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Distinct Paths for Basic Amino Acid Export in *Escherichia coli*: YbjE (LysO) Mediates Export of L-Lysine

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ABSTRACT

In *Escherichia coli*, *argO* encodes an exporter for L-arginine (Arg) and its toxic analogue canavanine (CAN), and its transcriptional activation and repression, by Arg and L-lysine (Lys), respectively, are mediated by the regulator ArgP. Accordingly *argO* and *argP* mutants are CAN supersensitive (CAN^{ss}). We report the identification of *ybjE* as a gene encoding a predicted inner membrane protein that mediates export of Lys, and our results confirm the previous identification with a different approach of YbjE as a Lys exporter, reported by Ueda and coworkers (T. Ueda, Y. Nakai, Y. Gunji, R. Takikawa, and Y. Joe, U.S. patents 7,629,142 B2 [December 2009] and 8,383,363 B1 [February 2013] and European patent 1,664,318 B1 [September 2009]). *ybjE* was isolated as a multicopy suppressor of the CAN^{ss} phenotype of a strain lacking ArgO. The absence of YbjE did not confer a CAN^{ss} phenotype but instead conferred hypersensitivity to the lysine antimetabolite thialysine and led to growth inhibition by the dipeptide lysylalanine, which is associated with elevated cellular Lys content. YbjE overproduction resulted in Lys excretion and syntrophic cross-feeding of a Lys auxotroph. Constitutive overexpression of *argO* promoted Lys cross-feeding that is indicative of a latent Lys export potential of ArgO. Arg modestly repressed *ybjE* transcription in an ArgR-dependent manner, and ArgR displayed Arg-sensitive binding to the *ybjE* promoter region *in vitro*. Our studies suggest that the reciprocal repression of *argO* and *ybjE*, respectively, by Lys and Arg confers the specificity for basic amino acid export by distinct paths and that such cross-repression contributes to maintenance of cytoplasmic Arg/Lys balance. We propose that YbjE be redesignated LysO.

IMPORTANCE

This work ascribes a lysine export function to the product of the *ybjE* gene of *Escherichia coli*, leading to a physiological scenario wherein two proteins, ArgO and YbjE, perform the task of separately exporting arginine and lysine, respectively, which is distinct from that seen for *Corynebacterium glutamicum*, where the ortholog of ArgO, LysE, mediates export of both arginine and lysine. Repression of *argO* transcription by lysine is thought to effect this separation. Accordingly, ArgO mediates lysine export when repression of its transcription by lysine is bypassed. Repression of *ybjE* transcription by arginine via the ArgR repressor, together with the lysine repression of *argO* effected by ArgP, is indicative of a mechanism of maintenance of arginine/lysine balance in *E. coli*.

Bacteria possess membrane exporters for a variety of compounds. Their activities to a large extent are thought to play an adaptive role in mitigating the detrimental effects on bacterial growth caused by the presence of biotic stresses, such as those imposed by antibiotics, heavy metals, and other toxic compounds, in their natural environments. While it is easy to come to terms with the existence of proteins that mediate export of compounds described above, the presence of specific export systems for cellular metabolites such as sugars and amino acids appears somewhat enigmatic. For example, *Escherichia coli* encodes multiple proteins for the export of sugars, such as glucose and lactose (1), and for arabinose (2, 3). In addition, the occurrence of proteins mediating export of amino acids, such as alanine (4), arginine (5), aromatic amino acids (6), cysteine (7, 8), leucine (9), threonine (10, 11) and valine (12), in *E. coli* has been reported. While the physiological basis for the existence of amino acid exporters is not clear, their occurrence is relatively widespread in bacteria (13, 14). It is thought that an amino acid exporter may serve to contribute to the fitness of an organism during conditions of metabolic imbalance resulting from excessive levels of its amino acid substrate in the cytoplasm (13, 14). An alternative view could be that export of the amino acid occurs by chance, with the cognate substrate being a naturally occurring structurally related antimetabolite present in the environment. As an example of the latter, one mechanism contributing to the resistance of *E. coli* to the plant-derived antimetabolite canavanine (CAN), an L-arginine analogue, involves its export by ArgO, the ortholog of the basic amino acid exporter LysE of *Corynebacterium glutamicum*, following its uptake (5). Harnessing the amino acid export potential of a given bacterium has found widespread application in the commercial production of amino acids (reviewed in references 13 and 14).

In *C. glutamicum*, LysE mediates export of the basic amino acids L-arginine (Arg) and L-lysine (Lys) (15, 16), whereas its ortholog in *E. coli* ArgO so far has been thought to promote export

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TABLE 1 *E. coli* strains and plasmids

Strain or plasmid[a]	Genotype or description[b]
Strains	
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 spoT1 araD139 flbB5301 deoC1 ptsF25*
GJ4823	MC4100 *argO205*::Tn*10*dTet
GJ9026	MC4100 Δ*ybjE*::Kan
GJ9028	GJ4823 Δ*ybjE*::Kan
GJ9029	MC4100 Δ*argR*::Kan
G9048	BL21(DE3) Δ*argR*::Kan
GJ9060	MC4100 Δ*lysA* Δ*dppB* Δ*oppB* Δ*tppB*::Kan
Plasmids	
pHYD915	pCL1920, encoding wild-type ArgP (5)
pHYD2606	pCL1920, encoding ArgP bearing the P274S amino acid substitution (39)
pHYD2833	pACYC184 containing a segment of chromosomal DNA extending from coordinates 912287 to 914782, bearing *ybjE*, present in the BamHI site of *tetA*; the coordinate 914782 is proximal to the *tetA* promoter
pHYD2833.1	Identical to pHYD2833
pHYD2833.2	Identical to pHYD2833
pHYD2834	pACYC184 containing a segment of chromosomal DNA extending from coordinates 3063431 to 3067219, bearing *argO*, present in the BamHI site of *tetA*; the coordinate 3063431 is proximal to the *tetA* promoter
pHYD3025	Derivative of the plasmid pTrc99A in which an NcoI site was converted to an NdeI site by site-specific mutagenesis following the elimination by end filling of a preexisting NdeI site in pTrc99A
pHYD2835	pHYD3025 containing *argO* that abuts a DNA sequence encoding the hemagglutinin tag in the NdeI and SalI sites at its 3′ end
pHYD2836	pHYD3025 containing *ybjE* in the NdeI and HindIII sites
pHYD2847	pET21b containing *argR* that abuts a DNA sequence encoding the hexahistidine tag in the NdeI and XhoI sites at its 3′ end
pHYD2885	pMU575 containing the *cis* regulatory region of *ybjE* extending from −456 to +55 in the SalI and XbaI sites
pHYD2888	pHYD2885 bearing the T-to-G substitution at −58 in *ybjE*
pHYD2889	pHYD2885 bearing a deletion in the *cis* regulatory region of *ybjE* from −333 to −302
pHYD2890	pHYD2889 bearing a deletion in the *cis* regulatory region of *ybjE* from −85 to −68
pHYD2891	pHYD2889 bearing a deletion in the *cis* regulatory region of *ybjE* from −64 to −47
pHYD2892	pHYD2889 bearing a deletion in the *cis* regulatory region of *ybjE* from −43 to −26
pHYD2893	pHYD2889 bearing nucleotide substitutions at −42 (T to G), −41 (T to G), −40 (G to T), and −37 (A to C) in the *cis* regulatory region of *ybjE*

[a] All strains listed are *E. coli* K-12 strains except GJ9048, which is *E. coli* B. MC4100 and BL21(DE3) were from our laboratory collection.
[b] The molecular characterization of the *argO205*::Tn*10*dTet insertion has been described previously (5). The Δ*argR*::Kan and the Δ*ybjE*::Kan deletion insertion alleles were sourced from strains of the Keio collection (21) and introduced into appropriate strains by P1 transduction. The Δ*lysA*::Kan, Δ*dppB*::Kan, Δ*oppB*::Kan, and Δ*tppB*::Kan mutations were obtained from strains of the Keio collection and introduced sequentially into MC4100 by P1 transduction followed by the removal of the antibiotic cassette using the procedure described in reference 22, prior to the introduction of the next deletion insertion, to generate strain GJ9060 (indicated with the symbol Δ). The ancestral plasmids used in this study are described in references 25 (pCL1920), 23 (pACYC184), 24 (pTrc99A), and 26 (pMU575).

only of Arg (5), and evidence for ArgO-mediated Lys export is as yet unavailable. In the present study, we ascribe a Lys export function to the product of *ybjE*, present on the *E. coli* chromosome. *ybjE* was isolated on the basis of its ability to suppress the canavanine-supersensitive (CAN^{ss}) phenotype of a strain lacking ArgO (5) when present on a multicopy plasmid. A strain lacking YbjE was impaired for growth when challenged with the presence of the lysine antimetabolite thialysine in the medium and displayed reduced fitness under the condition of elevated cytoplasmic Lys content, achieved by the inclusion of a Lys-containing dipeptide in the medium. In addition, experiments on the genetic regulation of *ybjE* implicated the involvement of Arg in mediating transcriptional repression of *ybjE* via the arginine repressor ArgR. Lastly, we describe the Lys export potential of ArgO, which establishes a premise for the existence of distinct pathways of basic amino acid export in *E. coli*, as opposed to the situation in *C. glutamicum*, where one protein, LysE, promotes the export of both. Our results are consistent with previous reports by Ueda and coworkers, who employed an approach different from ours and have described the identification of YbjE as a Lys exporter in multiple patents (17, 18, 19).

