, pMG033 (Fig. 1), was used to transform strain 1A1 to phleomycin resistance. The deletion-insertion within the chromosomal *cypA* gene arising from a double-crossover recombination event was confirmed by Southern blot analysis (data not shown).

TABLE 2. Similarities of *azlB* region products to other proteins

Gene name	Coordinates[a] (nucleotides)	Protein size (aa)	Molecular mass (kDa)[b]	pI[b]	Similar genes	Level of identity (%)[c]	Accession no.[d]
'orf182	1–548	>182			*yddQ*, *B. subtilis*	30	AB001488
					orf264 (*N*-carbamoylsarcosine amidohydrolase), *Arthrobacter* sp.	27	P32400
cypA	1192–2421	410	47.4	7.7	*orf405*, *Saccharopolyspora erythraea*	42	P33271
					bioI, *B. subtilis*	39	P53554
orf91	2696–2968	91	10.7	4.0	*orf89* (barstar), *Bacillus amyloliquefaciens*	47	P11540
azlB	3305–3775	157	17.5	4.9	*prp*, *Agrobacterium tumefaciens*	35	U39263
					lrp, *E. coli*	33	P19454
azlC	3791–4552	254	28.3	10.3	*orf244* (HI1738), *H. influenzae*	51	P44302
					orf245, *E. coli*	23	ECAE000353
azlD	4552–4881	110	12.0	10.5	*orf109* (HI1737), *H. influenzae*	54	P44301
					orf111, *E. coli*	20	ECAE000353
azlE (*brnQ*)	5049–6368	440	47.0	10.3	*braB*, *Pseudomonas aeruginosa*	42	P19072
					brnQ, *Lactobacillus delbrueckii*	41	P54104
azlF (*orf105*)	6329–6643	105	12.3	9.3	NA[e]		

[a] Coordinates are given with respect to the *azlB* region sequence.
[b] Calculated molecular mass or pI.
[c] The Bestfit program of the Genetics Computer Group package (12) was used to determine similarity of proteins.
[d] Accession numbers for the GenBank or Swiss-Prot database.
[e] NA, not available.

Construction of an *orf91* null mutant. The 0.6-kb *Eco*RI$_2$-*Pvu*II fragment (Fig. 1) was ligated to pBLE1 (21) that had been cleaved with *Eco*RI and *Sac*I (filled in) to create pMG031, and then the 1.2-kb *Acc*I$_1$ (filled in)-*Hin*dIII$_3$ fragment (Fig. 1) was ligated to pMG031 cleaved with *Hin*cII and *Hin*dIII. The 2.9-kb *Eco*RI-*Hin*dIII fragment of the resulting plasmid, pMG035, was subcloned in pHV32 (36) to yield pMG041 (Fig. 1). The latter plasmid was used for transformation of strain 1A1, with selection for phleomycin resistance and screening for chloramphenicol sensitivity. The double-crossover recombination event replacing the chromosomal *orf91* gene with the *ble* gene in one of the transformants, LUW7, was confirmed by Southern blot analysis (data not shown).

Disruption of *'orf182*. Plasmid pBB404 (Fig. 1), containing an internal 0.28-kb *Hin*dIII$_2$-*Eco*RI$_1$ fragment of an open reading frame corresponding to *'orf182*, was integrated into the chromosome of strain SMY by transformation via a single-crossover, homologous recombination event.

DNA sequencing. DNA fragments containing the *azlB* region were sequenced on both strands by the dideoxy chain termination method of Sanger et al. (40), using vector- or *azlB* region-specific oligonucleotides as primers. Nested deletions in pMG001 (Fig. 1) were obtained by using exonuclease III and mung bean nuclease as described elsewhere (1). Plasmid double-stranded DNA to be sequenced was purified by a modification of the boiling lysis protocol (24). A Sequenase reagent kit (Amersham Life Science) was used according to the protocol of the manufacturer.

DNA and protein sequences were analyzed by using DNA Strider, the package of programs provided by the University of Wisconsin Genetics Computer Group (12), and the BLAST program (2).

Enzyme assays. β-Galactosidase activity was determined as described previously (5). The specific activity was expressed in Miller units (32).

Nucleotide sequence accession number. The nucleotide sequence of the *azlB* region reported here has been assigned GenBank accession no. Y11043.

