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Two Genes for Carbohydrate Catabolism Are Divergently Transcribed from a Region of DNA Containing the *hexC* Locus in *Pseudomonas aeruginosa* PAO1

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The *hexC* locus of *Pseudomonas aeruginosa* PAO1 was localized to a 247-bp segment of chromosomal DNA on the multicopy broad-host-range vector pRO1614. The presence of this plasmid (pPZ196) in strain PAO1 produced the so-called "*hexC* effect," a two- to ninefold increase in the activities of four carbohydrate catabolism enzymes, glucokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase. The extent of the *hexC* effect was restricted, since three independently regulated metabolic enzymes were not affected by the presence of the *hexC* plasmid. Furthermore, the *hexC*-containing plasmid did not suppress catabolite repression control. Nucleotide sequence analysis of the segment of DNA encompassing *hexC* revealed a 128-bp region rich in adenosine-plus-thymine (AT) content separating two divergent open reading frames (ORFs). Transcriptional start sites for these two genes were mapped to the intergenic region, demonstrating that this sequence contained overlapping divergent promoters. The intergenic region contained potential regulatory sequences such as dyad symmetry motifs, polydeoxyadenosine tracts, and a sequence matching the integration host factor recognition site in *Escherichia coli*. One of the ORFs encoded a 610-amino-acid protein with 55 to 60% identity to 6-phosphogluconate dehydratase from *E. coli* and *Zymomonas mobilis*. The second ORF coded for a protein of 335 amino acids that displayed 45 to 60% identity to the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAP) family of enzymes. The NAD-dependent GAP gene on the *P. aeruginosa* chromosome was previously unmapped. GAP was found to exhibit the *hexC*-dependent increase in its basal activity, establishing it as a fifth catabolic enzyme in the multioperonic *hex* regulon.

Pseudomonads utilize a number of alternative pathways for the catabolism of carbohydrates to carbon and energy (reviewed in reference 37). In *Pseudomonas aeruginosa*, the coordinately regulated enzymes glucokinase (GLK), glucose-6-phosphate dehydrogenase (ZWF), and the Entner-Doudoroff pathway enzymes 6-phosphogluconate dehydratase (EDD) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EDA) play a central role in the dissimilation of glucose to pyruvate and glyceraldehyde-3-phosphate (Fig. 1). These enzymes are coinduced by growth of *P. aeruginosa* on such diverse carbohydrates as glucose, gluconate, mannitol, and glycerol (33, 37). Also, their expression is subject to catabolite repression control (CRC) by succinate or other intermediates of the tricarboxylic acid (TCA) cycle (40, 58).

The genes encoding these four enzymes (*glk*, *zwf*, *edd*, and *eda*) were mapped by transductional analysis to 39 min on the PAO1 chromosome (14, 30, 47). Cloning and complementation analyses revealed, however, that the four genes were expressed from separate promoters (15, 57), indicating that the genes were not contained in a single operon. Two regulatory effects further defined this group of genes. The first was the *hexR* mutation, thus far inseparable from *edd*, which caused loss of inducibility of *glk*, *zwf*, *edd*, and *eda* (15, 47, 57). The second was a locus designated *hexC* (15), also closely linked to *edd*, revealed by its presence on a multicopy plasmid (pPZ190) which did not complement mutations in *glk*, *zwf*, *edd*, and *eda* (57). Wild-type strain PAO1 containing pPZ190 exhibited the so-called "*hexC* effect," a two- to ninefold increase in activities of GLK, ZWF, EDD, and EDA in extracts from cells grown without inducer (57). Taken together, these genetic and physiologic data indicated that *glk*, *zwf*, *edd*, and *eda* constituted a multioperonic regulatory unit, which was designated the *hex* regulon (50).

In experiments described here, *hexC* was further localized to a 247-bp *Xho*I-*Xma*III DNA fragment encompassing a 128-bp region positioned between the 5′ ends of two divergent open reading frames (ORFs). The ORFs were identified as the gene for EDD and the previously unmapped gene (*gap*) for NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAP). Also, coregulation of GAP with the other enzymes of the *hex* regulon was established. Finally, the transcriptional start sites for *gap* and *edd* were mapped to the intergenic sequence, demonstrating that this region contained divergent promoters for *gap* and *edd*, and potential regulatory sequences within the sequence were identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* PAO1 was used throughout this study for measurement of enzyme activities. Plasmid pRO1817 (15) was constructed by insertion of a 6.0-kb *Eco*RI chromosomal fragment of *P. aeruginosa* PAO1 into pRO1614 (44). This plasmid complemented mutants deficient in GLK, EDD, and glucose transport activity. Plasmid

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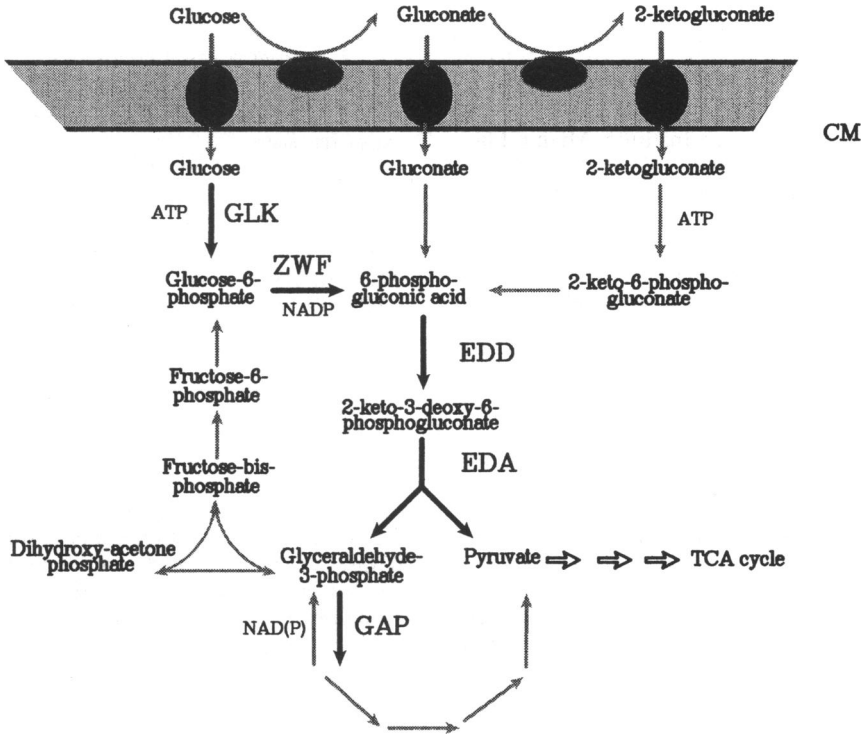


