Transcriptional Cross-Regulation between Gram-Negative and Gram-Positive Bacteria, Demonstrated Using ArgP-*argO* of *Escherichia coli* and LysG-*lysE* of *Corynebacterium glutamicum*

Carmelita N. Marbaniang and J. Gowrishankar

Laboratory of Bacterial Genetics, Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India

The protein-gene pairs ArgP-*argO* of *Escherichia coli* and LysG-*lysE* of *Corynebacterium glutamicum* are orthologous, with the first member of each pair being a LysR-type transcriptional regulator and the second its target gene encoding a basic amino acid exporter. Whereas LysE is an exporter of arginine (Arg) and lysine (Lys) whose expression is induced by Arg, Lys, or histidine (His), ArgO exports Arg alone, and its expression is activated by Arg but not Lys or His. We have now reconstituted in *E. coli* the activation of *lysE* by LysG in the presence of its coeffectors and have shown that neither ArgP nor LysG can regulate expression of the noncognate orthologous target. Of several ArgP-dominant (ArgP^d) variants that confer elevated Arg-independent *argO* expression, some (ArgP^d-P274S, -S94L, and, to a lesser extent, -P108S) activated *lysE* expression in *E. coli*. However, the individual activating effects of LysG and ArgP^d on *lysE* were mutually extinguished when both proteins were coexpressed in Arg- or His-supplemented cultures. In comparison with native ArgP, the active ArgP^d variants exhibited higher affinity of binding to the *lysE* regulatory region and less DNA bending at both *argO* and *lysE*. We conclude that the transcription factor LysG from a Gram-positive bacterium, *C. glutamicum*, is able to engage appropriately with the RNA polymerase from a Gram-negative bacterium, *E. coli*, for activation of its cognate target *lysE in vivo* and that single-amino-acid-substitution variants of ArgP can also activate the distantly orthologous target *lysE*, but by a subtly different mechanism that renders them noninterchangeable with LysG.

A common mechanism for activation of gene expression in all organisms is that involving recruitment by a transcription factor of RNA polymerase (RNAP) to a promoter so that the latter can then engage in productive transcription (9, 24, 58). RNAP recruitment occurs by a specific protein-protein interaction between the transcription factor, typically after its binding to DNA in the vicinity of the gene's promoter, and one or more of the RNAP subunits. The overall structures of the multisubunit housekeeping RNAPs also exhibit evolutionary conservation across all the three kingdoms of life (20, 71). In Gram-positive and Gram-negative bacteria, the core RNAP (represented by the subunit composition $\alpha_2\beta\beta'\omega$) associates with one of several σ subunits to constitute the RNAP holoenzyme.

The family of LysR-type transcriptional regulators (LTTRs) is widely distributed across both Gram-positive and Gram-negative bacteria, with multiple paralogs being represented even within a single organism (38, 45). Taken together, these proteins are involved in modulating an extremely diverse set of metabolic functions, which in most cases is achieved by their binding to coeffector ligands. Considerable specificity exists in the interactions between an individual protein, its coeffector(s), and cognate target(s); nevertheless, a limited extent of cross talk has been identified between LysR-type paralogs in a single bacterium that control genes of related function, such as those for catabolism of aromatic compounds (35, 52, 53). Structural studies have indicated that although the LysR-type transcriptional regulators share common protein folds, they differ in their oligomerization properties and the mechanisms by which they both bind to DNA regulatory regions and contact RNAP (45).

The proteins LysG and ArgP are orthologous members of the LysR family from, respectively, the Gram-positive bacterium *Corynebacterium glutamicum* (6, 68) and the Gram-negative bacterium *Escherichia coli* (12, 47). LysG is a transcriptional regulator of

LysE expression (6), and likewise ArgP regulates expression of ArgO, which is a LysE ortholog (47). LysE and ArgO belong to the family of amino acid exporters in bacteria; however, whereas LysE exports both lysine (Lys) and arginine (Arg) (6, 68), ArgO is an exporter only of Arg (47). Similarly, LysG activates *lysE* transcription in the presence of Arg, Lys, or histidine (His) (6), while ArgP activates *argO* in the presence of Arg but not Lys or His (47); indeed, Lys-bound ArgP actively shuts off *argO* transcription by a mechanism involving RNAP trapping at the *argO* promoter (34), so that *argO* is not transcribed even in the simultaneous presence of both Arg and Lys (34, 47). ArgP also serves to mediate repression by Lys of at least seven other genes in *E. coli* and related bacteria (8, 26, 39, 48, 62). Several gain-of-function dominant *argP* mutations (*argP^d*) have been identified that direct the elevated expression *in vivo* of *argO* and of the other transcriptional target genes (12, 39, 47).

In this study, we have reconstituted LysG regulation of *lysE* in *E. coli*, thereby demonstrating that appropriate and productive interactions can indeed occur between a Gram-positive transcription factor and a heterologous Gram-negative RNAP—that is, between two proteins from bacteria that are proposed to have diverged from a common ancestor more than 2 billion years ago (22). While neither native ArgP nor LysG cross-activated their respective noncognate targets *lysE* and *argO*, several ArgP^d variants were, like LysG, able to activate *lysE* transcription *in vivo*; in

combination, however, ArgP^d and LysG were mutually dominant negative for *lysE* expression under some growth conditions. *In vitro*, the ArgP^d proteins exhibited higher affinity of binding to the *lysE* regulatory region than did native ArgP, and induced less DNA bending at the *argO* and *lysE* loci. Thus, heterologous activation of *C. glutamicum lysE* is possible with certain *E. coli* ArgP variants that apparently share a set of altered characteristics of binding to their regulatory DNA regions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two pairs of isogenic *argP*$^+$ and Δ*argP E. coli* strains that have been described earlier (39) were employed in the study: MC4100 (*argP*$^+$) and GJ9602 (Δ*argP*) and their *ara*$^+$ derivatives GJ9650 and GJ9651, respectively. *E. coli* strain BL21(DE3), used for overexpression of native ArgP and the ArgP^d proteins, has been described previously (66). *C. glutamicum lysE* and *lysG* loci were PCR amplified from strain ATCC 13032, whose genome sequence has been determined (30). Unless otherwise indicated, the growth temperature for bacterial cultures was 37°C and the routine defined and rich growth media were, respectively, glucose minimal A medium and LB medium (43), with amino acids and antibiotics supplemented as necessary at the concentrations previously described (39). L-Arabinose (Ara) supplementation was at 0.2%. Dipeptides with L-alanine (Ala) plus Arg, Lys, and His (Arg-Ala, Lys-Ala, and His-Ala, respectively) were used as culture supplements at 1 mM.