MATERIALS AND METHODS

Growth media, bacterial strains, and plasmids. In this study, LB medium and glucose minimal A medium (MA medium) were routinely used as rich and defined synthetic media, respectively (20). The antibiotics ampicillin (Amp), chloramphenicol (Cm), kanamycin (Kan), tetracycline (Tet), spectinomycin, and trimethoprim and the inducer of the P_{trc} promoter isopropyl-β-D-thiogalactoside (IPTG) were used at appropriate concentrations, and antibiotic selection for plasmid maintenance was employed where required. The *E. coli* K-12 and *E. coli* B strains employed in this study and their genotypes are listed in Table 1. Strain construction was performed using P1 transduction (20) from appropriate strains of the Keio collection (21). The routine temperature for growth of strains was 37°C. If required, the kanamycin resistance determinant marking the null mutation sourced from the Keio collection was excised by treatment with the plasmid pCP20 as described earlier (22). The molecular characterization of the *argO*::*205*Tn*10*dTet insertion in *argO* that abolishes ArgO function has been described previously (5). The plasmids used in this study are derivatives of the plasmids pACYC184 (23), pTrc99A (24), pCL1920 (25), and pMU575 (26), and their construction is described in Table 1. Standard procedures for PCR, cloning, and overlap extension PCR-based site-directed mutagenesis were followed for their construction (27). The oligonucleotide primers used in this study are listed in Table S1 in the supplemental material.

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To study the transcriptional regulation of *ybjE*, segments of DNA bearing the 5′ promoter/regulatory region of *ybjE* and those bearing modifications in the aforementioned DNA sequence, generated by overlap extension PCR, were placed upstream of a promoterless *lacZ* present on the single-copy promoter probe plasmid pMU575.

Phenotypic tests and other procedures. The levels of tolerance to canavanine (CAN) and thialysine were assessed by spotting 10-fold serial dilutions of cultures of appropriate strains on the surfaces of MA plates containing defined concentrations of CAN and thialysine. Tolerance to CAN was also used as a phenotypic indicator of the functionality of hemagglutinin-tagged ArgO and hexahistidine-tagged ArgR expressed from the plasmids pHYD2835 and pHYD2847, respectively. Presence of the plasmid pHYD2835 complemented the CAN^{ss} phenotype of an *argO*-null mutant (5), indicating that the epitope-tagged ArgO retained its normal function. Similarly, an *argR argO* double mutant is rendered more resistant to CAN than an *argO* mutant, owing to elevated levels of intracellular Arg in the former strain due to the absence of ArgR (28), which mitigates to some extent the growth-inhibitory effects of CAN. The presence of pHYD2847 in an *argR argO* double mutant impaired its resistance to CAN in MA medium containing 2 μg/ml CAN, indicating that ArgR encoded on the plasmid pHYD2847 was biologically active.

Growth measurements (see Fig. 3) were undertaken by inoculation of stationary-phase cultures of the chosen strains grown in MA medium at a dilution of 1:100 in the appropriate medium, and the A_{600} of the cultures was measured over time. Representative curves obtained from two independent experiments are shown. β-Galactosidase specific activities in Miller units in exponential-phase cultures were measured by the method of Miller (20), and values reported herein are means ± standard errors (SE) of values obtained from two independent measurements performed in duplicate. Plasmid clones able to suppress the canavanine-supersensitive (CAN^{ss}) phenotype of the *argO* mutant strain GJ4823 were isolated following the transformation of GJ4823 with a plasmid library bearing Sau3AI-digested *E. coli* K-12 chromosomal DNA inserts present within the BamHI site located in *tetA* of pACYC184 (23). Transformants exhibiting Cm- and CAN-resistant (CAN^{r}) phenotypes were isolated on MA Cm plates containing 2 μg/ml CAN. Following the demonstration that the CAN^{r} phenotypes were plasmid borne, junction sequences in the chosen plasmids were determined with a pair of vector-based *tet* primers, 5′-CG CCGAAACAAGCGCTCATGAGCC-3′ and 5′-CTATGCGCACCCGTT CTCGGAGCAC-3′. The coordinates of chromosomal segments of DNA present in the plasmids isolated from the plasmid library are as per reference 29.

The Lys export phenotype of *ybjE* was assessed by a Lys cross-feeding assay which involved the construction of a Lys auxotroph of MC4100 bearing the *lysA*-null mutation, GJ9060, that was also rendered defective for the activities of the three peptide uptake systems due to the presence of null mutations in *dppB*, *tppB*, and *oppB*. Thus, GJ9060 grew on MA agar containing Lys but did not grow in Lys-Ala (1 mM)-supplemented MA agar. Lys export was visualized by the assay of halo formation of GJ9060 bearing the plasmid pHYD3025 (vector), representing its syntrophic growth, when seeded into an MA agar plate containing Amp, 1 mM IPTG, and 1 mM Lys-Ala, on the surface of which 10^6 cells of MC4100 bearing the plasmid pHYD2836 or the vector control pHYD3025 were spotted. The plates were photographed after 30 h of incubation.

Measurements of cellular and extracellular Lys content. For measurement of cellular Lys content, stationary-phase cultures of the appropriate strains grown in LB medium were washed with MA medium, subcultured in the same medium, and grown until early log phase. The cultures obtained were each inoculated, at an A_{600} of 0.02, into two flasks containing 30 ml MA medium, one of which was also supplemented with the lysylalanine (Lys-Ala) dipeptide at 1 mM, and the cultures were grown further for 3.5 h. A cell suspension with an adjusted A_{600} of 7 was generated from these cultures, and the cells were separated from the medium by centrifugation of 1 ml of the cell suspension at 13,000 rpm at room temperature for 5 min, through 300 μl of an organic layer comprising bis-(2-ethylhexyl) phthalate and dibutyl phthalate at a ratio of 1:2 (vol/vol). The supernatant above the organic layer was removed, and the likelihood of contamination with Lys either originating from the growth medium or present in Lys-Ala was minimized by washing the organic layer seven times with water. Following a final centrifugation, the organic layer was removed and the cell pellet was treated with 200 μl of 20% perchloric acid, vortexed, and sonicated in Eppendorf tubes immersed in an ice-water mixture using a Diagenode Bioruptor UCD-200 on a low-wave output power of 160 W using alternate (30-s) on/off cycles for 10 min. Following a centrifugation of this solution at 13,000 rpm at 4°C for 5 min, its supernatant was collected, and its acidity was neutralized by the addition of 10 N KOH. After a clarification by centrifugation, the supernatant was concentrated by vacuum desiccation at 30°C to a volume of 120 μl, and half of it was used to estimate cellular Lys content on the Agilent Technologies (model 1200 series) HPLC system after a precolumn derivatization with phenylisothiocyanate (PITC) using Waters Pico-tag chemistry to detect the product. Appropriate PITC-derivatized standards of Lys monohydrochloride were also processed.

For measurement of extracellular Lys content, stationary-phase cultures of the parent MC4100 bearing the vector pHYD3025 and the plasmid pHYD2836 were washed with MA medium and subcultured in the same medium containing 1 mM IPTG and 1 mM Lys-Ala with the appropriate antibiotic selection and grown for 8 h at 37°C. Three hundred microliters of each culture was passed through an organic layer [30 μl; bis-(2-ethylhexyl) phthalate and dibutyl phthalate in a ratio of 1:2 (vol/vol)]. Two hundred microliters of the supernatant above the organic layer was aspirated and processed for HPLC detection of Lys as described above. Samples that did not contain cells were processed similarly, and the amount of contaminating Lys amounting to 33 ± 1.3 nmol per ml in the preparation of Lys-Ala was determined. The area under the HPLC peak corresponding to contaminating Lys was subtracted from the Lys peaks obtained from grown cultures prior to normalization by A_{600} of the cultures. The values reported for cellular and extracellular Lys content are means ± SE from two independent experiments.