RESULTS

DNA sequence of the *azlB* region. The cloning of a 12.7-kb DNA segment from the 233° region of the *B. subtilis* chromosome and the sequence of a 3.7-kb part of this segment, containing the *brnQ* and *gltR* genes, were recently reported (5). The previously identified *azlB* locus (46) was shown to reside within this region of DNA, to the left of the *brnQ* gene (Fig. 1) (5). The sequence of the 4,958-bp *Hin*dIII$_2$-*Hin*dIII$_4$ chromosomal fragment (Fig. 1) from strain 168, immediately adjacent to the previously sequenced segment and overlapping the site of the *azlB101* mutation, was determined in the present work. Partial sequences of this region from strain SMY were identical to corresponding sequences from strain 168 (4).

The five complete open reading frames and one partial open reading frame found in this region are described in Table 2. All open reading frames in this region are transcribed in the same direction, counterclockwise with respect to the standard *B. subtilis* chromosomal map (3).

The 1.9-kb *Pst*I$_1$-*Nsi*I$_2$ fragment that was able to rescue the *azlB101* mutation (5) contained all or parts of four of these open reading frames. To localize more precisely the position of the mutation, we showed that it could not be rescued by the 1.5-kb *Eco*RI$_2$-*Eco*NI fragment of pBB580 (Fig. 1), placing the *azlB101* mutation within the 0.4-kb interval between the *Eco*NI and *Nsi*I$_2$ sites or upstream of and very close to the *Eco*NI site (Fig. 1). The appropriate region of the chromosomal DNA from the *azlB101* mutant was cloned and sequenced to show that the mutation represented a deletion of one C nucleotide between positions 3583 and 3585, ~50 bp downstream of the *Eco*NI site. An open reading frame encoding 157 amino acids preceded by a putative ribosome binding site overlapped the site of the *azlB101* mutation. Computer analysis of the deduced AzlB sequence showed that it belongs to a family of proteins that includes *E. coli* transcription regulators AsnC (29) and Lrp (9, 35) and their homologs. Phylogenetic analysis of this family separates it into several distinct groups and shows that AzlB is not particularly close to either the Lrp or AsnC subfamily (Fig. 3). Several archaeal members of the family are close to the AsnC subfamily or belong to the AsnC subfamily (Fig. 3).

Phenotype of an *azlB* null mutant. A large DNA segment internal to the *azlB* gene was deleted without altering the reading frame of the distal part of the gene (see Materials and Methods and Fig. 1). This deletion removed 61% of the *azlB* coding region, including codons 22 to 43, encoding the putative helix-turn-helix DNA binding motif (13) of AzlB. When introduced into the *B. subtilis* chromosome, the Δ*azlB2* mutation had no apparent effect on growth of cells in broth or in defined media. The deletion conferred resistance to 4-azaleucine, the level of which was indistinguishable from that of the *azlB101* mutant (46). A similar but out-of-frame deletion mutation, Δ*azlB1*, conferred an identical phenotype. We conclude that *azlB101* is also a loss-of-function mutation, reflecting a frameshift starting from codon 94 and premature translation termination of the mutant AzlB protein. A Δ*azlB*::*ble* mutation (see Materials and Methods and Fig. 1) conferred an intermediate level of 4-azaleucine resistance on minimal glucose-ammonia