FIG. 1. Routes of glucose and gluconate metabolism in *P. aeruginosa*. Steps mediated by enzymes of the *hex* regulon are represented by the larger dark arrows. CM, cytoplasmic membrane.

pRO1836 (57) was constructed from pRO1817 by deletion of a *Sal*I fragment from pRO1817 (Fig. 2). Plasmid pPZ196 (2.9 kb) was derived from pRO1836 and contained a 247-bp *Xho*I-*Xma*III fragment of DNA (Fig. 2). Plasmid pPZ421 contained the same 6.0-kb *Eco*RI fragment as pRO1817 inserted into pPZ375, a broad-host-range vector constructed by insertion of the *oriV* of pRP1 (44) into pGEM-3Z(+) (Promega Corp., Madison, Wis.). Plasmid pPZ432 (5.4 kb) was constructed by insertion of an *Nco*I-*Xho*I fragment from pPZ421 into pPZ375. When possible, plasmids were maintained in *Escherichia coli* DH5α. *E. coli* and *P. aeruginosa* strains containing plasmids were maintained on media containing ampicillin (100 μg/ml) and carbenicillin (500 μg/ml), respectively.

Cell growth, extract preparation, and enzyme assays. Basal salts medium (BSM), growth conditions, and extract preparation have been described previously (33). For cell extracts, bacteria were disrupted either by sonication or by use of a French pressure cell.

Activities of the following enzymes were assayed spectro-

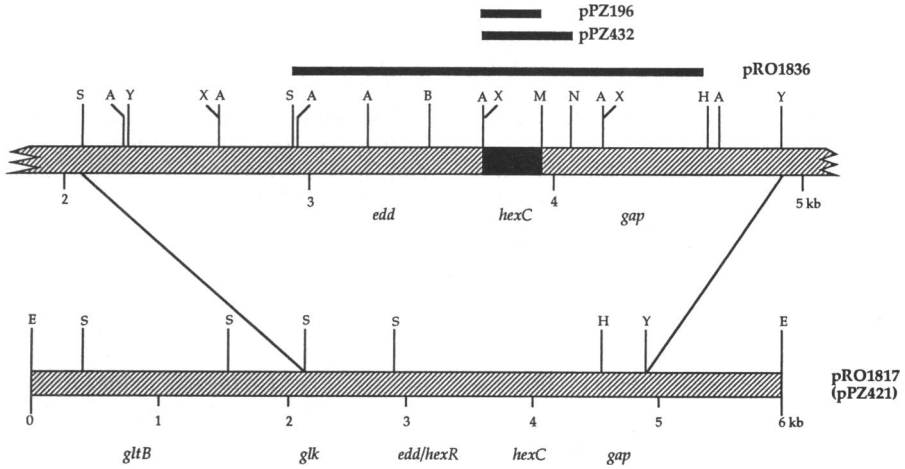


FIG. 2. Partial restriction map of the 6.0-kb insert of pRO1817 (also of pPZ421) with the region encompassing *gap*, *hexC*, and *edd* expanded. The respective locations of the fragments contained in pRO1836, pPZ432, and pPZ196 are delineated by black bars. Abbreviations: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; A, *Ava*I; X, *Xho*I; N, *Nco*I; M, *Xma*III; Y, *Sph*I; B, *Bcl*I.

photometrically by measuring the change in A_{340} of reduced pyridine dinucleotide as previously described (33, 57, 58): ZWF, EC 1.1.1.44; GLK, EC 2.7.1.2; EDD, EC 4.2.1.12; EDA, EC 4.1.2.14; and gluconokinase, EC 2.7.1.12. 2-Keto-3-deoxy-6-phosphogluconate was kindly supplied by H. Paul Meloche of the Papanicolaou Cancer Research Institute, Miami, Fla.

NAD- and NADP-dependent GAP (EC 1.2.1.12 and EC 1.2.1.13, respectively) activities were measured by monitoring the increase in A_{340} of reduced pyridine dinucleotide (9). Reaction mixtures consisted of 50 mM Tris-HCl (pH 8.0), 10 mM (each) 2-mercaptoethanol and $MgCl_2$, 0.3 mM NAD or NADP, 0.25 mM glyceraldehyde-3-phosphate, and 5.7 mM Na_2HAsO_4. After a 5-min preincubation, reactions were started by addition of cell extract. Amidase transferase (EC 3.4.1.5) activity was measured in whole-cell suspensions by monitoring the production of acetohydroxamate from acetamide and hydroxylamine hydrochloride (6).

The protein content of cell extracts was determined spectrophotometrically by the method of Kalb and Bernlohr (35) or by the method of Lowry et al. (39).

DNA preparation, manipulation, and transformation. Plasmid DNA was isolated by the alkaline lysis method (51). DNA manipulations were performed as recommended by the suppliers of enzymes, Bethesda Research Laboratories (Gibco-BRL), Rockville, Md., International Biotechnologies Inc., New Haven, Conn., and Promega.

Transformation of *E. coli* was done according to the procedure outlined by Sambrook et al. (51). Transformation of *P. aeruginosa* cells was done according to a previously described modification (44) of the procedure of Mercer and Loutit (41).

DNA sequencing and analysis. The Sanger dideoxy chain termination method was used for all sequencing (52). For the region encompassing the 1.7-kb insert of pRO1836 (Fig. 2), *Hpa*II, *Taq*I, *Ava*I, *Hin*dIII, and *Sal*I fragments were cloned into the M13mp8 and M13mp9 vectors, which were then used to prepare single-stranded DNA (56). Areas of *edd* and *gap* outside the *Sal*I-*Hin*dIII insert of pRO1836 were cloned from pPZ421 as *Sal*I, *Sph*I, or *Hin*dIII-*Eco*RI fragments into pGEM-3Z(−) (Promega). The resulting constructs were used to make single- or double-stranded template. The M13 forward and reverse universal sequencing primers were used whenever possible. Additional oligonucleotide primers were obtained from the Nucleic Acid Core Facilities of the Massey Cancer Center (MCV/VCU) or the ECU School of Medicine Biotechnology Program. Sequencing reactions were carried out with the Taq-track system of Promega. Addition of 20% (vol/vol) deionized formamide to the polyacrylamide gels facilitated the clarification of the regions of sequence that were high in G+C content. Nucleotide sequences were analyzed by using the University of Wisconsin Genetics Computer Group sequence analysis software package (24).