Purification of ArgR. Strain GJ9048 bearing the plasmid pHYD2847 was inoculated into 5 ml of LB Amp broth. Following overnight growth, the culture was inoculated into 1 liter of LB Amp broth and grown to an A_{600} of 0.4, after which 1 mM IPTG was added to the culture, and the culture was further incubated with shaking for 6 h. Following centrifugation of the culture at 6,000 rpm for 15 min at room temperature, the cell pellet obtained was resuspended in 20 ml of lysis buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM $MgCl_2$, 10 mM imidazole, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Cell lysis was performed by subjecting the cell suspension to sonication, and the cell lysate obtained was centrifuged at 13,000 rpm at 4°C for 20 min. The supernatant was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) (Qiagen) chromatographic column preequilibrated with lysis buffer. After passage of the lysate, the chromatographic column was washed five times with 10 ml of washing buffer (lysis buffer with 30 mM imidazole), and the bound ArgR was eluted with 10 ml of elution buffer (lysis buffer with 250 mM imidazole). The purity of ArgR was determined by SDS-PAGE to be greater than 95%. Purified ArgR was dialyzed against buffer A (lysis buffer with 100 mM imidazole and 10 mM β-mercaptoethanol) at a protein-to-dialysis buffer ratio of 1:200 (vol/vol) for 1 h with the Tube-o-dialyzer system with a 15-kDa cutoff (G-Biosciences). Another dialysis was performed for 1 h against buffer B (buffer A but with 50 mM imidazole). The purified protein solution was subjected to a final dialysis for 12 h against buffer C (buffer A lacking imidazole). Protein concentration was estimated by using the Bradford reagent (Sigma), and the purified ArgR was stored at −80°C in a buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 45% glycerol.

EMSAs with ArgR. DNA templates for electrophoretic mobility shift assays (EMSAs) for determination of binding of ArgR to the *cis* regulatory region of *ybjE* were generated by PCR. In the study described here, the proficiency of binding of ArgR to DNA templates bearing specific dele-

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TABLE 2 Effect of Arg and ArgR on *ybjE-lac* expression from plasmid pHYD2885 and in derivatives bearing deletions or substitutions in the *cis* regulatory region of *ybjE*

Plasmid	*ybjE cis* regulatory region[a]	β-Galactosidase activity (Miller units)[b]					
		argR$^+$			Δ*argR*		
		Nil	Arg	Lys	Nil	Arg	Lys
pHYD2885	−456 to +55	133 ± 2	81 ± 0.4	148 ± 1	144 ± 4	140 ± 5	135 ± 7
pHYD2888	−456 to +55; T	153 ± 2	156 ± 1.5	ND	150 ± 0	147 ± 2	ND
pHYD2889	−456 to +55; ΔIR	139 ± 9	86 ± 3	151 ± 8	131 ± 2	144 ± 7	139 ± 6
pHYD2890	−456 to +55; ΔIR, Δ1	193 ± 8	135 ± 5	ND[j]	190 ± 7	195 ± 9	ND
pHYD2891	−456 to +55; ΔIR, Δ2	111 ± 4	111 ± 3	ND	108 ± 4	107 ± 8	ND
pHYD2892	−456 to +55; ΔIR, Δ3	21	21	ND	19 ± 0.5	18 ± 0.1	ND
pHYD2893	−456 to +55; ΔIR, 4S	60 ± 0.3	52 ± 0.8	ND	54 ± 2	55 ± 1	ND

[a] Additional features of the plasmid employed are as follows: T, contains the T-to-G substitution at −58 of *ybjE*; ΔIR, deletion of the inverted repeat, removing a region of *ybjE* from −333 to −302; Δ1 to Δ3, deletions in *ybjE* from −85 to −68, −64 to −47, and −43 to −26, respectively; 4S, substitutions of 4 bases from −43 to −26 of *ybjE*.
[b] β-Galactosidase activities in Miller units were determined in exponential-phase cultures of parent MC4100 (*argR*$^+$) and in its Δ*argR*::Kan derivative GJ9029, bearing the plasmid pHYD2885, and its derivatives grown in MA medium (nil) and MA medium supplemented with Arg and Lys at 10 mM. ND, not determined.

tions and substitutions in the *cis* regulatory region of *ybjE* was also tested. In both instances, the desired region was first amplified either from pMU575 bearing the wild type *cis* regulatory region or from its derivatives (Table 2) bearing the aforementioned modifications with the primer pair JGPMUFP (5′-TCCCCACATCACCAGCAA-3′) and JGPMUGALKRP (5′-CAGAGATTGTGTTTTTTCTTTCAG-3′), which primes DNA synthesis from sites located upstream and downstream of the cloned region present on pMU575 in the various plasmids. The PCR products were gel purified and used as templates for PCR amplification with the primer pair JGLYBJEPFA (5′-ACCCCCGGGCTCTGGCGAAC-3′) and JGLYBJEDRP (5′-TATGCTCTAGAAACAGCCCAGAAAACATGA-3′), a segment of the *cis* regulatory region of *ybjE* from −301 to +55 relative to the start site of *ybjE* transcription that was determined by primer extension. For EMSA reactions, PCR products that were generated as described above were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and the EMSA reaction mixtures (20 μl) consisted of 0.5 nM labeled DNA, 1 μg of bovine serum albumin (BSA), and 1 μg of poly(dI·dC) present in the EMSA binding buffer, which comprised 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 22.5% glycerol, and various hexamer concentrations of ArgR. Where required, 5 mM Arg (Arg monohydrochloride) was included in the binding reaction mixture. EMSA reaction mixtures of labeled DNA and protein were incubated on ice for 30 min, and DNA-protein complexes were resolved by electrophoresis on a 4% native polyacrylamide gel that was cast with 1× Tris-borate buffer (30) and 1 mM Arg with the electrophoresis buffer comprising 1 mM Arg and 0.5× Tris-borate. The electrophoresis was performed at constant current for 4 to 6 h at 4°C. Radioactive bands following gel drying were visualized with a Fujifilm FLA-9000 scanner.

RESULTS

Identification of *ybjE* as a multicopy suppressor of the CAN^{ss} phenotype of an *argO* mutant. As described above, a previous study showed that a strain lacking ArgO is severely impaired for growth in MA medium containing CAN, yielding a CAN^{ss} phenotype (5). In order to identify new genes mediating Arg export, we sought multicopy suppressors of the CAN^{ss} phenotype of an *argO* mutant. For this purpose, an *E. coli* genomic library constructed on the backbone of the plasmid pACYC184, with chromosomal DNA inserts present within the BamHI site, located in *tetA* of pACYC184, was introduced into the *argO* mutant derivative of MC4100, GJ4823, by transformation. Twenty-three transformants from a total pool of approximately 10^5 Cm-resistant transformants, exhibiting a CAN^r phenotype, were isolated on MA agar plates supplemented with Cm and 2 μg/ml CAN. Of these, 20 yielded an *argO*-specific PCR product when PCR was performed on plasmid DNA isolated using a primer pair specific for *argO*, indicating that the CAN^r phenotype they exhibited resulted from the presence and expression of a plasmid-borne wild-type *argO*. One such plasmid, designated pHYD2834, that upon retransformation into GJ4823 rendered it CAN^r was saved, and DNA junction sequencing showed that it carried a 3.7-kb insert spanning the chromosomal coordinates 3063431 to 3067219 (Table 1). This showed that the chromosomal insert in pHYD2834 encoded a functional ArgO.

Plasmids designated pHYD2833, pHYD2833.1, and pHYD2833.2, isolated from the remaining three transformants, upon retransformation into GJ4823 also rendered it CAN^r. DNA junction sequencing showed that they all bore an identical segment of chromosomal DNA extending from chromosomal coordinate 912287 to 914782 bearing a complete *ybjE* ORF, flanked by two incomplete *ybjW* and *aqpZ* ORFs. In addition, the chromosomal insert on the three plasmids was present in the same orientation within the BamHI site of pACYC184 (Fig. 1A and Table 1). Derivatives of GJ4823 bearing the plasmid pHYD2836, which contained a minimal *ybjE* ORF under the expression control of the P_{trc} promoter, were rendered CAN^r in the presence of 1 mM IPTG, whereas those bearing the vector pHYD3025 remained CAN^{ss} (data not shown). The CAN^r phenotype yielded by the presence of the plasmid pHYD2833 was partial in that the CAN^r phenotype was prominent on MA agar plates containing 2 but not 20 μg/ml CAN (Fig. 1B). Expression of *ybjE* from the P_{trc} promoter of the plasmid pHYD2836 with IPTG also conferred a partial CAN^r phenotype to GJ4823 (data not shown). Derivatives of GJ4823 bearing the plasmid pHYD2834, however, displayed a robust CAN^r phenotype in MA agar plates containing CAN up to 20 μg/ml (Fig. 1B). We found that in MC4100 (parent), whereas the absence of ArgO led to a CAN^{ss} phenotype, the absence of YbjE did not lead to any discernible sensitivity to CAN on MA agar plates containing 20 μg/ml CAN. However, in a medium with a lower CAN concentration (1 μg/ml), the *argO ybjE* double mutant was less resistant to CAN than the *argO* mutant (Fig. 2). Lastly, examination of the amino acid sequence of YbjE suggested that it was a protein of a predicted membrane location bearing eight putative transmembrane segments.