Plasmids. The plasmids used in this study as previously described include the following (with salient features and selection markers given in parentheses): pMU575 (IncW single-copy-number replicon with promoterless *lacZYA* operon; trimethoprim) (2) and its derivative, pHYD1723, carrying an *argO-lac* fusion (34); pBAD18 (pMB9 replicon for Ara-induced expression of target genes; ampicillin) (27); pET21b (pMB9 replicon for T7 RNAP-based overexpression of proteins with C-terminal His$_6$ tag; ampicillin) (Novagen, Darmstadt, Germany) and its derivative bearing the *argP*$^+$ gene (34); and the vector pCL1920 (pSC101 replicon; streptomycin and spectinomycin) and its derivatives bearing *argP*$^+$ or any of the *argP*^{d} alleles, as described previously from this laboratory (39, 47).

The following plasmids were constructed in this study by the cloning of PCR fragments with primer pairs bearing appropriate restriction site sequences (denoted in italics). Plasmid pHYD2676 is a pBAD18 derivative carrying, downstream of P$_{ara}$ of the vector, the *C. glutamicum lysG* open reading frame on a 939-bp EcoRI-HindIII fragment obtained with the primer pair 5′-ACAAG*GAATTC*GGTTCTTAACATGGT-3′ and 5′-ACAA *AAGCTT*GCGAAGAAGTGAAA. Plasmid pHYD2677 is a pMU575 derivative carrying, upstream of the *lacZ* reporter gene of the vector, a 334-bp PstI-BamHI fragment with the *C. glutamicum lysE* regulatory region (from −289 bp to +45 bp with respect to the start site of transcription taken as +1) obtained with the primer pair 5′-TAGTTT*CTGCAG*G CAGCAACAC-3′ and 5′-GTCC*GAT*GGAT*CC*TAAAAGACTGG-3′. Plasmids pHYD2678 and pHYD2680 are derivatives of pET21b for expression of the ArgP^d variants ArgP^d-S94L and -P274S, respectively, the genes for which were obtained on NdeI-XhoI fragments from the corresponding pCL1920 plasmid derivatives with the primer pair 5′-AGCAG ACAACA*CATATG*AAACGCCCGGA-3′ and 5′-ATTATTTGAT*CTCGA* GATCCTGACGAAG-3′. The insert regions of all plasmids described above were verified by DNA sequencing.

Protein methods. C-terminally His$_6$-tagged derivatives of native ArgP and the ArgP^d variants ArgP^d-S94L and -P274S were overexpressed from the corresponding pET21b plasmid derivatives in strain BL21(DE3) and purified as described previously (34, 66), with the modifications that the buffers for imidazole elution and storage contained NaCl at 300 mM instead of 150 mM and the latter also contained glycerol at 40% instead of 20% (Fig. 1). Gel filtration chromatography was performed at room temperature on a BioLogic LP protein purification system (Bio-Rad, Hercules, CA) with an in-house packed Sephadex G-100 column with a size of
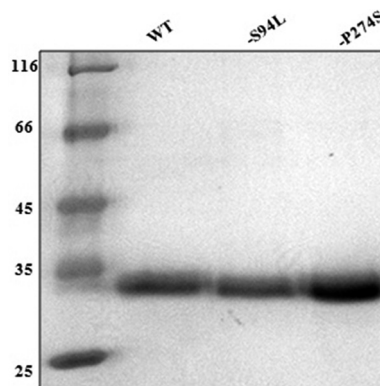
FIG 1 Purified preparations of C-terminally His$_6$-tagged wild-type (WT) ArgP, ArgP^d-S94L, and ArgP^d-P274S proteins demonstrated by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide). The left lane contains protein molecular mass markers of the sizes (kilodaltons) indicated.

1.5 × 43 cm; each protein sample was loaded in a 0.8-ml volume, and the buffer used for chromatography was 20 mM Tris-Cl (pH 8) with 200 mM NaCl at a flow rate of 0.1 ml per min, with 1.5-ml fractions being collected for analysis. Native isoelectric focusing was done at 15°C with precast gels (pH 3 to 9 range) on a Phast Gel apparatus (GE Healthcare, United Kingdom) essentially as described earlier (50).

EMSAs. The DNA templates used in the electrophoretic mobility shift assays (EMSAs) included the 427-bp *argO* fragment from −293 to +109 bp reported earlier (34) and the PCR fragment (359 bp) encompassing the *lysE* regulatory region obtained with the primer pair described in the preceding section. Other DNA templates are listed in Table 1, along with the primer sequences that were used to obtain them by PCR. The protocols for EMSA were described previously (39). For EMSAs to determine DNA bending, coeffector if any was added not to the binding reaction mixture but to both the gel and running buffer at 0.1 mM (1).

Other methods. Procedures for PCR, *in vitro* DNA manipulations, and transformations were performed as described previously (63). β-Galactosidase assays were performed by the method of Miller, and specific activity values are reported as Miller units (43); each value is the average of at least three independent experiments, and the standard error was <10% of the mean in all cases.

RESULTS

Absence of cross-regulation between *E. coli* ArgP-*argO* and *C. glutamicum* LysG-*lysE*. ArgP has previously been shown to activate *argO* in the presence of Arg or citrulline but not in the presence of Lys (34, 39, 47, 55). On the other hand, LysG activates *lysE* expression in the presence of any of the coeffectors Arg, Lys, citrulline, or His (6). The two proteins share 35% identity and 53% similarity, and we wished to determine if they could cross-regulate their noncognate targets *lysE* and *argO*, respectively, in *E. coli*.