RNA isolation. Total RNA was isolated by the acid lysis method described by Von Gabain et al. (63). Prior to extraction, cells were mixed with chloramphenicol (100 μg/ml) to improve message recovery (38). After initial isolation, RNA was treated with DNase I to remove any contaminating DNA. Purified RNA was stored at −70°C in 50% formamide.

RNase protection analysis. ^{32}P-labelled runoff transcript was synthesized by using the Gemini II Core System (Promega) and [α-^{32}P]CTP (Dupont, NEN Research Products, Boston, Mass.). Transcript specific for *gap* message was made by utilizing *Hin*dIII-digested pPZ432 as template and T7 polymerase. *Eco*RI-digested pPZ432 and SP6 polymerase were used in preparing *edd*-specific runoff transcript. Total RNA isolated from log-phase cultures of *P. aeruginosa* PAO1 was hybridized overnight at 65°C with 500,000 cpm of runoff transcript. The hybridization mixture was precipitated, dissolved in RNase digestion buffer (Promega), and treated with RNases A and T1, and the products were purified and resuspended in running buffer (8 μl of 80% formamide, 1 μg of bromophenol blue per ml, xylene cyanol). Samples were resolved by polyacrylamide gel electrophoresis alongside a sequence ladder prepared from pPZ432 as template and reverse or forward primers by using the Taq-track system of Promega.

TABLE 1. Effect of plasmids pRO1614, pRO1817, and pPZ196 on activities of carbohydrate catabolism enzymes in extracts of *P. aeruginosa* PAO1 grown without carbohydrate inducer

Strain (Plasmid)[a]	Enzyme specific activity (nmol/min/mg of protein)[b]					
	GLK	ZWF	EDD	EDA	GAP	
					NAD dependent	NADP dependent
PAO1	18	10	4	29	4	21
PAO1(pRO1614)	17	6	4	ND[c]	7	32
PAO1(pRO1817)	97	34	84	ND	210	30
PAO1(pPZ196)	50	50	36	96	24	22

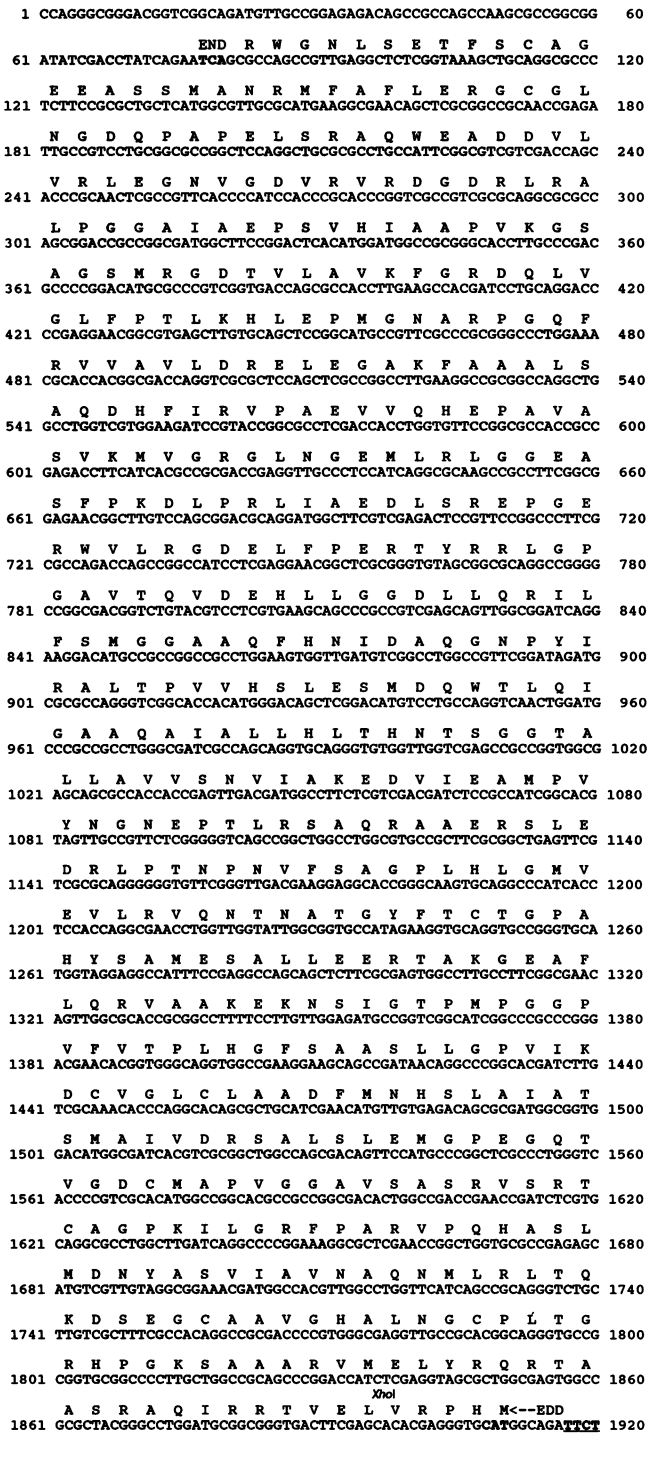
[a] Bacteria were grown to late log phase in BSM–20 mM lactate. Crude cell extracts were prepared for use in enzyme assays as described in Materials and Methods.
[b] Results are from one representative experiment.
[c] ND, not determined.

Primer extension analysis. Primer extension analysis was generally done according to the procedure described by Sambrook et al. (51). Two synthetic oligonucleotide primers were used for mapping of the transcriptional start sites of *gap* and *edd*. The primers for *gap* were CATGGACGCTGTCTGAC TGG and CGCGGTAGTGGCCGGTATAC, and the primers for *edd* were CGCAGCCCGGACCATCTCGA and GCGCT ACGGGCCTGGATGCG. RNA isolated from log-phase cells was hybridized at 42°C overnight with primer (50,000 cpm) that was end labelled with [δ-^{32}P]ATP (Dupont, NEN) by using polynucleotide kinase (Gibco-BRL). Extension reactions were carried out on the resulting DNA-RNA hybrids by using the reverse transcription system of Promega. Actinomycin D (50 μg/ml), 2 mM deoxynucleoside triphosphates, and 50 U of avian myeloblastosis virus reverse transcriptase were used, and the reaction mixtures were incubated at 42°C to minimize artifacts (51). After extension, the RNA was digested with RNase A, and the single-stranded DNA extension product was isolated, dissolved in tracking buffer, and resolved on a polyacrylamide gel alongside a sequencing ladder prepared from pPZ421 and the same respective primers.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL and GenBank nucleotide sequence databases under accession number M74256.