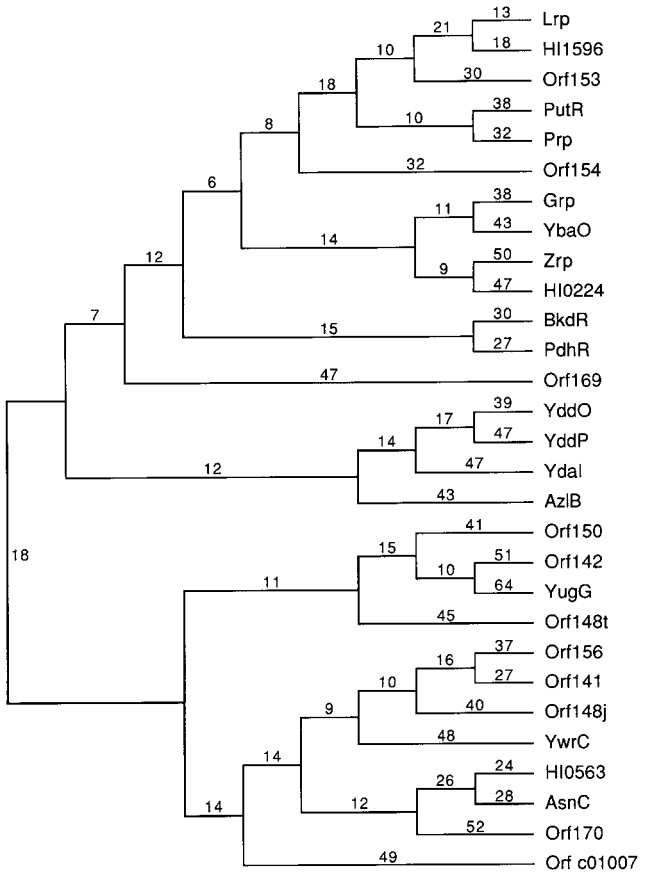


FIG. 3. Phylogenetic relationships among proteins of the AsnC-Lrp family. Protein sequences were aligned by using the Genetics Computer Group PileUp program (12). The aligned segments corresponding to positions 5 to 122 of AzlB were analyzed by using the PAUP 3.1.1 program (44). Midpoint rooting was employed; the branch lengths are shown. The sources and accession numbers of the sequences are as follows (with the sizes of the proteins in parentheses): *E. coli* AsnC, P03809 (152 amino acids [aa]); *B. subtilis* AzlB, Y11043 (157 aa); *Pseudomonas putida* BkdR, P42179 (161 aa); *Zymomonas mobilis* Grp, S52279 (164 aa); *H. influenzae* HI0224, P44580 (168 aa); *H. influenzae* HI0563, P44337 (150 aa); *H. influenzae* HI1596, P45265 (166 aa); *E. coli* Lrp, P19494 (164 aa); *Sulfolobus solfataricus* Orf c01007, Y08256 (155 aa); *Pyrococcus furiosus* Orf141, M97860; *Agrobacterium rhizogenes* Orf142, M61151; *Methanococcus jannaschii* Orf148j, U67472; *Mycobacterium tuberculosis* Orf148t, Z79702; *M. tuberculosis* Orf150, Z92771; *Bradyrhizobium japonicum* Orf153, U85623; *Rhodobacter sphaeroides* Orf154, L07247; *M. jannaschii* Orf156, U67519; *Rhizobium meliloti* Orf169, AE000097; *Rhodococcus* sp. Orf170, U42216; *Ralstonia eutropha* PdhR ('Orf147), X91878; *Agrobacterium tumefaciens* Prp (PutR), U39263 (156 aa); *Rhodobacter capsulatus* PutR, X78346 (154 aa); *E. coli* YbaO, D82943 (152 aa); *B. subtilis* YdaI, AB001488 (reported as 231 aa, likely 144 aa); *B. subtilis* YddO, AB001488 (136 aa); *B. subtilis* YddP, AB001488 (149 aa); *B. subtilis* YugG, Z93934 (166 aa); *B. subtilis* YwrC, Z93767 (158 aa); and *Z. mobilis* Zrp, L34333 (163 aa). Apparent frameshift mutations were corrected for L07247 and M97860.

plates (MIC, 25 to 50 μg/ml compared with 5 to 10 μg/ml for a wild-type strain and >200 μg/ml for the Δ*azlB2* and *azlB101* mutants [data not shown]).

Role of AzlB in expression of the *azlB* gene. Transcriptional fusions of the *azlB* gene to the *lacZ* reporter gene were constructed as described in Materials and Methods and inserted by double-crossover recombination at the *amyE* locus. The *azlB-lacZ* fusion, containing the entire intergenic region between the *orf91* and *azlB* genes (as in pBB525 [Fig. 2A]), was not significantly expressed in a wild-type background of strain BB831 in glucose-ammonia minimal medium (Fig. 4A) and was moderately induced during the exponential growth phase in nutrient broth medium (Fig. 4B). Addition of leucine or a mixture of leucine, isoleucine, and valine to minimal medium did not affect *azlB-lacZ* expression (data not shown). Replacement of ammonia by 0.2% proline in minimal glucose medium enhanced *azlB-lacZ* expression three- to fourfold; addition of 0.2% Casamino Acids (Difco) to glucose-ammonia medium activated expression of this fusion sixfold (data not shown).