For this purpose, a *lysE-lac* fusion and an *in vivo* Ara-inducible LysG expression system were constructed following PCR amplification of the corresponding DNA fragments from *C. glutamicum* genomic DNA (30) and their cloning, as described above. Upon Ara-induced expression of LysG in a Δ*argP* strain, no activation of *argO-lac* was observed in either the absence or presence of coeffectors such as Arg, Lys, or His (Table 2) or their corresponding dipeptides (with Ala) (data not shown). Neither did ectopic LysG expression in an *argP*$^+$ strain interfere with the ability of ArgP to regulate *argO-lac* in the absence or presence of the coeffectors as either free amino acids (Table 2) or dipeptides (data not shown).

TABLE 1 DNA templates used in EMSAs[a]

Template	Length (bp)	Positions	Sequence of:	
			PCR primer 1	PCR primer 2
argO (D)	335	−305–+30	GTGCGCCTGAACGAACTTGGTG	CACGTTGGATATTCCGAATT
argO (M)	333	−212–+121	CTGGAGCGTATTAAACGTGA	GTATGCCCTGATTCATCACAAAG
argO (U)	332	−105–+227	CGCTGAGGCCAGATAATACT	CACGGCGACTGCATCAATAA
lysE (D)	289	−268–+21	CTGCTTGCACAAGGACTTCACC	ACCTGTAATGAAGATTTCCAT
lysE (M)	289	−188–+101	TCGAGAGCTTTAACGCGCTGAC	CCTTCGCGCTTAATTCCTTGTT
lysE (U)	289	−88–+201	CCAGTTGAATGGGGTTCATGA	CACGATCGGCGCGGCATTGGAC

[a] The parenthetical qualifications D, M, and U for the *argO* and *lysE* templates indicate that the LTTR-binding site is located, respectively, toward the downstream end, the middle, and the upstream end of the corresponding DNA fragments. The LTTR-binding site on *argO* has been experimentally determined (34), while that on *lysE* had been predicted earlier (6) by alignment with consensus sequence motifs.

Similarly, the *lysE-lac* fusion directed a low level of β-galactosidase expression that was not different between *argP*+ and Δ*argP* strains in all media tested (data not shown) (see Tables 4 and 5), indicating that native ArgP cannot also cross-activate *lysE*.

Reconstitution of *C. glutamicum* LysG-*lysE* regulation in *E. coli*. We then asked whether LysG could activate *lysE* in *E. coli* and found this to be the case (Table 3). In a Δ*argP* strain with Ara-induced LysG, *lysE-lac* expression was significantly upregulated in the presence of Arg, Lys, or His, in comparison with the level of expression observed in the absence of any coeffector.

For Lys and His, activation was observed upon their supplementation either as free amino acids or as dipeptides (with Ala), in a defined medium both without and with fortification by the other 17 amino acids. For Arg, however, it was only the dipeptide and not the free amino acid that activated *lysE* expression. Although the possibility exists that it is the Arg-containing dipeptide that is the coeffector for LysG-mediated activation of *lysE*, we believe it more likely, for the following reasons, that Arg is indeed the coeffector and that uptake of exogenously provided Arg is not as efficient as that of the dipeptide in generating the intracellular concentration of amino acid needed for *lysE* induction for the following reasons. (i) It is known that the *E. coli* Arg uptake systems are feedback repressed in the presence of Arg (11). (ii) Since the data indicate that the effects of Lys-Ala and His-Ala on *lysE* are mediated by the free amino acids Lys and His, respectively, it is reasonable to assume that the Arg-Ala effect is also mediated by Arg. (iii) LysG's ortholog, ArgP, possesses a single binding pocket for competitive binding of the coeffectors Arg and Lys (34, 73). (iv) Finally, ArgP-mediated *argO-lac* induction was also 4-fold higher with Arg-Ala than it was with Arg (data not shown).

Thus, our results recapitulate, at least qualitatively, the regulation in *C. glutamicum* itself of *lysE* by LysG (6).

Some *E. coli* ArgP[d] proteins can activate *C. glutamicum* *lysE* in vivo. Several gain-of-function single-amino-acid substitutions in ArgP are known that mediate high and constitutive expression of *argO* in *E. coli* (39, 47), and we tested seven such ArgP[d] variants for their ability to cross-regulate *lysE*. While four of them (ArgP[d]-A68V, -V144M, -P217L, and -R295C) were unable to activate *lysE-lac* in *E. coli*, three (ArgP[d]-S94L, -P108S, and -P274S) were able to do so to different extents (Table 4). ArgP[d]-P274S and -S94L were the most effective for *lysE* activation, which in both cases was independent of coeffector addition and comparable to the maximal activation obtained with LysG itself upon coeffector supplementation.

Mutual antagonism between LysG and ArgP[d] variants for *lysE* activation. Since the typical LTTR is dimeric in solution and assembles on DNA as a dimer of dimers or as higher-order oligomers to activate transcription (38, 45), we tested the effects of the combined presence of LysG and ArgP or its variants on *lysE* regulation in *E. coli*. Activation of *lysE* transcription in the presence of LysG and its coeffectors Arg, Lys, or His (added as their respective dipeptides with Ala) was unaffected by native ArgP (i.e., in the *argP*+ strain). Interestingly, however, strains in which LysG was coexpressed with any one of the three *lysE* activation-proficient ArgP[d] variants (ArgP[d]-S94L, -P274S, or -P108S) displayed significantly lower *lysE* expression than those with only one of the

TABLE 2 Absence of LysG effect on *argO-lac* expression[a]

	β-Galactosidase sp act (Miller units)			
	Δ*argP*		*argP*+	
Supplement	−LysG	+LysG	−LysG	+LysG
None	25	29	29	33
Lys	30	27	36	14
His	26	30	28	20
Arg	24	30	150	174
Citrulline	28	27	135	198

[a] Derivatives of strains GJ9650 (*argP*+) and GJ9651 (Δ*argP*) carrying (i) the *argO-lac* fusion plasmid pHYD1723 and (ii) either plasmid vector pBAD18 (−LysG) or its derivative pHYD2676 bearing P*ara*-*lysG* (+LysG) were grown in minimal A medium with 0.2% each glycerol and Ara and without (None) or with Arg, Lys, His, or citrulline supplementation at 1 mM, as indicated, for β-galactosidase assays.