RESULTS

Further definition of *hexC*. Previously, *hexC* had been localized to a 0.6-kb *Bcl*I-*Nco*I chromosomal fragment (Fig. 2) which failed to complement mutants deficient in GLK or EDD activity (57). To further delineate the *hexC* locus, plasmid pPZ196 (Fig. 2) containing a 247-bp *Xho*I-*Xma*III chromosomal fragment was constructed and tested for the *hexC* effect (56). Activities of GLK, ZWF, EDD, and EDA in crude extracts of *P. aeruginosa* PAO1(pPZ196) grown in BSM–20 mM lactate were two- to ninefold higher than those of the same enzymes in bacteria without pPZ196 (Table 1), demon-



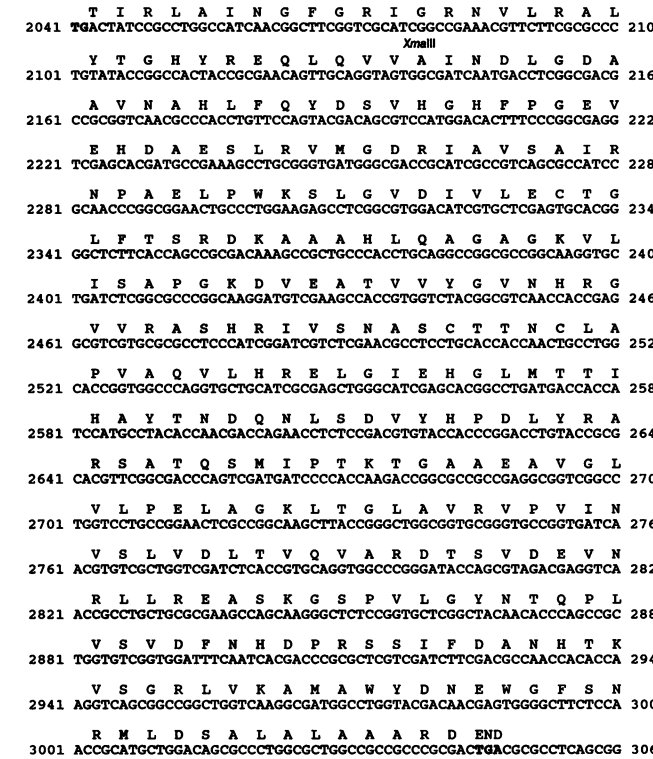


FIG. 3. Nucleotide and predicted amino acid sequences of the 3,060-bp region of DNA encompassing *gap*, *hexC*, and *edd*. The ORF for *edd* extends from bp 1910 to 78 (complementary strand); *gap* extends from bp 2040 to 3047. ATG start and TGA stop sites are in bold. Putative Shine-Dalgarno sequences are in bold and underlined. Transcriptional start sites for *gap* and *edd* are indicated by arrows with either gap^P or edd^P. *Xho*I and *Xma*III restriction sites delineate the smallest cloned DNA fragment to which *hexC* has been localized.

strating that the 247-bp *Xho*I-*Xma*III insert of pPZ196 contained the entire *hexC* locus.

The effect of the *hexC* locus had been analyzed only with respect to the four carbohydrate catabolism enzymes, GLK, ZWF, EDD, and EDA. The question of whether *hexC* might affect other catabolic enzymes was addressed by determining activities of the following independently regulated enzymes: gluconokinase, a gluconate catabolism enzyme (37); NADP-dependent GAP, a constitutively expressed gluconeogenic enzyme (46, 57); and amidase, an enzyme involved in the catabolism of aliphatic amides (19, 20). None of these enzyme activities was affected by the presence of pPZ196 in strain PAO1 (Table 1 and data not shown), demonstrating that the *hexC* effect may be restricted in scope to similarly regulated carbohydrate catabolism enzymes.

GLK, ZWF, EDD, and EDA expression is subject to strong CRC caused by growth in the presence of succinate or other intermediates of the TCA cycle (40). To probe the relationship between CRC and *hexC*, we asked whether pPZ196 suppressed succinate-dependent CRC in strain PAO1. Activities of GLK, ZWF, and EDD in extracts from cultures of PAO1, PAO1(pPZ196), and PAO1(pRO1836) grown in minimal media with glucose, in the presence or absence of succinate, were compared. No differences in the degrees of succinate-dependent repression of the enzymes in the bacteria grown in glucose-succinate were found, demonstrating that *hexC* does not suppress CRC (data not shown). Thus, the *hexC* effect appears to be separate from and recessive to CRC.

Nucleotide sequences of *edd* and *gap*. Figure 3 presents the nucleotide sequence of the entire region encompassing *gap*, *edd*, and *hexC* and shows the predicted GAP and EDD amino acid sequences.