In a strain carrying the in-frame Δ*azlB2* mutation, the same fusion turned out to be highly active in minimal glucose-ammonia medium (Fig. 4A) or in nutrient broth medium (Fig. 4B), indicating negative autoregulation of *azlB*. Similar dere-

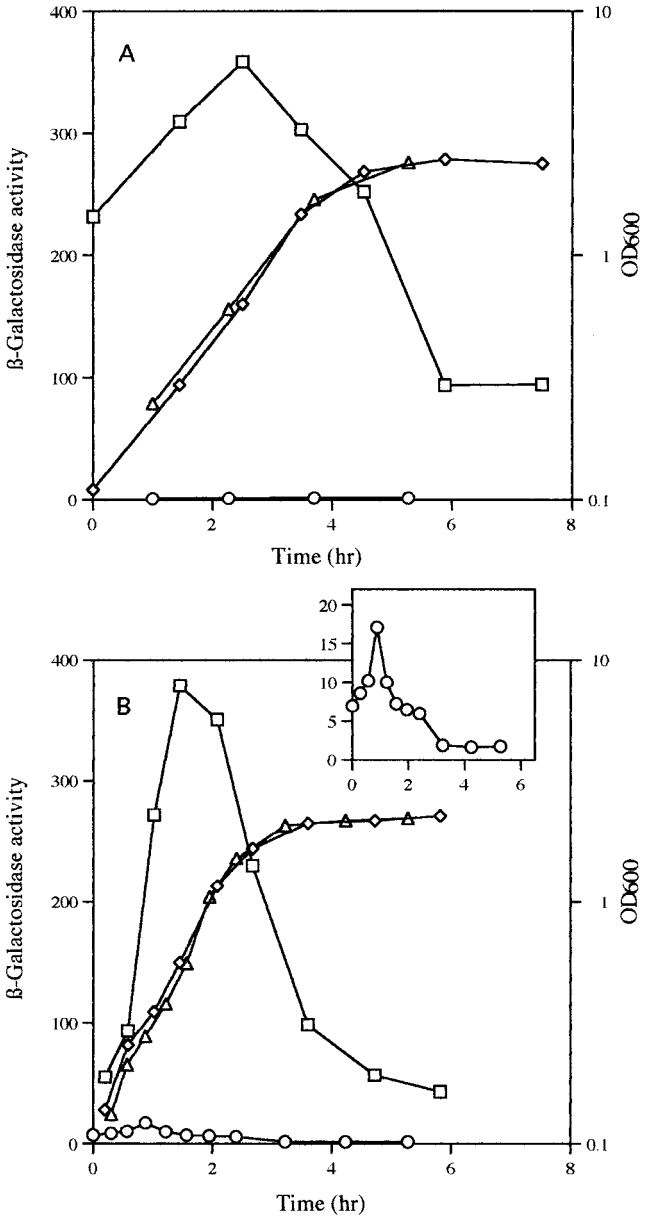


FIG. 4. Expression of an *azlB-lacZ* fusion. Strains BB831 (*azlB*$^+$) and BB834 (Δ*azlB2*) were grown in TSS glucose-ammonia medium (A) or in DS nutrient broth medium (B). β-Galactosidase activity in *azlB*$^+$ cells (circles) and Δ*azlB2* (squares) is expressed in Miller units as described in Materials and Methods. Growth curves of BB831 (triangles) and BB834 (diamonds) reflect the optical density at 600 nm (OD_{600}). β-Galactosidase activity in *azlB*$^+$ cells is also shown on a different scale (inset). Each experiment was repeated at least twice, with very similar results (in each case, data from a single experiment are presented).

pression of the *azlB-lacZ* fusion was observed in the *azlB101* mutant (data not shown). Other members of the AsnC-Lrp family (10, 28, 29, 31, 38, 45) are also negative autoregulators. In the absence of AzlB, expression of *azlB-lacZ* in nutrient broth medium was still induced during the exponential growth phase (Fig. 4B); this effect was much less apparent, if present at all, in minimal medium (Fig. 4A). Expression of the *azlB-lacZ* fusion was shut off at the beginning of stationary phase (Fig. 4). This decline of β-galactosidase activity was not dependent on the presence of AzlB (Fig. 4B) and was not prevented by introduction of a *spo0A* mutation abolishing sporulation (data not shown).