TABLE 3 *lysE-lac* activation by LysG in *E. coli*[a]

	β-Galactosidase sp act (Miller units)			
	MM		MM + 17 aa	
Supplement	Free	Dipeptide (with Ala)	Free	Dipeptide (with Ala)
None	22	18	16	18
Lys	110	95	98	85
His	95	89	87	95
Arg	22	111	24	105
Citrulline	21	ND[b]	20	ND

[a] A derivative of strain GJ9651 with plasmids pHYD2676 and pHYD2677 bearing P*ara*-*lysG* and *lysE-lac*, respectively, was grown for β-galactosidase assays in minimal A medium with 0.2% each glycerol and Ara and the following two categories of supplements as indicated: (i) without (MM) or with (MM + 17 aa) addition of a mixture of 17 amino acids other than Arg, Lys, or His; and (ii) without (None) or with addition of 1 mM Lys, His, Arg or citrulline either as free amino acid or as a dipeptide with Ala (for all but citrulline; i.e., Lys-Ala, His-Ala, or Arg-Ala).
[b] ND, not determined.

TABLE 4 *lysE-lac* activation by certain ArgP[d] variants[a]

	β-Galactosidase sp act (Miller units)		
argP genotype	None	Arg	Lys
Δ*argP*	15	15	14
argP[+]	15	19	18
argP[d]-A68V	25	24	19
argP[d]-S94L	84	85	67
argP[d]-P108S	35	36	18
argP[d]-V144M	15	16	14
argP[d]-P217L	16	19	14
argP[d]-P274S	126	138	118
argP[d]-R295C	11	12	14

[a] Derivatives of the Δ*argP* strain (GJ9602) carrying (i) the *lysE-lac* plasmid pHYD2677 and (ii) plasmid vector pCL1920 (Δ*argP*) or its derivatives with *argP*[+] or the different ArgP[d]-encoding variants as indicated, were grown for β-galactosidase assays in glucose-minimal medium A without (None) or with 1 mM Arg or Lys supplementation.

LTTRs present, indicative of a mutual antagonism or reciprocal dominant-negative effect (Table 5). For example, such mutual interference was observed for LysG with either ArgP[d]-S94L or ArgP[d]-P274S in cultures supplemented with either Arg-Ala or His-Ala, and LysG was also dominant negative over all three ArgP[d] variants in cultures without coeffector supplementation. On the other hand, in cultures supplemented with Lys-Ala, *lysE* expression in the combined presence of LysG and ArgP[d] was similar to that with either LTTR alone, and no antagonistic regulation was observed.

When the free amino acids were used (Table 5), His behaved like His-Ala in provoking the mutually antagonistic effect, and likewise Lys behaved like Lys-Ala in activating *lysE-lac* in the presence of both LTTRs. With Arg supplementation, the results were indistinguishable from those obtained in the unsupplemented cultures, which is consistent with the data above that exogenously provided Arg does not activate *lysE* in the presence of LysG.

ArgP[d] proteins differ from native ArgP in their binding to *argO* and *lysE* in vitro. Earlier studies (26, 34, 39, 62) have indicated that the classical model of transcription factor binding to a regulatory DNA site followed by RNAP recruitment to the promoter (58) explains the process of ArgP activation of its target genes in *E. coli*. Lys-mediated repression of the ArgP-regulated genes occurs either by RNAP trapping at the promoter in the case of *argO* (34) or by reduced binding of the protein to its operator sites in the case of the other targets (8, 26, 39).

To determine if the increased activation at *argO* and *lysE* by the

ArgP[d] variants ArgP[d]-S94L and -P274S (relative to that by native ArgP) was due to alterations in either the affinity or pattern of binding of the proteins to the corresponding regulatory regions, we performed EMSAs with the purified (His$_6$-tagged) proteins and upstream regulatory regions of *lysE* or *argO*. Attempts to use purified LysG as well in these experiments were unsuccessful, since the protein could not be recovered in active form from insoluble inclusion bodies following its overexpression (data not shown).

At *argO*, the K_ds (dissociation constants) of binding of ArgP and its variants ArgP[d]-S94L and -P274S were not significantly different from one another (around 10 nM in all cases); however, significant differences were observed in migration rates of the DNA-protein complexes, in decreasing order of ArgP[d]-P274S, ArgP[d]-S94L, and native ArgP (Fig. 2A). Neither the K_d of binding nor the migration rate of the DNA-protein complexes at *argO* was affected by Arg or Lys addition (data not shown).

At *lysE*, in contrast, native ArgP and the ArgP[d] variants showed significant differences in their binding affinities, whereas native ArgP displayed negligible binding even at 300 nM, the ArgP[d]-S94L and -P274S variants exhibited K_ds of ~300 nM and <60 nM, respectively (Fig. 2B). As with *argO*, the complexes of *lysE* DNA with ArgP[d] variants also migrated faster than the complex of *lysE* with wild-type (WT) ArgP. The EMSA features described above were unaltered by Arg or Lys supplementation (data not shown).