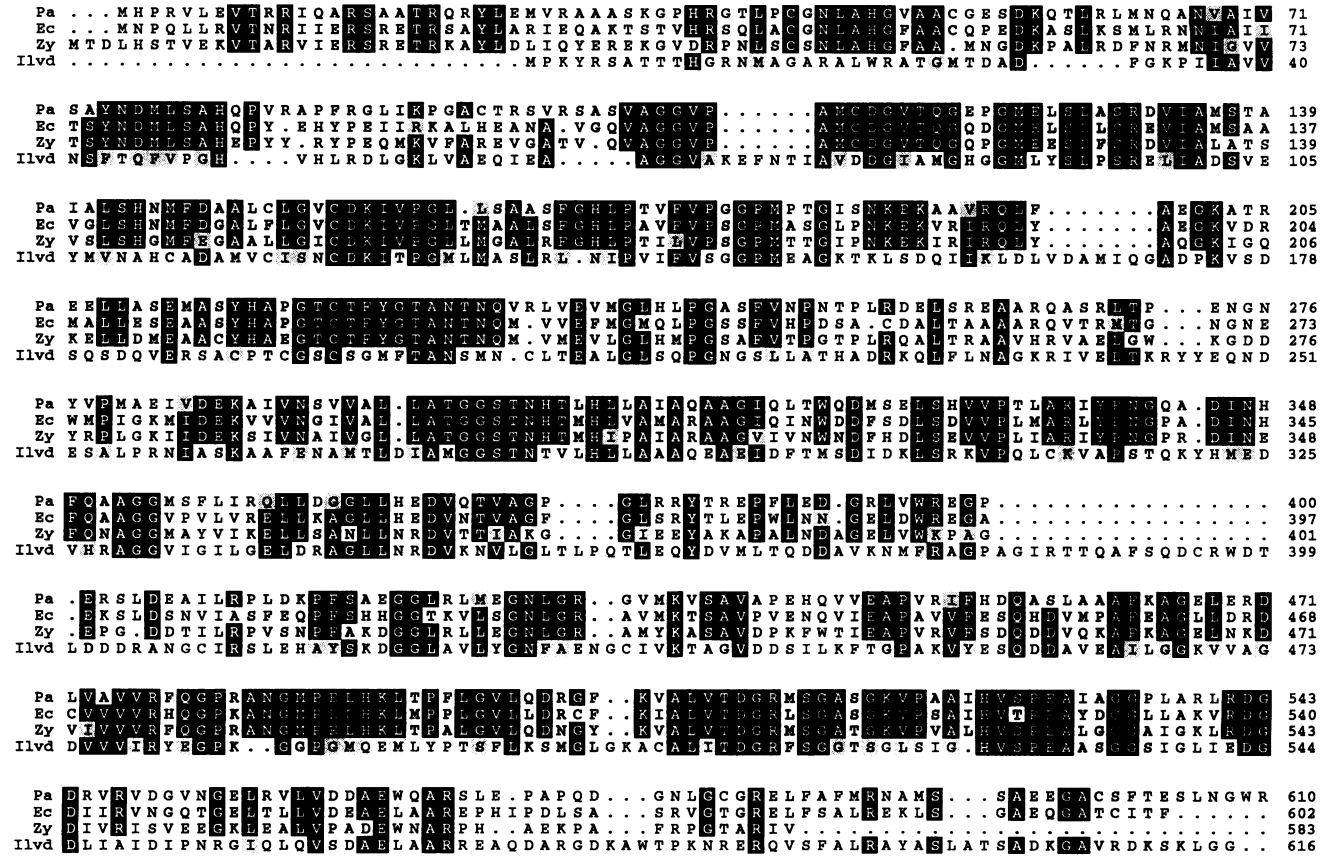


FIG. 4. Comparison of the peptide sequences of EDD from *P. aeruginosa* (Pa), *E. coli* (Ec) (21), *Z. mobilis* (Zy) (3), and dihydroxy-acid dehydratase of *E. coli* (Ilvd) (13) (made by using the PILEUP program of the University of Wisconsin Genetics Computer Group [24] and the PRETTYBOX sequence display program [64a]). Identical residues are in black boxes; similar residues are in gray boxes.

(i) *edd*. Computer analysis of initial sequence data revealed an ORF extending from within the 247-bp fragment in pPZ196 whose predicted amino acid sequence exhibited a high degree of homology with the EDD sequences from *E. coli* (21) and *Zymomonas mobilis* (3). The complete *edd* sequence from *P. aeruginosa* consisted of 1,833 bp encoding a 610-amino-acid protein with a predicted molecular weight of 65,140 (Fig. 3, bases 1910 to 78). A putative Shine-Dalgarno sequence which displayed 7 bases complementary to the 3′ end of *P. aeruginosa* 16S rRNA (55) was identified 13 bases upstream of the ATG start codon. No terminator-like sequences were apparent downstream of the TGA stop codon. The G+C content of *edd* (69.1%) was similar to that of the *P. aeruginosa* genome (67.8%). Codon usage also was found to be similar to that of other *P. aeruginosa* genes (64).

The amino acid sequence predicted by the ORF exhibited 60% identity with and 76% similarity to the amino acid sequence of EDD from *E. coli* (21) and 55% identity with and 71% similarity to that of EDD from *Z. mobilis* (3) (Fig. 4). Like EDD from *E. coli* and *Z. mobilis*, *P. aeruginosa* EDD exhibited close resemblance (51% similarity and 30% identity) to the product of *E. coli ilvD*, dihydroxy-acid dehydratase (13). The most striking conservation among all four peptide sequences consisted of the two segments ALVTDGRMSG (residues 507 to 516) and HVSPEA (residues 526 to 531).

(ii) *gap*. An ORF divergently oriented to the EDD gene displayed a high degree of homology at both the nucleotide and amino acid levels with the GAP family (7, 11, 12). The ATG start codon was located within the 247-bp *Xma*III-*Xho*I fragment, and the ORF extended 1,011 bp to a TGA stop codon (Fig. 3, bases 2040 to 3047). A 6-bp potential Shine-Dalgarno sequence (55) was present 13 bases upstream of the ATG start codon. No terminator sequences were found in the few nucleotides sequenced downstream from the TGA stop codon. The G+C content of *gap* (66.7%) was similar to that of genomic *P. aeruginosa* DNA (67.8%), and codon usage was consistent with that of other transcribed genes in *P. aeruginosa* (64).

A 335-amino-acid protein with a molecular weight of 36,238 was predicted by the ORF (Fig. 3). Comparison of the amino acid sequence with GAP sequences from a number of organisms produced a range of identity values from 45 to 60%, the highest being that for *Rhodobacter sphaeroides* (Fig. 5) (11). NAD-binding (residues 1 to 154) and catalytic (residues 149 to 336) domains exhibited equivalent homology. Residues 153 to 160 in the *P. aeruginosa* peptide, ASCTTNCL, were found to be identical to the catalytic site consensus motif of the GAP family (A)SC(T)Txx(L), as determined by comparison with the Prosite protein motif database of GenBank (24). Other residues thought to be involved in NAD binding and the catalytic reaction carried out by GAP, including D-38, H-183, T-185, T-213, R-237, and Y-308 (7), were also conserved in the predicted sequence of the peptide.

Identification of NAD-GAP as the *gap* gene product. The

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Rs        1 MTIRVAINGFGRIGRNVLRAIVESG.RTDIEVVAINDLGQVETNAHLLRF 49
Pa        1 MTIRLAINGFGRIGRNVLRALYTGHYREQLQVVAINDLGDAAVNAHLFQY 50

Rs       50 DSVHGRFPAKVTSGDDWIDVGRGPIKVTAIRNPAELPWA..GVDMAMECT 97
Pa       50 DSVHGHFPGEVEHDAESLRVMGDRIAVSAIRNPAELPWKSLGVDIVLECT 100

Rs       98 GIFTTKEKAAAHLQNGAKRVLVSAPCDGADRTIVYGVNH.ATLTADDLVV 146
Pa      101 GLFTSRDKAAAHLQAGAGKVLISAPGKDVEATVVYGVNHRGVVRASHRIV 150

Rs      147 SNASCTTNCLSPVAKVLHDAIGIAKGFMTTIHSYTGDQPTLDTMHKDLYR 196
Pa      151 SNASCTTNCLAPVAQVLHRELGIEHGLMTTIHAYTNDQNLSDVYHPDLYR 200