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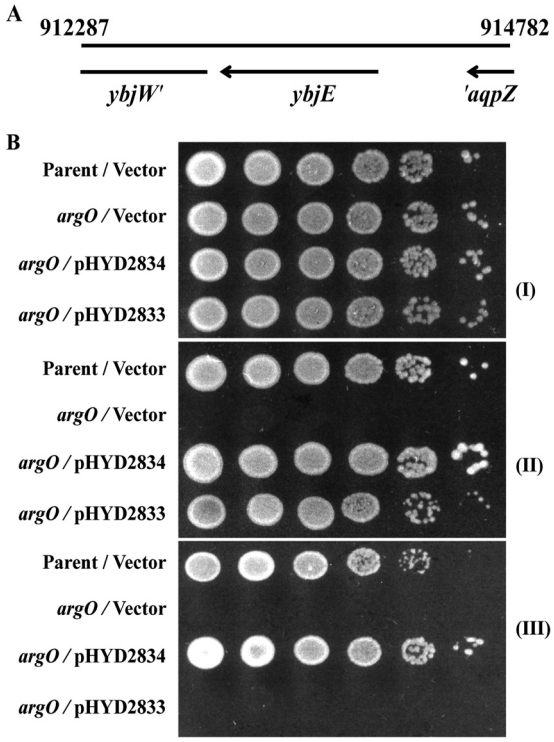

FIG 1 Suppression of the CAN^{ss} growth phenotype of an *argO*-null mutant by increased dosage of *ybjE*. (A) Schematic representation (to scale) of the extent of chromosomal DNA borne on plasmids pHYD2833, pHYD2833.1, and pHYD2833.2. The chromosomal coordinates marking the extremities of the insert and the extents of the 3′- and 5′-truncated *ybjW* and *aqpZ* genes, respectively, are indicated. (B) Suppression of the CAN^{ss} growth of an *argO* mutant by the plasmid pHYD2833. Tenfold serial dilutions of cultures of the parent (MC4100) and its *argO*::*205*Tn*10*dTet derivative GJ4823 (*argO*) bearing the indicated plasmids were spotted on the surfaces of an MA agar plate (I) and MA agar plates containing CAN at 2 (II) and 20 (III) μg/ml.

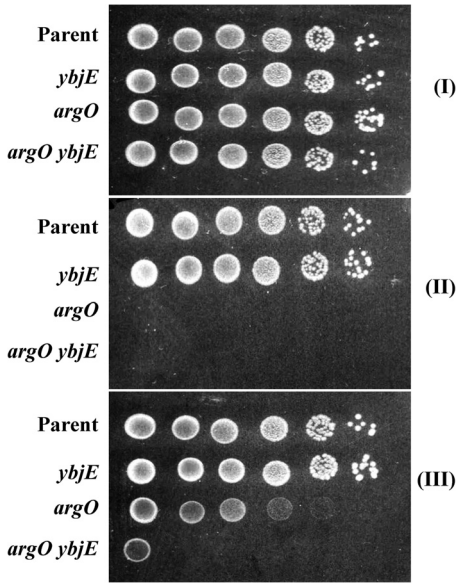

FIG 2 Growth phenotype of the *ybjE*-null mutant in the presence of CAN. Tenfold serial dilutions of cultures of MC4100 (parent), GJ9026 (Δ*ybjE*::Kan *ybjE*), GJ4823 (*argO205*::Tn*10*dTet *argO*), and GJ9028 (*argO205*::Tn*10*dTet Δ*ybjE*::Kan *argO ybjE*) were spotted on the surface of an MA agar plate (I) and MA agar plates containing CAN at 20 (II) and 1 (III) μg/ml.

Evidence that *ybjE* mediates Lys export. The aforementioned observation that increased dosage of YbjE mediated resistance to CAN in the absence of a functional ArgO hinted that the mechanism by which it did so may involve enhanced export of CAN or that an increased dosage of *ybjE*, perhaps by mediating the export of another basic amino acid, competed with CAN uptake. In order to clarify this, we first examined effects of lysylalanine (Lys-Ala) and arginylalanine (Arg-Ala) dipeptides on the growth of MC4100 and its derivatives bearing single or double deficiencies of ArgO and YbjE. Dipeptides provide a means to increase the intracellular level of an amino acid, as their entry into the cytoplasm occurs via the peptide uptake systems. The Lys-Ala dipeptide has previously been employed as a means to increase the intracellular levels of Lys in *C. glutamicum* (31). We found that the *ybjE* mutant was impaired for growth in MA medium containing Lys-Ala but not in MA medium containing Arg-Ala or the histidinylalanine (His-Ala) dipeptides (Fig. 3A to D). The *argO* mutant grew at rates comparable to that of MC4100 in all the above-mentioned media, and the *argO* mutation did not exacerbate further the impaired growth of the *ybjE*-null mutant in a medium containing the Lys-Ala dipeptide.

We measured cellular Lys content in MC4100 and its derivatives bearing single or double null mutations in *argO* and *ybjE* following their transient exposure to Lys-Ala by HPLC. Upon exposure to Lys-Ala, the *ybjE* mutant (and its *argO* derivative) displayed an approximately 3-fold elevation in cellular Lys content in comparison to MC4100 and its *argO* derivative, and the absence of ArgO did not cause any alteration in cellular Lys levels (Fig. 3E). Assuming that an A_{600} of 1 corresponds to 10^9 cells (20) and the cellular volume is 1 μm^3 (32, 33), estimates of the intracellular Lys concentrations in the parent and the *ybjE* mutant in the presence of Lys-Ala can be arrived at, namely, 6.8 and 21 mM, respectively. The level of another basic amino acid, Arg, was comparable in cultures of the above-mentioned strains that were treated with Lys-Ala and those that were untreated (data not shown). In addition, overexpression of *ybjE* from the plasmid pHYD2836 in MC4100 led to increased cross-feeding of the *lysA dppB tppB oppB* derivative of MC4100, GJ9060, in comparison to the vector-bearing control, which was indicative of increased *ybjE*-mediated Lys export (Fig. 4A). Lastly, we estimated Lys in the culture medium following 8 h of growth of MC4100 bearing the vector or pHYD2836 in a medium containing Amp, 1 mM IPTG, and 1 mM Lys-Ala and found that overexpression of *ybjE* from the plasmid pHYD2836 mediated increased export of Lys in comparison to its haploid *ybjE*$^+$ (vector-bearing) counterpart (Fig. 4B). From Fig. 4B, the normalized extracellular Lys concentration yielded by cultures of MC4100 bearing the vector or the plasmid pHYD2836 can be estimated to be 92 and 162 μM, respectively.

Thialysine phenotypes associated with deficiency or overexpression of *ybjE*. Thialysine (*S*-aminoethyl-cysteine) is a toxic analogue of Lys, and given the evidence above that YbjE mediates export of Lys, we gauged the growth of MC4100 and its derivatives that were singly and doubly deficient for ArgO and YbjE on MA agar plates containing thialysine and found that severe impairment of growth was seen only in strains bearing the *ybjE*-null mutation and that the absence of ArgO did not cause any sensitivity to thialysine (Fig. 5A). Furthermore, overexpression of *ybjE*