Differences in DNA bending induced by native ArgP and ArgP[d] proteins at *argO* and *lysE*. In the case of other LTTRs, differences in protein pIs (35) or in degree of DNA bending upon protein binding (1, 15, 18, 31, 33, 40, 52, 69) had earlier been invoked to explain differences in migration rates of the protein-DNA complexes in EMSA experiments. Since native ArgP is a homodimer (4, 34, 73), we also considered a third possibility—that the ArgP[d] proteins may function as monomers to therefore exhibit faster migration after binding to DNA. Results from gel filtration chromatography experiments indicated, however, that native ArgP and the ArgP[d] variants are all of the same size within error, that is, there is no difference in their oligomeric status in solution (Fig. 3A). Similarly, isoelectric focusing experiments indicated that the pI of the ArgP[d] proteins (~6.6) was not lower than that of native ArgP itself (~6.3) to explain the faster migration of DNA-bound complexes of the former under the buffer conditions (pH 8.3) of the EMSAs (Fig. 3B).

We then examined the role of DNA bending in explaining differences in EMSA migration rates between ArgP[d] and native ArgP,

TABLE 5 Antagonism between ArgP[d] and LysG for *lysE-lac* activation[a]

	β-Galactosidase sp act (Miller units)													
	−LysG							+LysG						
argP genotype	None	Arg	Arg-Ala	Lys	Lys-Ala	His	His-Ala	None	Arg	Arg-Ala	Lys	Lys-Ala	His	His-Ala
Δ*argP*	25	27	27	24	24	29	24	21	24	155	100	115	96	116
argP[+]	25	28	22	27	23	28	21	19	25	118	106	131	108	111
argP[d]-S94L	95	111	97	106	109	120	93	**21**	**21**	***19***	91	110	**55**	**56**
argP[d]-P274S	181	162	185	188	175	174	179	**23**	**23**	***20***	96	103	**45**	**43**
argP[d]-P108S	45	51	40	31	20	46	39	**19**	**23**	106	114	104	111	105

[a] Derivatives of the Δ*argP* strain GJ9651 carrying three plasmids, namely, (i) pHYD2677 (*lysE-lac*), (ii) vector pBAD18 (−LysG) or its derivative pHYD2676 with P$_{ara}$-*lysG* (+LysG), and (iii) vector pCL1920 (Δ*argP*) or its derivatives with *argP*[+] (WT) or ArgP[d]-encoding variants as indicated, were grown for β-galactosidase assays in minimal A medium with 0.2% each glycerol and Ara, and without (None) or with amino acid or dipeptide supplementation as indicated. Values indicative of a dominant-negative effect of LysG are in boldface, and those indicative of mutual dominant negativity of LysG and ArgP[d] are in italic boldface.
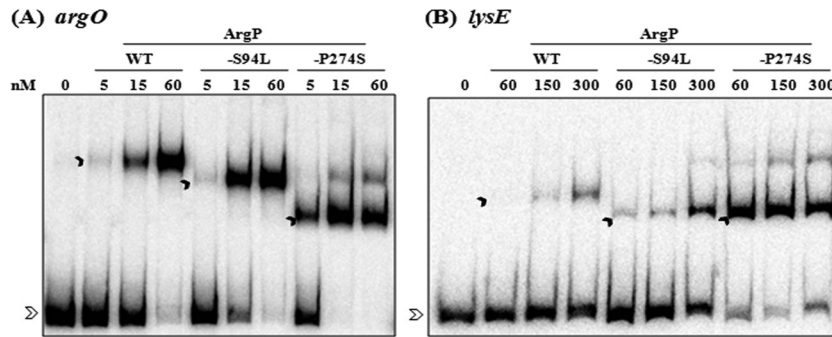
FIG 2 EMSAs with native ArgP (WT) or its variants ArgP^d-S94L and -P274S at the indicated protein concentrations and *cis* regulatory regions of *argO* (427-bp fragment) (A) and *lysE* (359-bp fragment) (B), in the absence of any coeffector. Bands corresponding to free DNA and to DNA in a binary complex with each of the ArgP proteins are marked by open and filled arrowheads, respectively.

since faster migration with ArgP^d proteins was consistent with data from other LTTRs that less bending of target DNA upon protein binding *in vitro* (leading to faster migration in EMSA) is correlated with promoter activation *in vivo* (1, 15, 18, 31, 33, 40, 52, 69).

The degree of electrophoretic mobility retardation of a bent DNA fragment depends on the position of the bend with respect to the fragment ends, so that retardation is most pronounced when the bend is located near the center of the fragment (69, 72). Accordingly, we determined the migration rates of *argO*-DNA complexes using three fragments of nearly identical sizes but in which the −80 to −20 *argO* segment (which is the region footprinted by ArgP) was located near the upstream end, middle, or downstream end of the fragment. Although the fragment sequences were not permuted with respect to one another, it was reasoned that the different neighboring sequences in the three fragments would not affect their mobility under the experimental conditions.

The results from these experiments indicated the following. (i) In the absence of any coeffector, the differences in migration noted earlier were reproduced for the standard *argO* fragment (with the ArgP binding site at middle) in complexes with the different ArgP proteins, so that the complexes with native ArgP and ArgP^d-P274S migrated the slowest and fastest, respectively (Fig. 4A; compare the circled bands in the different lanes labeled M). (ii) These migration differences between the different ArgP proteins were largely abolished for *argO* fragments in which the pro-

tein binding sites were located near the upstream or downstream ends, so that all complexes now migrated at the higher rate (Fig. 4A, see lanes labeled U and D, respectively). These data therefore strongly suggest that *argO* DNA is bent upon native ArgP binding and that there is considerably less bending in the case of binding by the two ArgP^d variant proteins. When the data obtained in the absence of a coeffector (Fig. 4A) were compared with those in the presence of Arg (Fig. 4B) or Lys (Fig. 4C), one significant finding was that there was a speeding up of the *argO* complex with the ArgP^d-S94L variant but not that with native ArgP, suggesting that the bend induced only by the former is reversed upon Arg addition (at least at the concentration used in the experiments).

When similar experiments were undertaken with binding by the different ArgP proteins to *lysE* fragments carrying the putative binding sites at three different locations relative to the fragment ends, it was once again observed (as for *argO*) that native ArgP binding to *lysE* is associated with more pronounced DNA bending than is binding of either of the ArgP^d variants (Fig. 5, compare lane M for native ArgP with all other lanes).