Deletion analysis of the *azlB* promoter region showed that only 135 bp upstream of the *azlB* coding region (as in pBB529 [Fig. 2]) are necessary to completely preserve the activity and regulation of this promoter (Fig. 2). Expression from another truncated promoter region containing only 80 bp upstream of the *azlB* coding region (as in pBB532 [Fig. 2]) was significantly derepressed in minimal medium and slightly increased in nutrient broth medium (Fig. 2A). In the Δ*azlB2* mutant, expression from this promoter was increased an additional 1.5- to 2.4-fold (Fig. 2A). This shows that major sites required for negative autoregulation of the *azlB* promoter are located upstream of the −80 position, with an additional site (or a partial site) downstream of this position (Fig. 2). In accordance with this conclusion, when a truncated, derepressed *azlB* promoter governed expression of an *azlBC′-lacZ* fusion containing a full-length *azlB* gene (as in pBB536 [Fig. 2A]), it resulted in intermediate expression of this fusion (Fig. 2A). This is apparently due to the ability of overproduced AzlB to negatively regulate this truncated promoter, though with low efficiency. The apparent moderate overproduction of AzlB in such a strain had no other observable consequences.

The truncated, derepressed *azlB* promoter (as in pBB532 [Fig. 2A]) was still subject to growth phase-dependent regulation in both *azlB*$^+$ and Δ*azlB2* cells (data not shown), consistent with these effects being independent of *azlB* negative autoregulation (Fig. 4). Activity of this promoter did not reach the same level as with the full-length *azlB-lacZ* fusion even in the Δ*azlB2* mutant (Fig. 2A), indicating that some upstream sequences are important for maximal promoter efficiency. Thus, the deletion analysis localized the *azlB* promoter within 80 bp upstream of its coding region. This segment contains sequences with similarity to −10 and −35 regions of σ^A-type promoters (Fig. 2B). Other sequences important for negative autoregulation, growth rate-dependent regulation, and high promoter activity are localized within 135 bp upstream of the *azlB* coding region.

Organization of the *azl* operon. Downstream of *azlB*, we found two open reading frames, *azlC* and *azlD*, that might belong to an operon with *azlB*. A Δ*azlB2CD-lacZ* transcriptional fusion, containing an in-frame deletion within the *azlB* sequence (as in pBB530 [Fig. 2A]) and integrated at the *amyE* locus, was regulated according to the same pattern as the full-length *azlB-lacZ* fusion (Fig. 2A and data not shown). A Δ*azlB3CD-lacZ* fusion, with a more extensive deletion that removed the *azlB* promoter (as in pBB531 [Fig. 2A]), was not induced in the wild-type strain in nutrient broth medium and was not derepressed in the Δ*azlB2* mutant (Fig. 2A and data not shown). Thus, the promoter located upstream of *azlB* determines the bulk of *azlD*, and presumably *azlC*, expression, i.e., these three genes form an operon. In addition, the operon apparently has at least one internal promoter, downstream from the *Hin*dIII$_3$ site, because Δ*azlB3CD-lacZ* and *azl′CD-lacZ* fusions that lack the *azlB* promoter (as in pBB531 and pBB528 [Fig. 2A]) have residual activities in both minimal and nutrient broth media. Activity of this promoter, in contrast to the activity of the *azlB* promoter, did not depend on the allelic state of the *azlB* gene (Fig. 2A) and increased three- to fourfold in stationary phase in minimal medium (data not shown). *azlB* is the first example of a gene coding for an Lrp-like protein shown to be cotranscribed with other genes.

We also checked if the previously identified *brnQ* gene (5), located ~170 bp downstream from the *azlD* gene and transcribed in the same direction (Fig. 1), could belong to the *azl* operon. A Δ*azlB2CD-brnQ–lacZ* fusion (as in pBB533 [Fig. 2A]) was expressed at a much higher level in the Δ*azlB2* mutant than in a wild-type strain in both minimal and nutrient broth media (Fig. 2A). *orf105* (5), partially overlapping the 3′ end of the *brnQ* gene (Fig. 1), could also be part of the *azl* operon. A translational *orf105-lacZ* fusion (as in pBB535 [Fig. 2A]) was integrated at the *azl-brnQ* locus to reconstruct the intact chromosomal DNA context upstream of this fusion. This fusion was highly derepressed in the Δ*azlB2* mutant in minimal and nutrient broth media (Fig. 2A). Both Δ*azlB2CD-brnQ–lacZ* and *orf105-lacZ* fusions were subject to growth phase-dependent regulation (data not shown). These results indicate coregulation and, very likely, coexpression of the *brnQ* and *orf105* genes with the *azlBCD* operon.