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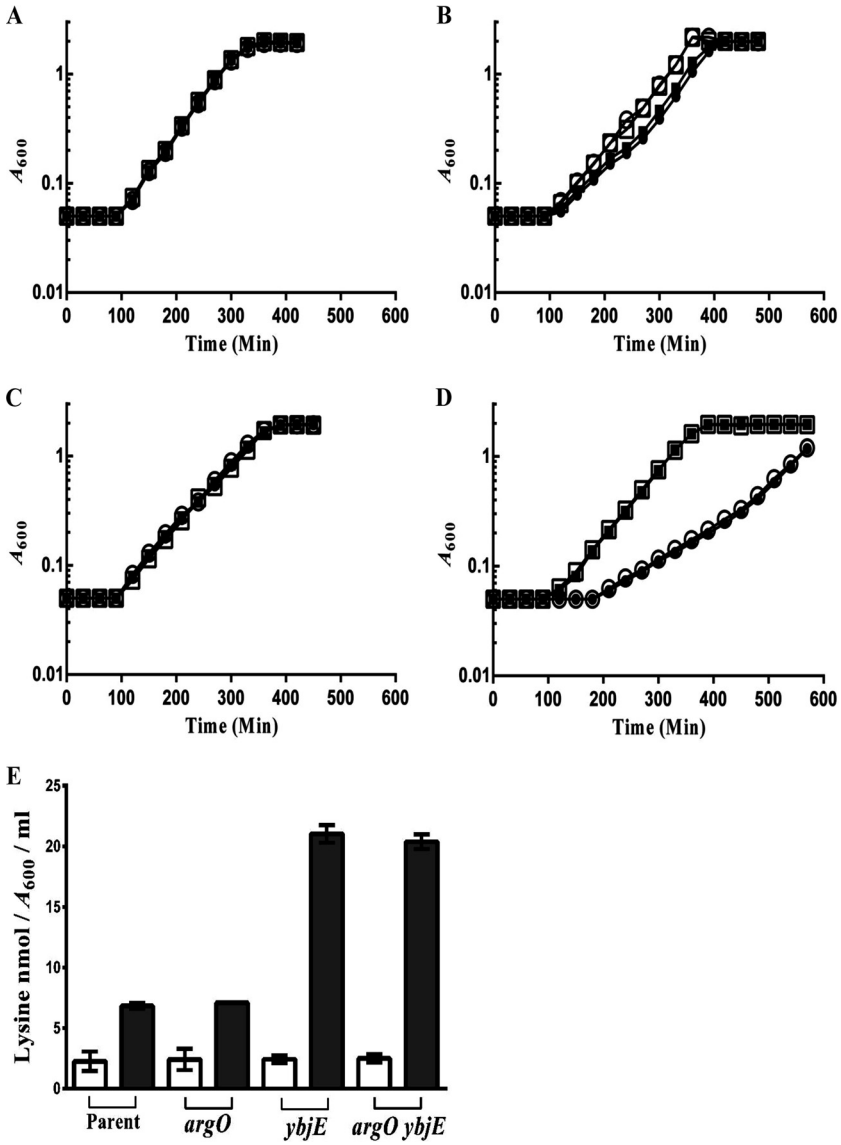

FIG 3 Growth impairment of the *ybjE* mutant by the Lys-Ala dipeptide and Lys-Ala-mediated elevation of cellular Lys content in the *ybjE* mutant. The parent MC4100 (open squares), GJ4823 (*argO205*::Tn*10*dTet; filled squares), GJ9026 (Δ*ybjE*::Kan; open circles), and GJ9028 (*argO205*::Tn*10*dTet Δ*ybjE*::Kan; filled circles) were grown in MA medium (A) and MA medium containing 1 mM Arg-Ala (B), His-Ala (C), and Lys-Ala (D) dipeptides, and the absorbance at 600 nm of the cultures was monitored. (E) Cellular Lys content in exponentially growing cultures of MC4100 (parent), GJ4823 (*argO*), GJ9026 (*ybjE*), and GJ9028 (*argO ybjE*) (white bars) and in their counterparts following their transient exposure to 1 mM Lys-Ala (black bars).

from the plasmid pHYD2836 conferred increased resistance to thialysine (Fig. 5B).

Characterization of the *ybjE cis* regulatory region. In order to delineate the core promoter elements and associated transcriptional regulation of *ybjE*, we initially performed primer extension and found that the *ybjE* message initiated from a G residue located 37 bases upstream of the predicted translational initiation codon of *ybjE* (Fig. 6A and data not shown). The *in vivo* expression level of the *ybjE* promoter was gauged by construction of the plasmid pHYD2885 in which the *cis* regulatory region of *ybjE* spanning a region from −456 to +55 was placed upstream of a promoterless *lacZ* in the single-copy plasmid pMU575 (Table 2). The location of the −10 and −35 promoter elements of *ybjE* was ascertained by measurements of *ybjE-lac* activity from derivatives of plasmid pHYD2885 bearing site-specific nucleotide substitutions that abolished *ybjE-lac* activity, engineered within a pair of hexameric sequences, 5′-TAGTGT-3′ and 5′-TTTACT-3′ (Fig. 6A and data not shown). Another feature of the *cis* regulatory region of *ybjE* that presented itself was the occurrence of an inverted repeat extending from −333 to −302, whose removal did not alter *ybjE-lac* activity (Table 2).

ArgR-mediated repression of *ybjE* expression by Arg. Upon testing the effects of external supplementation of MA medium with Arg and Lys at 10 mM, we found that the presence of Lys did not affect the magnitude of *ybjE-lac* expression, whereas Arg supplementation led to an approximately 1.5- to 2-fold repression of *ybjE-lac* (Table 2). *ybjE-lac* expression was unaffected by the presence of 10 mM Lys-Ala in MA medium, whereas it was repressed

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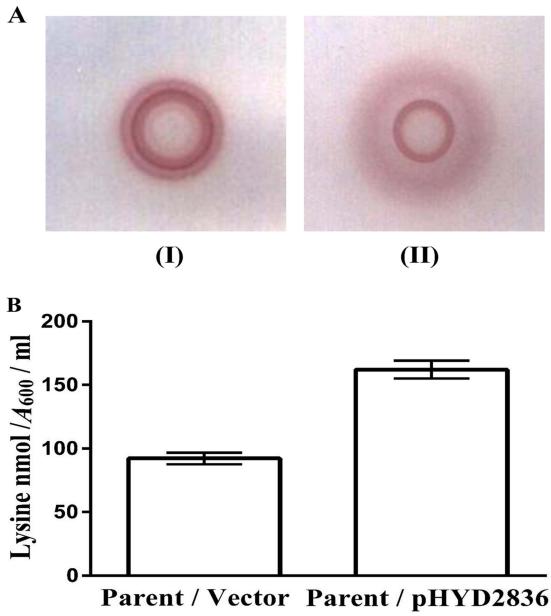

FIG 4 Visualization by cross-feeding of YbjE-mediated Lys export and extracellular Lys content following heterologous overexpression of *ybjE*. (A) Cultures of MC4100 bearing the plasmid pHYD3025 (vector) (I) and pHYD2836 (II) were spotted on the surface of an MA agar plate containing 1 mM Lys-Ala, 1 mM IPTG, and 1 μg/ml tetrazolium chloride. In addition, the plate was seeded with cells of strain GJ9060, which is a *lysA dppB oppB tppB* derivative of MC4100 bearing the vector and imaged after 30 h of incubation. (B) Lysine content in the medium following 8 h of growth of MC4100 (parent) bearing the vector or the plasmid pHYD2836 in MA medium supplemented with 1 mM Lys-Ala and 1 mM IPTG.

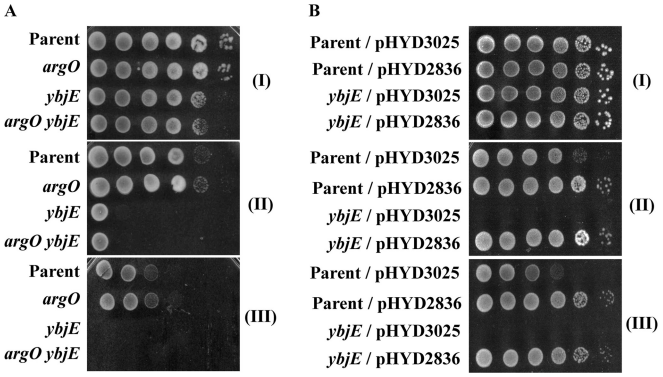

FIG 5 Hypersensitivity and enhanced resistance to thialysine caused by the absence and overexpression of *ybjE*. (A) Tenfold serial dilutions of cultures of MC4100 (parent) and its *argO205*::Tn*10*dTet (*argO*), Δ*ybjE*::Kan (*ybjE*), and *argO205*::Tn*10*dTet Δ*ybjE*::Kan (*argO ybjE*) derivatives, GJ4823, GJ9026, and GJ9028, respectively, were spotted on the surface of an MA agar plate (I) and MA agar plates containing thialysine at 1 (II) and 10 (III) μg/ml. (B) Tenfold serial dilutions of MC4100 (parent) and GJ9026 (*ybjE*) bearing the plasmids pHYD3025 (vector) and pHYD2836, in which expression of *ybjE* is under the IPTG inducible P_{trc} promoter, were spotted on the surface of an MA agar plate (I) and MA agar plates containing thialysine at 1 (II) and 10 (III) μg/ml. The indicated plates were also supplemented with 1 mM IPTG.

1.6-fold by the presence of 10 mM Arg-Ala (data not shown). Arg repression of *ybjE-lac* was absent in a strain lacking ArgR, the repressor protein of the Arg regulon (reviewed in references 34 and 35), and was independent of the presence or absence of the inverted repeat located in the *cis* regulatory region of *ybjE* (Table 2). The presence of both Arg and Lys in the medium did not alter β-galactosidase activity from the *gadA-lac* transcriptional fusion present on the plasmid pMU575, indicating that the repression by Arg was specific to the *ybjE* promoter (data not shown). The magnitude of *ybjE-lac* was not elevated by the absence of ArgR (Table 2), which is in contrast to that seen for genes of the Arg regulon that are significantly derepressed in an *argR* mutant (34, 35).