DISCUSSION

The major findings of this study were that (i) *C. glutamicum* LysG can activate its cognate target *lysE* in the heterologous milieu of *E. coli*; (ii) some ArgP^d variants of *E. coli* can activate *C. glutamicum lysE*, whereas native LysG and ArgP do not cross-regulate their noncognate targets (*argO* and *lysE*, respectively) *in vivo*; and (iii)
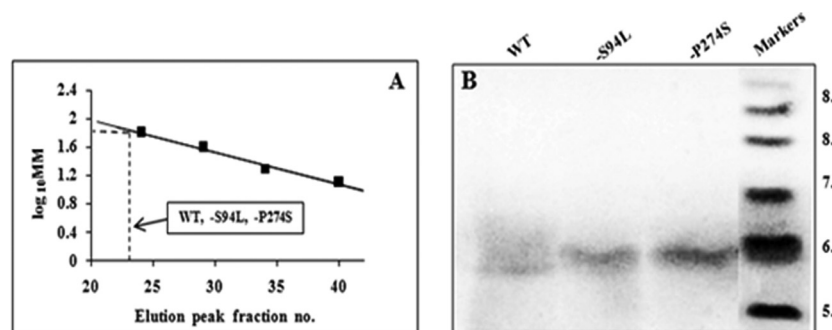
FIG 3 Subunit composition and pIs of native ArgP (WT) and its -S94L and -P274S variants. (A) Plot of $\log_{10}$ molecular masses (MM) in kilodaltons of protein standards (with solid squares denoting in descending order bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase A) against the fraction numbers representing their peaks of elution. The dashed lines show the fraction number representing the elution peak for each of the three ArgP proteins (WT or the ArgP^d-S94L and -P274S variants) and the corresponding $\log_{10}$ molecular mass value as intercepts of the *x* and *y* axes, respectively. (B) Native isoelectric focusing of the three proteins. The lane to the right depicts protein markers of the pIs indicated.
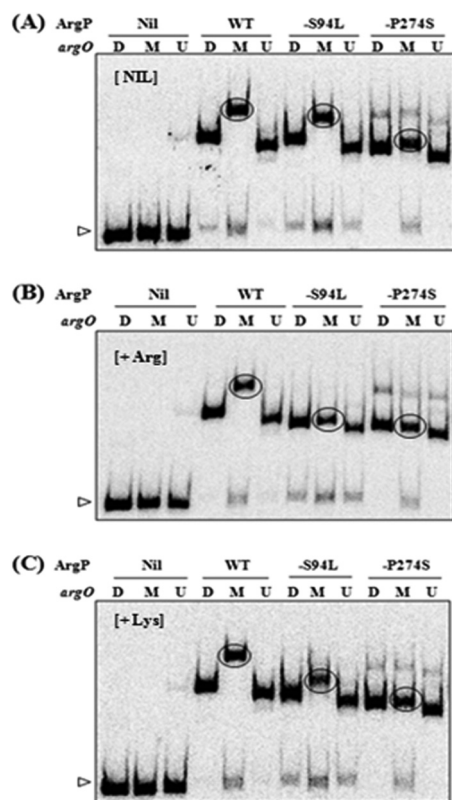
FIG 4 EMSAs with native ArgP (WT) or its variants ArgP^d-S94L and -P274S at 100 nM and the *argO* templates D, M, and U (with the LTTR-binding site at the downstream end, middle, and upstream end of the fragments, respectively, as described in Table 1). The experiments were done in the absence (A) or presence of the coeffector Arg (B) or Lys (C) at 0.1 mM. Nil, no supplementation. The open arrowhead in each panel denotes the unbound DNA probe, and bands corresponding to binary complexes of protein with template M are circled.

FIG 5 EMSAs with native ArgP (WT) or its variants ArgP^d-S94L and -P274S at 400 nM and the *lysE* templates D, M, and U (with the LTTR binding site at the downstream end, middle, and upstream end of the fragments, respectively, as described in Table 1), done in the absence of coeffectors. Nil, no supplementation. The open arrowhead denotes the unbound DNA probe, and bands corresponding to binary complexes of protein with template M are circled.

scriptional activation requires high-fidelity interactions across macromolecular interfaces, our results imply that such contacts (between LysG and RNAP) have been conserved across a vast evolutionary distance.

An alternative explanation would be that the genes coding for ArgP and LysG are not orthologs that have diverged from a single gene in the ancient common ancestor, but instead represent an example of horizontal gene transfer (HGT) that occurred more recently in evolution. One way to distinguish between these possibilities is to compare an organism's overall GC content with that of the gene in question, since it is a discordance between the two that is often taken as a signature of HGT (25). In the present case, we note that the GC content of the gene encoding the closest relative of *E. coli* ArgP in each of seven different bacteria more or less matches that for the entire genome of the corresponding bacterium (Table 6), which suggests that the genes are indeed orthologs rather than outcomes of HGT.

A few other instances have been reported of conserved interactions between Gram-positive and Gram-negative bacteria, which are manifested as successful cross-complementation of genes whose products (proteins or RNA) function within macromolecular complexes; the examples include F$_o$F$_1$ ATPase between *Bacillus megaterium* and *E. coli* (64), protein complexes involving YidC between *Streptococcus mutans* and *E. coli* (19) or Era/Bex

binding of the ArgP^d variant proteins to the regulatory regions of *argO* or *lysE* is associated with a reduced angle of DNA bending compared to that obtained with native ArgP. Each of these is further discussed below.

Reconstitution of *C. glutamicum* LysG-*lysE* regulation in *E. coli*. In the classical model for gene activation (58), transcription factor binding to a target gene regulatory region is followed by RNAP recruitment to enable productive transcription. The ability of *C. glutamicum* LysG to activate its cognate target, *lysE*, in *E. coli* in the presence of Lys, His, or Arg is remarkable since it indicates not only that *E. coli* RNAP recognizes the *C. glutamicum lysE* promoter for initiating transcription, but more importantly that a Gram-positive transcription factor is successful in recruiting the Gram-negative enzyme to make appropriate promoter contacts for this purpose. The LTTRs CatR and OccR from, respectively, *Pseudomonas putida* and *Agrobacterium tumefaciens*, had earlier been shown to activate transcription with *E. coli* RNAP *in vitro*— that is, within the Gram-negative kingdom itself (40, 69).

