JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 5261–5269 Vol. 177, No. 18
0021-9193/95/$04.00+0

Identification of the Transcriptional Activator Controlling the Butanediol Fermentation Pathway in *Klebsiella terrigena*

D. MAYER, V. SCHLENSOG, AND A. BÖCK*

Lehrstuhl für Mikrobiologie der Universität München, D-80638 Munich, Germany

Received 14 April 1995/Accepted 5 July 1995

The gene *budR*, whose product is responsible for induction of the butanediol formation pathway under fermentative growth conditions in *Klebsiella terrigena*, has been cloned and sequenced. This gene is separated from the *budABC* operon by a nontranslated region of 106 bp and transcribed in the opposite direction. *budR* codes for a protein of molecular weight 32,124, the sequence of which exhibits characteristics of regulators belonging to the LysR family. When transferred into the heterologous host *Escherichia coli*, *budR* activates expression of *budA′-lacZ* transcriptional and translational fusions with a regulatory pattern identical to that in *K. terrigena*, namely, induction by acetate, low pH, and anaerobiosis. Induction by acetate was specific, indicating that it is the physiological inducer. Primer extension analysis located the start site of transcription to two positions, 23 and 24 bp upstream of the *budR* initiation codon, and also showed that BudR strongly autoregulates its own expression. The products of *fhlA*, *arcA*, *hip*, *ntrA*, and *katF* did not influence expression of the *bud* operon. A mutation in *fnr*, however, led to a threefold increase in expression, indicating that Fnr acts as a repressor. The results support the notion that BudR coordinates the activity of the energy-conserving, nonreductive, but acidifying acetate formation pathway with the expression of the non-energy-conserving, reductive, but nonacidifying butanediol pathway.

Under fermentative conditions, *Escherichia coli* acidifies the medium by excreting a mixture of lactic, acetic, succinic, and formic acids in addition to ethanol and CO_2 plus H_2, whereas members of the *Klebsiella*, *Enterobacter*, and *Serratia* genera release predominantly neutral compounds, namely, ethanol and butanediol. To counteract lethal acidification, *E. coli* induces the formation of the formate hydrogenlyase system, which disproportionates formic acid into CO_2 and H_2 (for a review, see reference 40). The production of butanediol and ethanol by the members of the *Klebsiella* group, on the other hand, is thought to channel so much of the pyruvate into the butanediol pathway that the production of acidic end products from pyruvate is limited (18, 21).

Three enzymes are involved in the production of 1 mol of butanediol from 2 mol of pyruvate: α-acetolactate synthetase (ALS), α-acetolactate decarboxylase (ALDC), and acetoin reductase (AR) (for a review, see reference 21). In *Klebsiella terrigena*, they are encoded by the genes *budA* (ALDC), *budB* (ALS), and *budC* (AR), which are organized in an operon (7). The expression of the operon is optimal in the presence of acetate, at low medium pHs, and under anaerobic conditions (21). Although acetate is thought to play a major role in regulation of the butanediol fermentative pathway, it is not known whether it acts per se or via some follow-up product (9, 38, 39). It has been shown that regulation occurs at the transcriptional level (7), but the genetic elements involved have not been identified yet. In this communication, we report on the characterization of the regulatory gene *budR*, responsible for the control of the synthesis of the butanediol formation system, and on the physiological and genetic parameters involved in regulation.

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, D-80638 Munich, Germany. Phone: (089) 17919856. Fax: (089) 17919862.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, plasmids, and phages used in this study are compiled in Table 1.

Media and growth conditions. For use in enzyme assays and for the isolation of RNA, bacteria were cultivated at 37°C in rich medium buffered with potassium phosphate at the indicated pH (medium TGYEP [3]). To anaerobically grown cultures, glucose was added at 0.4% (wt/vol), sodium molybdate and selenite were added at 1 μM, and nickel chloride was added at 5 μM (final concentration). For induction of the cultures, sodium acetate was present at 40 mM unless noted otherwise. LB medium was used for all other cultivation purposes (22). Antibiotics were added at the following concentrations: ampicillin at 100 μg ml^{-1}; chloramphenicol at 30 μg ml^{-1} for plasmid-encoded and at 15 μg ml^{-1} for chromosomally encoded resistance; kanamycin sulfate at 50 μg ml^{-1}; and tetracycline at 20 μg ml^{-1}. For work with λ phages, the media given by Kleckner et al. (19) were used. If required, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to the plates at 1 mM, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 0.5 mM.

UV mutagenesis. A Lac^- derivative of *K. terrigena* DSM2687 was generated by UV mutagenesis (22). Lac^- colonies arose with a frequency of 10^{-4}. The stability of the mutation in one of the resulting strains, designated KT14, was checked in a reversion test with nitrosoguanidine (22).