To test whether the *cis* regulatory region of *ybjE* contains an ArgR binding site(s), we purified ArgR and showed that *in vitro* ArgR bound only in the presence of Arg to a DNA fragment extending from −301 to +55 of *ybjE*, bearing the *ybjE* core promoter (Fig. 6; also, see Fig. S1 in the supplemental material). Our ArgR preparation also displayed proficient and Arg-dependent binding to a DNA fragment containing the promoter/operator region of *argF*, known to bear a cognate binding site for ArgR (34, 35) (see Fig. S1 in the supplemental material). ArgR bound with greater avidity to the *argF* DNA template than the *ybjE* DNA template, indicating that the binding site for ArgR in the *cis* regulatory region of *ybjE* represented a weak ArgR binding site (see Fig. S1 in the supplemental material).

In *E. coli* ArgR represses the promoters of genes encoding enzymes of Arg biosynthesis, in the presence of Arg by binding in a hexameric state to a pair of 18-bp imperfect palindromes (ARG boxes) separated by a 3-bp spacer and overlapping the promoters of Arg biosynthesis genes to different extents (34, 35). Assuming that the mode of binding of ArgR to the DNA of the *cis* regulatory region of *ybjE* was similar to that seen in the case of its cognate targets in promoters of genes involved in Arg biosynthesis, we searched for a pair of 18-bp DNA sequences located in close proximity to the *ybjE* promoter. We identified an 18-bp sequence extending from −64 to −47 bearing an imperfect match to the consensus sequence of the ARG box (see Fig. S2 in the supplemental material), whose deletion (Δ2) (Fig. 6A), abolished ArgR repression of *ybjE-lac* (Table 2) and abolished the binding of ArgR to the *ybjE* promoter DNA fragment (Fig. 6C). An earlier study on ArgR/ARG box interaction has pointed to the importance of a T base at the seventh position (T7), with minor groove occupancy, which is highly conserved in DNA sequences of all ARG boxes, mediating contact with ArgR (36). Furthermore, in all ARG box sequences there is absence of a guanine base at the T7 position (36). Another study has shown that replacement of T7 with G led to loss of ArgR repression at the promoter of *hisJ* (37). Substitution of the corresponding T7 (at −58) to G in the probable ARG box located within −64 to −47 in *ybjE* led to loss of ArgR repression of *ybjE-lac* (Table 2) and impaired the association of ArgR with the *ybjE* promoter DNA fragment bearing the above-mentioned base substitution (Fig. 6B). Deletion of an 18-bp segment of DNA from −85 to −68 (Δ1) (Fig. 6A), did not alter ArgR repression *ybjE-lac* (Table 2), indicating that the second ARG box, if present and required for ArgR binding to the *ybjE* promoter, may lie downstream of −47 in *ybjE*. We engineered substitutions of 4 bases, A to C, G to T, T to G, and T to G at −37, −40, −41, and −42, respectively, of *ybjE* (4S) (Fig. 6A) and found that the presence of 4S led to an overall reduction in the magnitude of *ybjE-lac* and loss of repression by ArgR (Table 2) and impaired the binding of ArgR to the *ybjE* promoter DNA template bearing 4S (Fig. 6D). The substitution(s) of the 4S cluster that is responsible for mediating the reduction in *ybjE-lac* and for affecting ArgR binding to the

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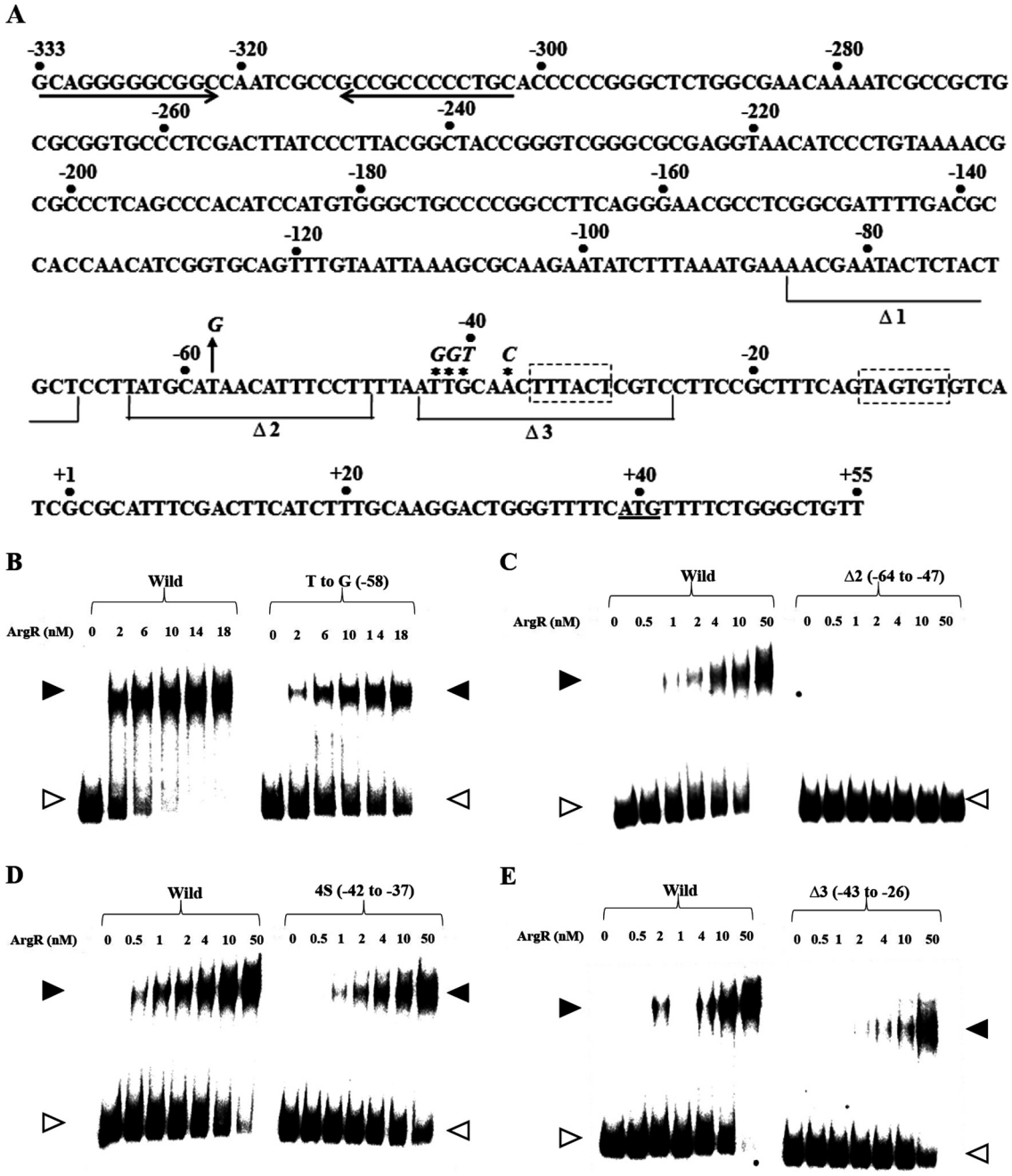

FIG 6 Nucleotide sequence of the *cis* regulatory region of *ybjE* and its interaction with ArgR *in vitro*. (A) The nucleotide sequence of *ybjE* shown extends from −333 to +55 relative to the start site of the *ybjE* message (indicated as +1), and the predicted initiation codon of *ybjE* is underlined. The −10 and −35 sequences of the *ybjE* promoter are shown as dashed boxes, and an inverted repeat extending from −333 to −302 is marked by a pair of convergent horizontal arrows. The extents of 18-bp deletions in the *cis* regulatory region of *ybjE* present in plasmids pHYD2890 (Δ1), pHYD2891 (Δ2), and pHYD2892 (Δ3) are indicated. The T-to-G substitution in the plasmid pHYD2888 and substitutions of 4 bases (4S) in the plasmid pHYD2893 are marked with an upward arrow and asterisks, respectively, and the base substitutions are italicized. Pairs of EMSA of ArgR with the *cis* regulatory region of *ybjE* (wild) and with its derivatives bearing the T-to-G base substitution at −58 (B), Δ2 (−64 to −47) (C), substitutions of 4 bases (4S) lying within −42 to −37 of *ybjE* (D), and Δ3 (−43 to −26) (E). The DNA templates used in the EMSA span a region of *ybjE* from −301 to +55 with the substitutions and deletions contained within, and the positions of the free DNA and the ArgR DNA complex are indicated with open and filled triangles, respectively. Hexamer concentrations of ArgR are indicated. Arg at 1 mM was incorporated in the electrophoresis buffer and gel, and the binding reaction mixture contained Arg at 5 mM.

ybjE DNA template remains unknown. Lastly, ArgR displayed impaired binding to a *ybjE* promoter DNA fragment lacking a region from −43 to −26 (Δ3) (Fig. 6A and E). Since the presence of Δ3 leads to the removal of the −35 sequence, the plasmid pHYD2892 (bearing Δ3) displayed very low levels of *ybjE-lac* (Table 2).