It is known that many *C. glutamicum* promoters are correctly recognized in *E. coli* (54), but there had been no report earlier of *in vivo* transcriptional activation across the divide between Gram-positive and Gram-negative bacteria, which some estimates indicate was established about 2 billion years ago (22, 51). Since tran-
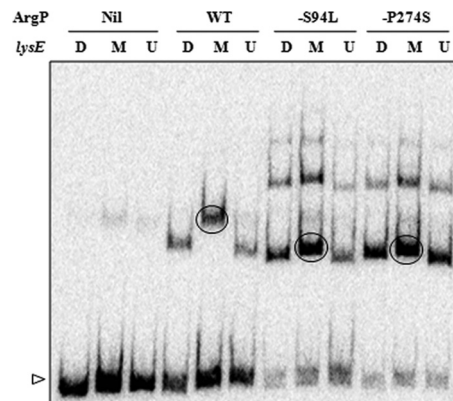
TABLE 6 Percentages of GC content of genomes and *argP* orthologs of different bacteria

Organism	Ortholog identification no.[a]	% GC content for:	
		Gene	Genome
Escherichia coli	NP_417391.1	54.4	50.8
Corynebacterium glutamicum	EGV41297.1	55.2	53.8
Mycobacterium tuberculosis	NP_216501.1	65.0	65.6
Streptomyces coelicolor	NP_631362.1	75.8	72.0
Klebsiella pneumoniae	YP_002236622.1	59.7	56.9
Haemophilus influenzae	ZP_01783604.1	33.4	38.0
Pseudomonas aeruginosa	YP_001350270.1	71.5	64.4

[a] As listed in the NCBI protein database.

between *Bacillus subtilis* and *E. coli* (44), and the protein-RNA components of RNase P between *B. subtilis* and *E. coli* (70). *In vitro* reconstitution of the 30S ribosome complex with protein subunits and rRNA intermixed between *E. coli* and *Bacillus stearothermophilus* has also been described (28, 49). However, several proteins of the *E. coli* and *B. subtilis* divisome complexes do not productively interact with one another, although they are orthologous (59), nor is the SecA homolog from either *B. subtilis* (42) or *Streptomyces lividans* (7) able to participate in protein translocation in *E. coli*.

Cross-regulation between ArgP-*argO* and LysG-*lysE*. Several examples of successful cross-regulation between different transcription factors have been described earlier, including those between BenR-XylS of the AraC family (16, 29), XylR-DmpR of the NtrC family (23), and CatR-ClcR and TfdR-TfdT-TcbR-ClcR of the LTTR family (35, 52, 53) of proteins. In all of these cases, the target genes are involved in catabolism of aromatic compounds in *Pseudomonas* species or related Gram-negative bacteria and are most often plasmid borne; hence, it is unclear whether the different regulator proteins are paralogs or merely orthologs that have been reunited through HGT (25). Among other LTTRs, limited or no cross-regulation is observed between NocR and OccR involved in the catabolism of nopaline and octopine, respectively, in *A. tumefaciens* (31); however, overexpressed GcvA (which regulates glycine cleavage) of *E. coli* is able apparently to partially substitute for *Citrobacter freundii* AmpR in regulation of the *ampC* gene encoding β-lactamase (21).

In this study, we observed no cross-regulation between the native ArgP and LysG proteins for their respective noncognate targets *lysE* and *argO*. For the LysG-*argO* combination, the failure to activate is not because of an inability of LysG to recruit *E. coli* RNAP, given that LysG activates *lysE* in *E. coli* (see above). Hence, we suggest that this failure reflects a decreased binding affinity of LysG to the *argO* regulatory region. Likewise, ArgP's inability to cross-regulate *lysE* is at least in part explained by the high K_d of protein binding to *lysE*, since the two ArgP^d variants (ArgP^d-P274S and -S94L) that were most effective for activation also exhibited improved binding affinities for *lysE* (concomitantly with decreased DNA bending; see below). The fact that neither native protein exerts a dominant-negative effect on the other's activating ability in *E. coli* (that is, LysG on *lysE* and ArgP on *argO*) would also suggest that the inability to cross-regulate is likely due to a defect in an early rather than late step in the process.

The ArgP^d single-amino-acid substitution variants used here had originally been selected to confer elevated *argO* expression (12, 39, 47); our finding that some of them have also become proficient for cross-regulation of *lysE in vivo* serves to strengthen the notion that ArgP-*argO* and LysG-*lysE* are indeed orthologous transcription factor-target pairs that are both functionally and evolutionarily related. The two ArgP^d derivatives that were most effective for *lysE* activation (ArgP^d-P274S and -S94L) are also those that conferred the highest constitutive levels of *argO* expression, but whether this is just a coincidence remains to be determined. Furthermore, an important distinction between native ArgP and LysG is that the former fails to activate its target in the presence of Lys, suggesting that the P274S and S94L ArgP^d variants are more akin to LysG in this regard.

The crystal structure of *Mycobacterium tuberculosis* ArgP has been determined (73) and may be described, by comparison with the structure of the LTTR CbnR of *Ralstonia eutropha* (46), as

comprising an N-terminal DNA-binding domain (DBD) followed sequentially by a linker helix and C-terminal regulatory domains I and II (RD-I and -II, respectively)—with hinge regions 1, 2, and 3 located between (i) DBD and the linker helix, (ii) the linker helix and RD-I, and (iii) RD-I and RD-II, respectively. Upon mapping of the seven ArgP^d substitutions (which render *E. coli* constitutive for *argO* expression) on the ArgP structure, we noted that none is in the DBD and that it is only the S94L and P274S substitutions that map to hinge regions 2 and 3, respectively. These hinge regions, therefore, appear to be crucial in conferring the flexibility needed for a single LTTR to recognize both the *argO* and *lysE* transcriptional targets. An alignment of some constitutive mutations of other LTTRs, such as NodD (10, 41), AmpR (5), AphB (67), and OxyR (32), indicates that they are also located in the corresponding hinge 2 regions (data not shown).