Rs      197 ARAAALSMIPTSTGAAKAVGLVLPELKGRLDGVSIRVPTPNVSVVDLVFE 246
Pa      201 ARSATQSMIPTKTGAAEAVGLVLPELAGKLTGLAVRVPVINVSLVDLTVQ 250

Rs      247 AARDTTVEEVNAAIEAAACGPLKGVLGFTTEPNVSSDFNHDPHSSVFHMD 296
Pa      251 VARDTSVDEVNRLLREASKGS..PVLGYNTQPLVSVDFNHDPRSSIFDAN 298

Rs      297 QTKVMEGRMVRILSWYDNEWGFSNRMADTAVAMGRLL   334
Pa      299 HTKV.SGRLVKAMAWYDNEWGFSNRMLDSALALAAARD  336
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FIG. 5. Comparison of the peptide sequences of GAP from *P. aeruginosa* (Pa) and *R. sphaeroides* (Rs) (11) (made by using the BESTFIT Program of the University of Wisconsin Genetics Computer Group [24]). The sequences exhibited 74.9% similarity and 60.1% identity. Lines indicate identity; dots mark similarity; single dots above sequence delineate lengths of 10 amino acids.

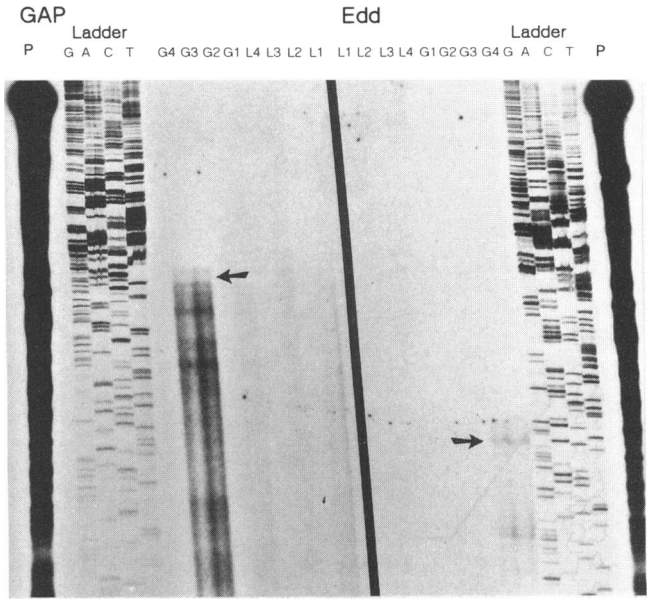

FIG. 6. RNase protection analysis of the transcriptional start sites of *gap* and *edd*. RNA was isolated at 30-min intervals from log-phase cultures of *P. aeruginosa* PAO1 grown in BSM–20 mM lactate (lanes L1 through L4) or BSM–20 mM lactate (lanes G1 and G2) plus 20 mM glucose added as an inducer (lanes G3 and G4). Total RNA (20 μg) from each sample was hybridized with runoff transcript specific for either *gap* or *edd* message and treated as described in Materials and Methods. Protected RNA fragments were resolved alongside a sequencing ladder to determine their sizes. Arrows indicate the largest protected fragments for each mRNA.

striking identity of the predicted protein with the GAP family of enzymes clearly suggested that this ORF encoded GAP. However, confirmation of the ORF's identity by complementation analysis was not possible because of a lack of *P. aeruginosa gap* mutant strains. Instead, GAP activities in extracts from noninduced cultures of strains PAO1, PAO1(pRO1614), and PAO1(pRO1817) were compared to determine whether GAP activity was subject to the same copy-number effect from pRO1817 as were GLK and EDD activities (15) (Table 1). Because inducible NAD-dependent and constitutive NADP-dependent GAP enzymes are found in *P. aeruginosa* (46), both activities were assayed (Table 1). NADP-dependent GAP activity did not vary significantly in any of these extracts. However, NAD-dependent GAP activity was elevated 20- to 30-fold in extracts from cells harboring pRO1817, demonstrating that the ORF encoded NAD-dependent GAP (Table 1).

Inclusion of *gap* in the *hex* regulon. Two observations, the physical proximity of *gap* to *hexC* and *edd* on the chromosome and the glucose inducibility and catabolite repression of GAP activity by succinate (46), suggested that *gap* was a fifth member of the *hex* regulon. To test this hypothesis, NAD-dependent GAP activity in extracts of noninduced *P. aeruginosa* PAO1 containing the *hexC* plasmid, pPZ196, was measured. NAD-dependent GAP activity was sixfold higher than that in extracts from strain PAO1 without a plasmid or with the vector alone (Table 1), indicating that GAP expression was affected by *hexC* in the same manner as GLK, ZWF, EDD, and EDA expression.

Identification of the transcriptional start sites of *edd* and *gap*. RNase protection analysis was carried out on RNA isolated from log-phase cultures of strain PAO1 to locate the transcriptional start sites of *gap* and *edd*. These studies localized both start sites to the intergenic region, at approximately bp 1980 for *edd* and bp 2000 for *gap* (Fig. 3 and 6).

Precise confirmation of the *gap* and *edd* transcriptional initiation sites was obtained by primer extension analysis. The start of *gap* transcription mapped 33 bases upstream of the ATG start site of the ORF (Fig. 7; Fig. 3, base 2007). Because a sufficient yield of the extension product for *edd* could not be produced by using RNA isolated from strain PAO1, RNA was isolated from strain PAO1 carrying the EDD structural gene on plasmid pPZ421. The resulting primer extension product mapped 87 bp upstream of the ATG translational start site of *edd* (Fig. 8; Fig. 3, base 1997). Thus, only nine bases separated the respective transcriptional start sites for *edd* and *gap* (Fig. 3).

The RNase protection studies were performed on RNA from cultures grown in either BSM–20 mM lactate alone or BSM–20 mM lactate to which 20 mM glucose was added as an inducer. Neither GAP nor EDD message was detectable in noninduced cultures of strain PAO1; quantities of both messages increased markedly after addition of glucose (Fig. 6). These data confirmed earlier Northern (RNA) analysis evidence suggesting that expression of GAP and EDD was controlled at the transcriptional level (56).

Analysis of the 128-bp *hexC* intergenic region. The 128-bp intergenic region exhibited 35.4% G+C content, compared with the 67.8% G+C content of *Pseudomonas* chromosomal DNA. Inspection of this region revealed the following features.