In addition, we tested the effects of absence of LysR the transcriptional regulator of *lysA*, whose product mediates the conversion of diaminopimelate to Lys (38), on the expression of *ybjE-lac*. Levels of *ybjE-lac* were unaffected by the absence of LysR. Furthermore, the repressive effect of Arg on *ybjE-lac* and the absence of

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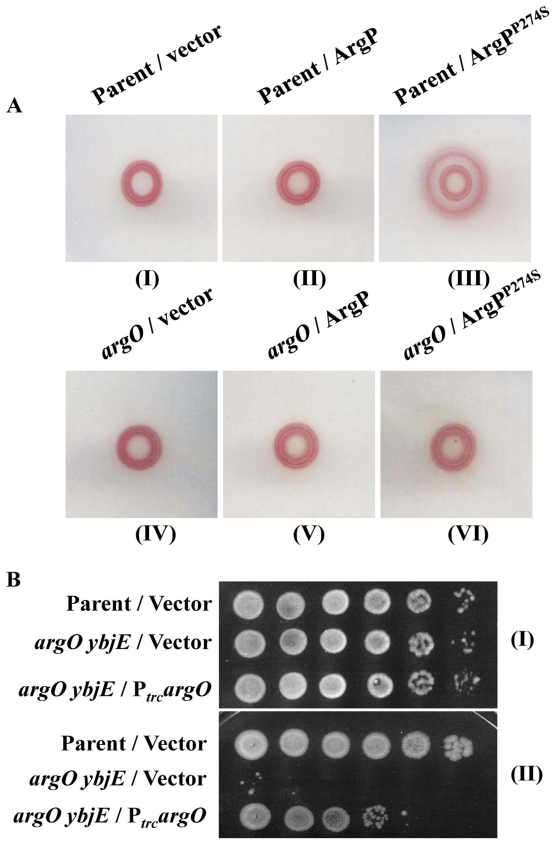

FIG 7 Lys export function of ArgO. (A) Cross-feeding of the *lysA dppB oppB tppB* mutant GJ9060 by constitutive overexpression of *argO* elicited by the ArgP^{P274S} variant of ArgP. Cultures of MC4100 (parent) bearing the plasmid pCL1920 (vector) (I) and its derivatives pHYD915, expressing ArgP (II), and pHYD2606, expressing ArgP^{P274S} (III) and of GJ4823 (MC4100 *argO205*::Tn*10*dTet) containing the same plasmids (IV, V, and VI) were spotted on the surface of an MA agar plate containing 1 mM Lys-Ala and 1 μg/ml tetrazolium chloride and imaged after 30 h of incubation. In addition, the plate was seeded with cells of the strain GJ9060 bearing the vector. (B) Functional complementation of the thialysine hypersensitive phenotype of an *argO ybjE* double mutant GJ9028 by heterologous overexpression of *argO*. Tenfold serial dilutions of cultures of MC4100 (parent) bearing the plasmid pHYD3025 (vector) and GJ9028 (*argO ybjE*), bearing the vector or the plasmid pHYD2835, which expresses *argO* from the P_{trc} promoter (P_{trc} *argO*), were spotted on the surface of an MA agar plate containing 10 μM IPTG (I) and an MA agar plate containing 10 μM IPTG and 0.6 μg/ml thialysine (II).

effect of Lys remained unaltered in the *lysR* mutant (see Table S2 in the supplemental material for additional details). *ybjE-lac* expression was similarly unaffected by the absence of ArgP, the Arg/Lys responsive transcriptional regulator of *argO* (5), and by the presence of two dominant mutations in *argP* that lead to high constitutive Arg/Lys-insensitive overexpression of *argO* (5, 39) (see Table S3 in the supplemental material). It may be noted that the repressive effect of Arg on *ybjE-lac* was not apparent (see Table S3 in the supplemental material) in strains bearing the dominant *argP* alleles but not the wild-type *argP*, which may be explained on the basis that constitutive overexpression of *argO* caused by dominant *argP* alleles (5, 39) may lead to enhanced cytoplasmic Arg export, thereby alleviating Arg repression of *ybjE-lac*.

Evidence for a latent Lys export capacity of ArgO. ArgP-*argO* of *E. coli* and LysG-*lysE* from *C. glutamicum* are thought to constitute orthologous protein-gene pairs (40). ArgP and LysG are transcriptional regulators of *argO* and *lysE*, respectively (5, 16), and the two proteins share 35% identity and 53% similarity at the amino acid level. ArgO and LysE have been shown to mediate export of Arg (5) and Arg/Lys (15, 16), respectively, with the two exporters sharing 35% identity and 50% similarity. One intriguing difference between the two orthologous protein-gene pairs is seen at the mode of transcriptional regulation effected by the two regulators. Whereas *lysE* transcription is activated by both Arg and Lys serving as coeffectors of LysG (16), *argO* expression is subject to activation by Arg and repression by Lys (5), with repression by Lys occurring via the formation in the presence of Lys (but not Arg) of an ArgP-Esig70 complex, reversibly trapped at the step of promoter clearance, following open complex formation (41). So far, ArgO has been shown to mediate export only of Arg, and in experiments described above, an *argO* mutant displayed fitness similar to that of its parent when subjected to cytoplasmic Lys stress imposed by Lys-Ala or thialysine (Fig. 3 and 5A).

We tested whether overexpression of *argO* could mediate export of Lys and alleviate the impaired growth of a *ybjE* mutant by the imposition of cytoplasmic Lys stress. In this study, we used an ArgP variant bearing a dominant mutation in *argP* encoding the ArgP^{P274S}, which, among a collection of similar *argP* mutations described earlier (5), is known to cause the highest magnitude of Lys-insensitive *argO* expression (39). Expression of ArgP^{P274S} but not ArgP in MC4100 promoted cross-feeding of the *lysA dppB tppB oppB* quadruple mutant GJ9060 and the ability of ArgP^{P274S} to cross-feed GJ9060 was absent in a strain lacking ArgO (Fig. 7A). In a related experiment, heterologous overexpression of *argO* from the IPTG inducible P_{trc} promoter of the plasmid pHYD2835 rendered the *argO ybjE* double mutant GJ9028 resistant to thialysine, though the resistance phenotype was best seen at a lower (0.6 μg/ml) concentration of thialysine (Fig. 7B). Lastly, expression of ArgP^{P274S} but not the wild-type ArgP rendered the *ybjE* mutant GJ9026 partially resistant to thialysine, and the ArgP^{P274S}-mediated resistance phenotype was absent in strain GJ9028 (see Fig. S3 in the supplemental material).

DISCUSSION

LysE mediated Arg/Lys export by *C. glutamicum* represents one of the earliest known examples of microbial amino acid export (42), and LysE belongs to a family of proteins known as the LysE superfamily (43). In *E. coli*, though, the Lys but not Arg export potential of ArgO is as yet unknown. In the present study, we obtained multiple lines of evidence which indicate that YbjE functions as a separate Lys exporter in *E. coli* and that under certain conditions, ArgO can mediate Lys export.

YbjE as a Lys exporter. Two phenotypes of the *ybjE* mutant, namely, its growth inhibition by elevated cytoplasmic levels of Lys attained by Lys-Ala dipeptide feeding (Fig. 3) and its hypersensitivity to thialysine (Fig. 5), fulfill the genetic criteria expected if its product were to mediate export of Lys. Lys excretion in *C. glutamicum* is thought to represent an example of limited catabolism as a physiological model that has been proposed to explain the biological need for amino acid export, since Lys export is required during growth in a medium containing lysyl-dipeptides, as *C. glutamicum* does not have the capacity to catabolize Lys (14, 31). Besides export, another way to redress the growth inhibition caused by elevated cytoplasmic content of an amino acid could be via the activation of its corresponding catabolic route. In *E. coli*,

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however, the pathway of degradation of Lys (and also of Arg) via its decarboxylation, followed by the export of the decarboxylated product, is fully operational only under conditions of anaerobiosis and low pH in Lys (or Arg)-containing complex medium (44). In addition the catabolism of Arg to glutamate by the arginine succinyltransferase pathway occurs only under conditions of nitrogen limitation (44). The requirement of YbjE function to mitigate the growth-inhibitory effects of a Lys stress imposed by Lys-Ala under the growth condition used for the experiments whose results are shown in Fig. 3D is thus compatible with the limited-catabolism model.