Preliminary Northern blot experiments with RNA isolated from cells grown in DS nutrient broth medium, using a DNA fragment corresponding to *azlB* and part of *azlC* as a probe, showed a transcript of approximately 3,500 nucleotides in *B. subtilis* BB34 (*azlB101*) (data not shown). This transcript has a length consistent with a polycistronic *azlBCD-brnQ-orf105* mRNA. A potential strong rho-independent transcription terminator was identified previously, immediately downstream of *orf105* (5) at positions 6678 to 6711 with respect to the present sequence. A slightly shorter transcript was detected with RNA from strain BB274 (Δ*azlB2*), presumably reflecting the 0.3-kb deletion within *azlB* (data not shown). We could not detect any transcript in the wild-type strain 1A1, which is in agreement with much lower expression of corresponding *lacZ* fusions (Fig. 4). Assuming that *azlBCD*, *brnQ*, and *orf105* constitute an operon, we renamed *brnQ* and *orf105* the *azlE* and *azlF* genes.

Role of the *azlCDE* genes in 4-azaleucine resistance and expression of the *azl* operon. Among four available mutations in which the *azlB* gene was completely inactivated, the Δ*azlB*::*ble* mutation was unusual because it conferred an intermediate level of 4-azaleucine resistance, compared to the *azlB101*, Δ*azlB1*, and Δ*azlB2* mutations. This property of the *ble* insertion suggested that expression of distal genes of the *azlBCDEF* operon may be involved in resistance to 4-azaleucine. An apparent candidate for such a role is the *azlE* (*brnQ*) gene, whose product is very similar to several proteins involved in branched-chain amino acid transport (43) (Table 2) and which could be responsible for 4-azaleucine transport. Nevertheless, inactivation of the *azlE* gene by plasmid integration (see Materials and Methods) neither affected growth of nor conferred 4-azaleucine resistance to an *azlB*$^+$ strain and did not alter the resistance of the Δ*azlB2* mutant (data not shown), indicating that *azlE* (and, most probably, the downstream *azlF* gene) is not involved in resistance to 4-azaleucine.

Inactivation of the *azlC* and *azlD* genes or of the *azlD* gene alone (see Materials and Methods) did not confer resistance to 4-azaleucine on an *azlB*$^+$ strain but abolished the resistance of the Δ*azlB2* mutant (data not shown). Since *azlC* and *azlD* are derepressed in the Δ*azlB2* mutant, it seems that overexpression of AzlD or both AzlC and AzlD causes 4-azaleucine resistance. Thus, the lower resistance of the Δ*azlB*::*ble* mutant may be due to (i) a partial polar effect of the *ble* gene insert reducing expression of the *azlCD* genes from the derepressed *azlB* pro-