(i) Upstream region of *gap*. A sequence perfectly matching the −10 *rpoD*-type consensus sequence of *P. aeruginosa* (48) occurred upstream of the *gap* transcriptional start (Fig. 9). Two sequences with 5 of 6 bases matching the −35 *rpoD*-type consensus sequence occurred at −35 and −45 bases upstream of the start of *gap* transcription at (Fig. 9). A sequence exhibiting near perfect homology with the integration host factor (IHF) consensus site of *E. coli* was located at bases 70 to 82 upstream of the transcriptional start site (Fig. 9). Several inverted repeats were noted in the sequence upstream of *gap*. Two of these motifs overlapped the −10 and −35 regions, and

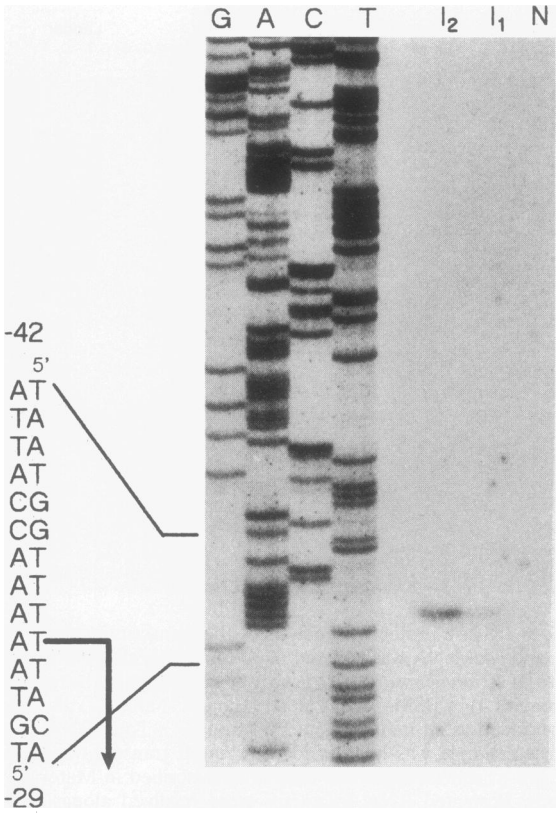

FIG. 7. Primer extension analysis of the transcriptional start site of *gap*. Total RNA was isolated from log-phase cultures of *P. aeruginosa* PAO1 grown in BSM–20 mM lactate to which 20 mM glucose was added. RNA was isolated from cells harvested prior to (lane N) and 30 (lane I_1) and 60 (lane I_2) min after the addition of glucose. RNA (20 μg) from each time point was hybridized overnight at 42°C with a [δ-^{32}P]ATP-end-labelled 20-mer (see Materials and Methods) and extended with avian myeloblastosis virus reverse transcriptase by using the Promega reverse transcription system. The extension product was then resolved alongside a sequencing ladder prepared by using the same oligonucleotide as a primer. The transcriptional start site is marked by the arrow.

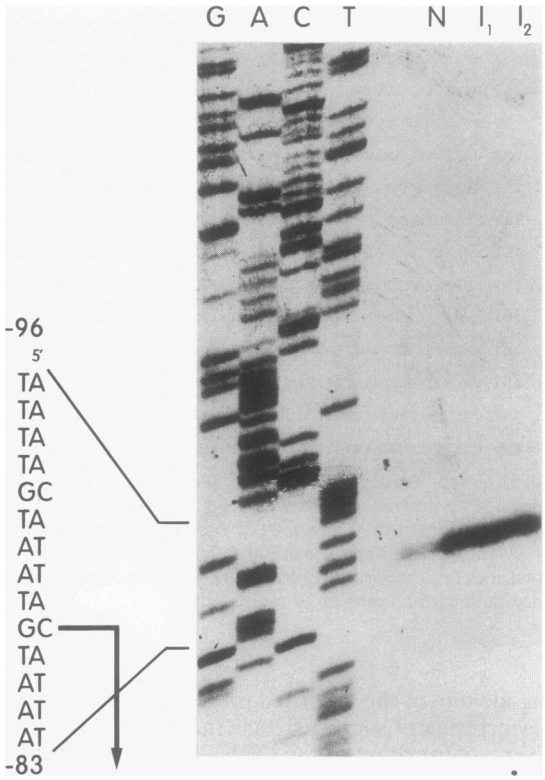

FIG. 8. Primer extension analysis of the transcriptional start site of *edd*. Total RNA was isolated from a log-phase culture of strain PAO1(pPZ421) grown on BSM–20 mM lactate prior to (lane N) and 15 (lane I_1) and 45 (lane I_2) min after addition of 20 mM glucose. RNA (50 μg) from each time point was hybridized overnight at 42°C with a [δ-^{32}P]ATP-end-labelled 20-mer (see Materials and Methods), extended, and resolved alongside a sequencing ladder as described previously. The transcriptional start site is marked by the arrow.

a third, closely resembling the Hut repressor protein binding site of the histidine utilization system in *Pseudomonas putida* (1, 32), overlapped the IHF-like site (Fig. 9).

(ii) Upstream region of *edd*. A match of 4 of 6 bp with *Pseudomonas rpoD*-type consensus sequences occurred at position −10, overlapping a deoxyadenosine-rich tract similar to the −10 region of *algB* (AAAACAA [65]) (Fig. 9). No sequences with close matches to *Pseudomonas rpoD*-, *rpoN*-, or *rpoF*-type −35 consensus sequences (48) were found upstream of *edd*, nor were dyad symmetry elements present.

DISCUSSION

A 3,060-bp segment of the *P. aeruginosa* PAO1 chromosome was sequenced, revealing the presence of *edd* and *gap*, two genes in the *hex* regulon. These genes diverged from within a 247-bp *Xma*III-*Xho*I fragment shown to carry the *hexC* locus. The *edd* and *gap* transcriptional start sites were mapped 9 bases apart within the 128-bp intergenic region, and sequences which may play roles in transcriptional control of *edd* and *gap* and in creation of the *hexC* effect were identified.

This report expands to three the number of conserved EDD genes from bacterial species that have been sequenced. The predicted molecular weight of the protein (65,000) agrees well with the estimated subunit M_r of the *Z. mobilis* enzyme (63,000) (53). The EDDs (53) and dihydroxy-acid dehydratase (22) belong to a subclass of hydrolases that are thought to contain iron-sulfur clusters. The amino acid sequence of dihydroxy-acid dehydratase contains three closely spaced cysteine residues (Fig. 4, residues 189, 192, and 195) reminiscent of the iron-sulfur binding signature of ferredoxins (8). The EDDs (Fig. 4) contain no such motif; however, there are four invariant cysteines (residues 42, 114, 156, and 222) and nine invariant histidines (residues 47, 81, 144, 171, 217, 305, 308, 490, and 526) which are potential ligand binding sites. Examples of proteins utilizing widely spaced cysteine or histidine residues complexed with iron include fumarate nitrate reductase regulatory protein (27), lipoxygenase (34), rubrerythrin (36), and prostaglandin endoperoxide synthase (54).