Mutual dominant negativity between LysG and ArgP^d variants for *lysE* regulation. The data from experiments examining the effects of the combined presence of LysG and ArgP^d variants on *lysE* expression *in vivo* indicate that both LysG on the one hand and ArgP^d-S94L or -P274S on the other mutually antagonize each other's ability to activate *lysE* in the Arg- or His-supplemented cultures. Furthermore, LysG prevents *lysE* activation by the ArgP^d proteins in media not supplemented with any coeffector. Our findings suggest that mixed oligomers between ArgP^d and LysG are being generated under these conditions, at the level either of the dimer itself or of the dimer of dimers upon DNA binding, which are inactive for appropriate RNAP recruitment and gene activation. The implication is that the homo-oligomers of LysG and ArgP^d themselves activate *lysE* by subtly different mechanisms that cannot be successfully integrated in the mixed oligomers.

In cultures with Lys supplementation, however, *lysE* activation was more or less the same when LysG and the ArgP^d variants were present either alone or together, indicating that in this case, gene regulation is mediated by a common or shared mechanism; an alternative explanation is that the LysG and ArgP^d protomers in their Lys-bound states exclude one another and assemble only as homo-oligomers both in solution and on DNA. Thus, although LysG mediates activation by all three amino acids of *lysE* expression, it appears to do so by at least two different mechanisms that can be distinguished by their interference or otherwise by the ArgP^d proteins.

Target DNA bending upon binding by ArgP and its variants. Promoter DNA bending can regulate transcription (56), and a common theme to emerge from studies with different LTTRs is that the target DNA undergoes bending upon protein binding. For many LTTRs, including CysB (15), OxyR (33), OccR (1, 31, 69), NocR (31), CatR (52), ClcR (40), and CbbR (18), DNA bending by the protein is more pronounced in the absence of the activating coeffector than in its presence, leading to the suggestion that bending is inversely correlated with gene activation. However, in a few cases, such as MetR (37), DntR (65), and TrpI (57), DNA bending that occurs upon protein binding is unaffected by coeffector addition, and for NodD (14), the bending is in fact increased in the presence of the coeffector. In the cases of CysB (15), TrpI (13), and MetR (37), the affinity of factor binding to target DNA is also increased upon coeffector addition, but this is not true for many of the other proteins. (The binding affinity is indeed reported to be reduced for OccR [69] and CbbR [18] under these conditions.)

Studies with LTTR mutants such as those for OxyR (33), CysB

(15, 36), OccR (1), and CbbR (18) indicate that constitutive activation can be achieved by different mechanisms even for a single protein-target pair. Some variants confer less DNA bending than their native counterparts, but others confer unchanged or even increased bending. Determinations of the structures of constitutive mutants of BenM (17, 61), CatM (17), and AphB (67) have also lent support to the idea of multiple mechanisms underlying constitutive activation. In some cases, such as OxyR, improved RNAP recruitment and a role for protein conformational changes following its binding to DNA have also been suggested to distinguish the constitutive mutants from the corresponding wild-type proteins (33). Binding to DNA can also dictate changes in LTTR oligomerization leading to gene activation, as has been demonstrated for some mutants of the NAC protein (60).

In this context, our studies indicate that native ArgP binding induces DNA bending at both *argO* and *lysE* and that the two active ArgP^d variants tested confer relaxation of bending (relative to native ArgP) at these target loci. Thus, our results are broadly consistent with those for other LTTRs on DNA bending induced by native protein binding and its alteration with substitutions conferring constitutivity. Bending by native ArgP at *argO* is unaffected by the activating coeffector Arg, which suggests that the coeffector acts by mechanisms other than (or in addition to) that related to ArgP-induced DNA bending. At *lysE*, the ArgP^d variants also show increased affinity of binding that is roughly proportionate to the magnitude by which they activate expression *in vivo*, suggestive of an induced-fit mechanism for protein-DNA binding.

The unanswered questions. Although LTTRs represent the largest family of bacterial transcription factors, our understanding of their mechanism of function continues to remain limited (38, 45). Structural studies of LTTRs have faced several technical impediments, and the few structures that have been determined suggest that there is no common theme in their mode of regulation (45). A similar conclusion is suggested also by analysis of constitutive variants of different LTTRs, as discussed above. The persisting uncertainties relate, among others, to the mechanisms by which coeffectors modulate LTTR function, the role in transcription activation of target DNA bending induced by LTTR binding, and the process of RNAP recruitment to the promoter by the LTTR.

The majority of LTTRs regulate just one or a very small number of operons, whereas ArgP is unusual in that it mediates, through more than one mechanism, Lys repression of at least eight transcription units in *E. coli* (8, 26, 39, 47, 62). At many of these loci, ArgP likely also interacts with other factors in mediating regulation (55, 62). Furthermore, ArgP is noncanonical in that it also exhibits specific binding to several DNA sites without an ostensible transcriptional regulatory role (4, 39), to the extent that it has also been referred to as a protein of the nucleoid IciA (4); however, the features distinguishing its regulatory and nonregulatory DNA binding roles are not known. It is possible that the two dimer interfaces on ArgP (73) engage in sequential alternating interactions to assemble as a polymeric scaffold, analogous to that which has been described for another nucleoid protein, H-NS (3); a similar infinite polymer arrangement has also been deduced for the LTTR BenM from structural studies (61). Thus, in the context of the present work, the different mechanisms of *argO* activation by the different ArgP^d variants, as well as the reason why only a subset of the ArgP^d variants are proficient for *C. glutamicum lysE* activation, remain to be determined.

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