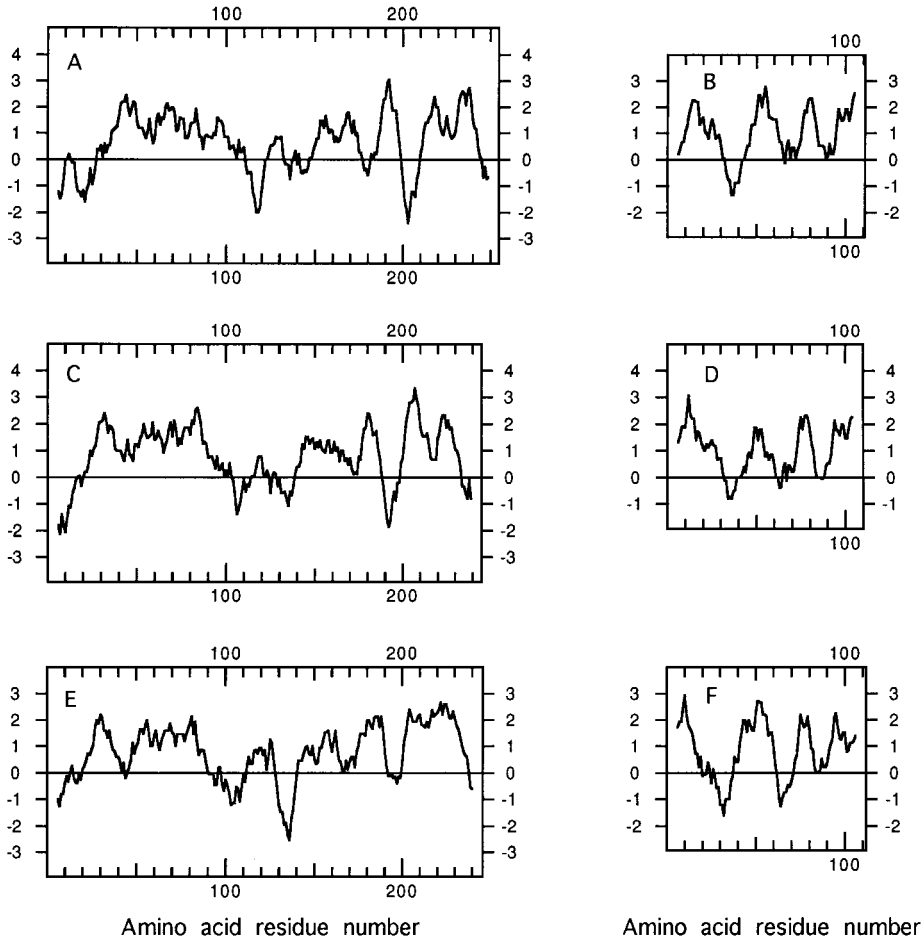


FIG. 5. Hydropathy plots for *B. subtilis* AzlC (A) and AzlD (B) and their counterparts from *H. influenzae* (C and D) and *E. coli* (E and F). Kyte-Doolittle profiles (30) were obtained by using the DNA Strider 1.2 program and a window of 11 aa.

moter, (ii) reduced expression of the derepressed *azlB* promoter due to convergent transcription from the *ble* promoter, or (iii) the presence of an unidentified promoter within the *ble* insert that allowed expression of the *azlCD* genes independently of the *azlB* promoter (probably at an intermediate level, compared to those of other *azlB* mutants).

In accordance with results presented above, a deletion extending from *azlC* to *azlE* (as in pBB598 and pBB599 [Fig. 1]) did not cause resistance to 4-azaleucine in an *azlB*$^+$ strain but abolished the resistance of the Δ*azlB2* mutant (data not shown). This deletion had no apparent effect on growth or expression of *azlB-lacZ*, *azl'CD-lacZ*, or Δ*azlB2CDE-lacZ* fusions (data not shown).

Dominance analysis of *azlB* alleles. As expected, the Δ*azlB2* and *azlB101* mutations were recessive to a wild-type *azlB* allele integrated at the *amyE* locus (as in pBB526 [Fig. 2A]) with respect to *azlB* autoregulation and resistance to 4-azaleucine (data not shown).

Lack of a role of AzlB in glutamate synthase regulation. No effect of the Δ*azlB1* mutation on *gltA-lacZ* expression was observed. The mutation also did not affect expression of *glnR-lacZ* or *citB-lacZ* fusions (data not shown).

Other genes in the *azlB* region. The open reading frame of 91 codons upstream of *azlB* is very similar to the *Bacillus amyloliquefaciens* barstar gene (Table 2), which codes for an inhibitor of an extracellular endoribonuclease (barnase). No homolog of barnase in *B. subtilis* has yet been identified. Expression of *B. amyloliquefaciens* barnase in *E. coli* cells is lethal unless barstar is coexpressed (23). Deletion of *orf91* by replacing the entire open reading frame with the *ble* gene did not affect growth of *B. subtilis* in minimal or rich media, raising the possibility that no barnase-like enzyme is present or expressed in *B. subtilis.*

The largest open reading frame in the *azlB* region, located upstream of *orf91*, codes for a protein that is very similar to bacterial P-450 cytochromes (Table 2). We called this gene *cypA*. No genes encoding putative electron carriers for P-450 cytochromes were found in the vicinity of *cypA*. Replacement of *cypA* with the *ble* gene did not affect growth in minimal or rich media.

No apparent growth defect was observed after disruption of *'orf182*, which codes for a protein similar to several bacterial hydrolases (Table 2).