This study is the first to map the *gap* locus on the *P. aeruginosa* chromosome. The gene for NADP-dependent GAP had been cloned separately on a cosmid and shown to be unlinked on the chromosome to the *zwf-edd-eda* locus (2). The predicted molecular weight of 36,238 for the *gap*-encoded protein agrees with the reported subunit M_r of 38,000; the enzyme in native form is a tetramer (29).

Divergent transcription of genes from a small regulatory area is not unusual in bacteria (4). Five examples of such

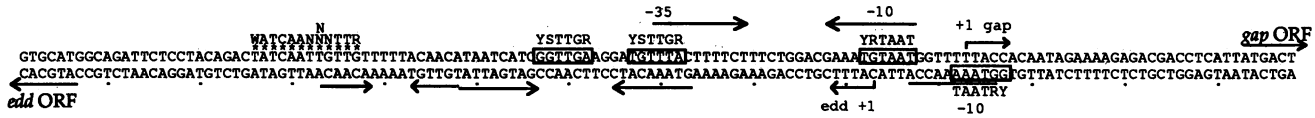

FIG. 9. Nucleotide sequence upstream of mRNA start sites for *edd* and *gap*. The sequence includes bases 1905 to 2045 as shown in Fig. 3; mRNA start sites are marked with labelled arrows. Sequences similar to *rpoD*-type *Pseudomonas* consensus sequences (48) are boxed at −10, −35, and −45 from the mRNA start site of *gap* and at −10 from the mRNA start site of *edd*. Dyad symmetry motifs are marked with pairs of arrows. The part of the sequence similar to the IHF recognition sequence is indicated with asterisks. IHF consensus sequences from *E. coli* (23) and *rpoD*-type consensus sequences (48) are shown above and below the sequence (Y equals C or T; R equals A or G; S equals G or C; W equals A or T; and N equals any nucleotide). Start sites of *edd* and *gap* ORFs are indicated on the ends by arrows.

transcription in *Pseudomonas* species have been described, those involving the *hutC* and *hutF* genes (1, 32), the *xylS* and *xylR* genes (31), and the *catR* and *catBC* genes (49) in *P. putida*; *trpI* and *trpBA* in *P. aeruginosa* (10); and the *tcbR* and *tcbCDEF* genes in *Pseudomonas* sp. strain P51 (62). In each case, at least one of the divergent genes encodes a regulatory protein. This is the first report of an arrangement in which the two divergent genes encode metabolic enzymes. The spacing of the transcriptional start sites of these two genes (9 bases apart) places the −10 regions of the divergent promoters on opposite sides of the DNA helix, separated by less than one turn. Effects on initiation resulting from the binding of two polymerase molecules to this small region are difficult to predict. However, preliminary results from Northern blot analysis of *edd* and *gap* messages indicate that *edd* may be expressed earlier than *gap* following induction by glucose. The fact that we had to clone *edd* onto a multicopy plasmid in order to detect the transcripts suggests a low level of expression relative to that of *gap*. Thorough kinetic studies will be needed to determine the transcriptional sequence of events at these closely spaced, divergent promoters.

Relatively little is known about transcription or the nature of promoter elements in *Pseudomonas* species (17, 48). However, the *edd-gap* promoter region exhibited several features similar to those of functionally significant sequences in other *Pseudomonas* promoters, including −10 and −35 consensus sequences, poly(A) tracts, an IHF recognition sequence, and inverted repeat sequences. Sufficient data concerning *P. aeruginosa* have emerged to suggest consensus sequences similar to those recognized by *E. coli* RNA polymerase sigma factors RpoD, RpoF, and RpoN (17, 59, 60). Close matches with RpoD consensus sequences occurred at −10, −35, and −45 bases upstream of *gap*; the spacing between the *rpoD*-type consensus sequences varies from 16 to 22 bases (48). A match of 4 of 6 bp with the −10 consensus sequence occurred upstream of *edd* (Fig. 9). The poly(A) tract upstream of *edd* bears resemblance to poly(A) tracts in the promoter regions of other *Pseudomonas* genes (5, 17, 25, 26, 31, 45, 65). Upstream poly(A)-rich tracts of nucleotides are associated with strong promoters in *E. coli* (18) and with DNA curving and bending (28). Although the relationship of these motifs to promoter activity in *Pseudomonas* spp. is largely unknown, such sequences have been shown to be important for *xylR* and *xylS* expression in *P. putida* (16, 25, 31, 43). IHFs are DNA-binding and -bending proteins, studied extensively as they occur in *E. coli* (23) and more recently as they occur in other species, including *P. aeruginosa* (31, 42, 61, 65). Expression of *algB* and *algD* in the alginate pathway of *P. aeruginosa* (42, 61, 65) and *xylS* and *xylR* in the toluene pathway in *P. putida* (16, 25, 31) has been shown to involve interaction of an IHF homolog with RpoN-type (σ^{54}) promoter sequences. The significance of an IHF recognition site upstream of the putative RpoD-type (σ^{70}) promoter for *gap* remains to be investigated.

The *hexC* effect probably results from titration of a repressor protein when the repressor binding site is present in multiple copies. Dyad symmetry motifs, strategically spanning the −10 and −35 regions upstream of *gap*, showed no homologies with documented repressor binding sites (Fig. 9). The symmetry element resembling the Hut repressor binding site, while matching the Hut sequence almost perfectly, was centered 70 bases upstream of *gap*, in a region unlikely to be involved in blocking RNA polymerase binding (Fig. 9). However, the overlap of this Hut-like potential binding site with the consensus IHF sequence is quite provocative, as protein binding to the IHF sequence could be blocked by a repressor protein. Commonalities between the promoters for *gap* and *edd* and the DNA sequences upstream of *glk*, *zwf*, and *eda*, the other genes under *hexC* control, should reveal potential repressor binding sites. This work is presently ongoing in our laboratory.

In summary, we have determined the sequences for *edd* and *gap*, the latter having previously been unmapped in the case of *P. aeruginosa*, and have expanded the *hex* regulon to include *gap*. The divergent promoters for these genes occur in a 128-bp intergenic region which contains key elements for understanding the *hex* regulon. The mechanisms by which gene expression from these promoters is controlled by the *hexC* locus, catabolite repression, and other regulatory signals are currently being investigated.

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