The engagement of amino acid export is thought to place the bacterial cell at a risk of performing an energy-consuming and futile cycle of export and reuptake of the amino acid (14, 45). In some instances, membrane proteins mediating amino acid efflux have been functionally categorized as exporters with very low affinity for their substrate, a property that is thought to mitigate the potential problem of futile cycling and ensure that export occurs only when the cytoplasmic level of the substrate amino acid is very high (reference 45 and references therein). In the current study, high-level expression of *ybjE* from the P_{trc} promoter in the parent did not lead to any discernible Lys requirement in glucose minimal medium (Fig. 5B), which suggests that the K_m for Lys export by YbjE may be high. Our studies that ascribe a Lys export function to YbjE are consistent with the previous studies of Ueda and coworkers, who reported in multiple patents the identification of YbjE as an exporter of Lys (17, 18, 19). They identified YbjE based on the observation that its elevated expression conferred resistance to growth-inhibitory concentrations of Lys in the medium, and a strain lacking YbjE displayed significant reduction in growth rate under the same growth condition. Ueda and coworkers reported that besides Lys, elevated expression of *ybjE* also conferred resistance to high concentrations of Arg, L-ornithine, L-isoleucine, L-glutamic acid, L-threonine, L-histidine, L-proline, L-phenylalanine, and L-cysteine. Among these, *ybjE* overexpression promoted significant resistance to extracellular L-ornithine and to a lesser extent to L-threonine, whereas for other amino acids, the resistance was marginal. In addition, Ueda et al. also showed that increased expression of *ybjE* led to an enhancement in the presence of extracellular Lys content, consistent with the notion that YbjE functions as an exporter of Lys in *E. coli*.

Premise for the identification of *ybjE*. Although our selection procedure for identification of *ybjE* points to an added Arg/CAN export potential of YbjE (Fig. 1B), an alternative explanation could be that the enhanced extracellular Lys concentration resulting from *ybjE* overexpression competitively inhibits CAN uptake through the common Arg, Lys, ornithine, and CAN uptake system (LAO [for "Lys/Arg/ornithine"]) (35). Consistent with this explanation, a previous study had noted that the MIC of CAN for the *argO* mutant was considerably increased when Lys was present in the medium (5). The observation that absence of YbjE to some degree exacerbated the Can^{SS} phenotype of an *argO* mutant (Fig. 2) may also be explained by the latter mechanism. It may be noted that overexpression of *ybjE* has been reported to mediate resistance to high extracellular concentrations of Arg (17, 18, 19); however, the resistance appears to be marginal at best, and an additional role for YbjE as an Arg/CAN exporter seems unlikely. The previously reported resistance of a strain overexpressing *ybjE* to elevated extracellular concentration of ornithine (17, 18, 19) may also be explained on the basis that enhanced Lys export via YbjE by competing with ornithine uptake through the LAO uptake system mediates resistance to ornithine. Another observation supporting a Lys (but not Arg) export property of YbjE comes from the experiments with dipeptides, wherein it was observed that the *ybjE* mutant grew at rates comparable to that of the parent in a medium containing the Arg-Ala dipeptide, whereas in a medium with the Lys-Ala dipeptide, its growth was considerably hindered (Fig. 3). It was surprising that in experiments with dipeptides, the *argO* mutant was not growth inhibited when challenged with the Arg-Ala dipeptide in the medium (Fig. 3), which is in contrast to the scenario in *C. glutamicum*, wherein the *lysE* mutant is rendered sensitive to both Arg- and Lys-containing dipeptides (15, 16). It appears that *E. coli* may bear an additional mechanism(s) to alleviate the potential growth-inhibitory effects of elevated cytoplasmic Arg levels generated by Arg containing dipeptides.

Separation of Arg and Lys export in *E. coli*. The existence of YbjE-mediated Lys export in *E. coli* represents a situation that is distinct from that seen in *C. glutamicum*, wherein a single protein, LysE, performs export of both Arg and Lys (15, 16). In addition, the genome of *C. glutamicum* does not possess an ortholog of YbjE. The finding that a Lys export potential could be demonstrated for ArgO only under conditions that bypassed the repressive effect of Lys on the expression of its cognate gene (Fig. 7) indicates that the Lys export capacity of ArgO is rendered cryptic, leading to a division of labor in the export of Arg and Lys in *E. coli*. We suggest that repression of *argO* by Lys in *E. coli* perhaps effects this division. Going solely by strength of phenotypes alone, it appears that ArgO possesses a weaker Lys export capacity than YbjE (Fig. 7). Given the absence of an ortholog of YbjE in *C. glutamicum*, we used its amino acid sequence to query the nonredundant database using the BLAST algorithm, which revealed a wide distribution of YbjE orthologs across Gram-negative bacteria (data not shown). Restricting the BLAST search to Gram-positive bacteria returned only two possible orthologs with significant coverage and E values, in the genomes of *Enterococcus gallinarum* EGD-AAK12 (100% coverage) and *Virgibacillus halodenitrificans* (90% coverage).

Repression of Lys exporter expression in *E. coli* by Arg. Transcription of genes encoding amino acid exporters in many instances is subject to stimulation by its amino acid substrate. Amino acid-mediated expression control is thought to be another means of avoiding futile cycling, since expression would occur only when the level of the amino acid reaches a certain threshold (14, 45). Stimulation of *brnFE* expression encoding a multi-amino-acid exporter, by methionine and the branched-chain amino acids in conjunction with an Lrp-like transcription factor (45, 46) in *C. glutamicum* and by Lrp and leucine of the inducible LeuE leucine exporter in *E. coli* (9), is another example, besides those of *lysE* and *argO* (described above), where the amino acid substrate exerts control over the expression of its exporter.

We tested the effects of mutations in genes encoding transcription factors with known roles in Arg/Lys metabolism on expression of *ybjE* and found that Arg exerted a modest repressive effect on *ybjE* via ArgR, with the ArgR binding site being likely to exist in an overlap with the *ybjE* promoter (Table 2 and Fig. 6). It appears therefore that ArgR at the *ybjE* promoter is likely to exert its repressive effects in a manner analogous to its role as a repressor of genes involved in Arg biosynthesis, that is, by occluding promoter binding of the RNA polymerase holoenzyme (34, 35). Further

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studies are needed to delineate the mechanism of ArgR repression at the *ybjE* promoter.

Our studies on the putative ARG boxes within the *cis* regulatory region of *ybjE* suggest that of the two boxes, the one absent in the DNA template of *ybjE* with the Δ2 deletion (from −64 to −47) represents a stronger binding site than the site that is absent in the Δ3 deletion DNA (from −43 to −26). If this is true, then *ybjE* would represent yet another example where ArgR imparts gene repression by interacting with imperfect low-affinity ARG boxes. The *cis* regulatory regions of *hisJ* (37) and the *gltBDF* operon (47) contain imperfect ArgR binding sites (see Fig. S2 in the supplemental material) located upstream of the core promoter elements. ArgR mediates repression of gene expression at *hisJ* and *gltBDF* in mechanistically distinct ways which in both cases are different from its mode of action on genes involved in Arg biosynthesis (37, 47). It may be noted that for the *gltBDF* operon, *hisJ*, and genes involved in Arg biosynthesis, the absence of ArgR leads to derepression of gene expression, which is not the case for *ybjE* (Table 2). Absence of derepression may be explained if in minimal glucose medium *ybjE-lac* expression is fully derepressed in the first place. Based on estimates of the number of ArgR molecules in the cell and the affinity of ArgR for Arg, it is believed that a fraction of total ArgR exists in the Arg-bound hexameric state capable of DNA binding (34). Since the affinity of the ArgR hexamer for the classical ARG box is very high, hexameric ArgR binds to the ARG box even during growth in minimal medium, and hence its absence causes derepression of expression of genes involved in Arg biosynthesis. Since the ArgR binding sites in the *cis* regulatory region of *ybjE* appear to be weak binding sites (see Fig. S1 in the supplemental material), during growth in minimal medium *ybjE-lac* expression may remain derepressed as a result of titration of hexameric ArgR by other stronger ARG boxes on the chromosome. Consequently, the repressive effect of ArgR would be seen upon increased ArgR hexamer formation, which would occur when cytoplasmic Arg level is elevated during growth in Arg-containing medium. Going by this, it would appear that the ARG boxes in the *cis* regulatory regions of *hisJ* and *gltBDF* may have higher affinities for ArgR than those in the promoter region of *ybjE*. Overall, though, the repression of the expression of the Arg exporter ArgO by Lys (5) and of the Lys exporter YbjE by Arg in *E. coli* is indicative of a mechanism for maintenance of an Arg/Lys balance. Lastly, given the evidence herein that *ybjE* encodes a functional Lys exporter in *E. coli*, we propose that *ybjE* be redesignated *lysO* (for Lys outward permease), and the energetics of Lys export through LysO remains a subject of future study.

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