DISCUSSION

Among the characterized members of the AsnC-Lrp family, only the Lrp protein of *E. coli* (and, presumably, nearly identical Lrp proteins of other enteric bacteria) is known to be involved in global regulation of cellular metabolism (9, 35). Other proteins of this family seem to have more-specific regulatory functions, related to transport or utilization of amino

acids (10, 28, 29, 31, 38), and this is apparently the case for the *B. subtilis* AzlB protein. The only role we have found for AzlB so far is to negatively regulate expression of the *azlBCDEF* operon, apparently by binding just upstream of and within the *azlB* promoter region. When AzlB is inactive, the operon is derepressed, and overexpression of AzlC and AzlD confers resistance to a leucine analog, 4-azaleucine. Unlike Lrp (8, 16), AzlB does not regulate glutamate synthase gene expression. We do not know at present what affects the activity of AzlB and have only limited information on what growth conditions alter expression of *azlB* and other genes of the *azl* operon.

The exact roles of the *azlC* and *azlD* genes in conferring resistance to 4-azaleucine remain mysterious. Induction of leucine biosynthesis enzymes could potentially explain this resistance, but no such induction was observed in an *azlB* mutant (46). We hypothesize that AzlC and AzlD, when overexpressed, interfere with transport of 4-azaleucine and probably other branched-chain amino acids. Alternatively, these two proteins may be involved in pumping 4-azaleucine out of the cells. Hydropathy profiles of these proteins indicate that both are likely to be integral membrane proteins (Fig. 5). Our results show that the target of their action is not the AzlE (BrnQ) product, though the facts that the three proteins are coexpressed and that proteins similar to AzlE are involved in branched-chain amino acid transport (25, 37, 43) suggest that AzlC or AzlD and AzlE may somehow interact functionally.

Other bacteria have multiple systems for branched-chain amino acid transport. Two such operons, *livJ* and *livKHMGF* of *E. coli*, are known to be regulated by the Lrp protein (22). The product of another, as yet undiscovered branched-chain amino acid transport system in *B. subtilis* may be a target of overproduced AzlC and AzlD. If this were the case, *azlE* (*brnQ*) mutants that also overproduce AzlC and AzlD might be severely deficient in branched-chain amino acid transport. However, when *liv*::Tn*917* or *leuB*::Tn*917* mutations were introduced into a Δ*azlB2 azlE* double mutant, a Δ*azlB2* Δ*azlCDE* double mutant, or Δ*azlB2*, *azlE*, and Δ*azlCDE* single mutants, we did not observe any growth defect on minimal medium plates supplied with appropriate branched-chain amino acids (data not shown).

Two genes highly similar to *azlC* and *azlD*, *orf244* and *orf109* of *Haemophilus influenzae* (Table 2), appear to be organized in an operon structure which includes a gene coding for a LysR-type transcription regulator as the promoter-proximal gene (19). The putative product of *E. coli orf245* (Table 2) is also similar to AzlC, albeit much less so. The product of the next downstream and overlapping *E. coli* gene, *orf111*, does not reveal any convincing sequence similarity to its *B. subtilis* and *H. influenzae* counterparts (Table 2). Nevertheless, the three pairs of proteins show striking similarity in their hydropathy profiles (Fig. 5), indicating that the products of these two-cistron units might have similar functions.

Despite the proximity of their coding regions, expression of the *azlC* gene apparently is not coupled to translation of *azlB* inasmuch as two frameshift deletion mutations within the *azlB* gene, *azlB101* and Δ*azlB1*, that cause premature termination of *azlB* translation confer the same level of 4-azaleucine resistance (and, presumably, the same level of *azlCD* overexpression) as an in-frame deletion within *azlB*. It is not known if expression of the *azlC* and *azlD* genes is translationally coupled, though their coding regions overlap by 1 bp. In a more unusual fashion, the coding regions of the *azlE* and *azlF* genes overlap by 40 bp.

While this work was in progress, five additional *B. subtilis* gene products that belong to the AsnC-Lrp family of transcription regulators were identified. Some of them apparently form a separate group within this family, whereas others are close to archaeal members of the AsnC subfamily (Fig. 3). Two of them, LrpA (YddO) and LrpB (YddP), may be involved in regulation of the *glyA* gene (11); the functions of the other proteins (YdaI, YugG, and YwrC) have not been described. It remains to be seen if any of these proteins is involved in glutamate synthase regulation in *B. subtilis.*

ACKNOWLEDGMENTS

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