Recombinant DNA techniques. Standard recombinant DNA techniques were adapted from Sambrook et al. (27). Plasmid DNA was isolated by the method of Holmes and Quigley (15), but with an additional phenol-chloroform extraction step. Chromosomal DNA was prepared by the method given in Ausubel et al. (2). Fragments from chromosomal DNA were amplified by PCR with *Pfu* DNA polymerase purchased from Pharmacia (Freiburg, Germany). Fragments for cloning purposes were amplified in a symmetrical reaction (20 pmol of each primer), and those for direct sequence analysis were amplified in an asymmetrical reaction (50 and 1 pmol concentrations).

Cloning of the *budA* 5′-flanking region. Chromosomal DNA from *K. terrigena* was isolated by symmetrical PCR with the following primers: oligonucleotide 1, 5′-GGGGATCCATCGTGGGCCGCCCGGG-3′, identical to the sequence between −142 and −125 of the coding strand; and oligonucleotide 2, 5′-GGG GATCCTGGCAGGTGCATTCAGG-3′, covering positions 68 to 49 of the noncoding strand. The sequence positions are given relative to the start site of transcription of the *budA* gene. The primers used carry *Bam*HI restriction sites at their 5′ ends, which were used to clone the 219-bp amplified fragment into *Bam*HI-linearized and dephosphorylated vector pUC19. The insert of the resulting plasmid, pBU1, was checked for authenticity by DNA sequencing (28) and comparison with the published sequence (7).

Construction of *budA′-lacZ* fusions. For the construction of *budA′-lacZ* fusions, the insert of plasmid pBU1 was isolated and cloned into *Bam*HI-linearized and dephosphorylated vectors pRS551 and pRS552. The orientation of the insert and the fusion joints of the resulting plasmids, pBTK142 (transcriptional fusion) and pBTL142 (translational fusion), were checked again by sequence analysis. The translational fusion carried by pBTL142 was integrated into the chromo-

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Genotype or phenotype	Reference or source
K. terrigena		
DSM2687	Wild type	DSM[a]
KT14	Lac$^-$	This work
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*)	41
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB5301 rpsL150* λ$^-$	10
FM420	MC4100 *srl*::ΔTn*10 recA56*	42
BL142	MC4100 λBTL142	This work
SA2	MC4100 Δ*fhlA*	34
RM101	MC4100 Δ*fnr*	31
BN450	MC4100 *ntrA208*::Tn*10*	5
RM3132	MC4100 *arcA2 zjj*::Tn*10*	16
RH90	MC4100 *rpoS359*::Tn*10*	20
MC252	*metB ara* Δ(*lac-pro*) *gyrA argE*(Am) *thi supF* Δ*3*(*himD*)::Cam^r	M. Chandler
Plasmids		
pUC19	Ap^r, *lac′*IPOZ′	41
pBU1	Ap^r, pUC19, 178 bp 5′ of the translational start site (TLS) from *budA* to 32 bp 3′ of TLS from *budA* from *K. terrigena*	This work
pBU2	Ap^r, pUC19, 32 bp 3′ of TLS from *budA* to 1068 bp 3′ of TLS from *budR*	This work
pSA32	Cm^r, pACYC184, *fhlA*$^+$	35
pRH320	Amp^r, pBR322, *rpoS*$^+$	20
pUFR-1	Amp^r, pUC19, *fnr*$^+$	G. Sawers
pUAA-1	Amp^r, pUC19, *arcA*$^+$	G. Sawers
pBN61	Cam^r, pACYC184, *ntrA*$^+$	4
p$P_L$hip.himA-5	Cam^r, *himA* and *hip* genes under control of the p_L promoter	23
pRS551	Km^r Ap^r *lacZ*$^+$ *lacY*$^+$ *lacA*$^+$	37
pRS552	Km^r Ap^r *′lacZ lacY*$^+$ *lacA*$^+$	37
pBTK142	pRS551, Φ(*budA′-lacZ*$^+$), 178 bp 5′ of TLS from *budA* to 32 bp 3′ of TLS from *budA*	This work
pBTL142	pRS552, Φ(*budA′-′lacZ*), 178 bp 5′ of TLS from *budA* to 32 bp 3′ of TLS from *budA*	This work
pRBT1	pRS551, Φ(*budR*, *budA′-lacZ*), 32 bp 3′ of TLS from *budA* to 931 bp 3′ of TLS from *budR*	This work
pBR322	Ap^r Tet^r	8
pBAK1	Ap^r *budR*, 1,800-bp *Sau*3A fragment from chromosomal DNA of *K. terrigena* cloned in *Bam*HI site of pBR322	This work
pBAK4	Ap^r *budR*, 3,850-bp *Sau*3A fragment from chromosomal DNA of *K. terrigena* cloned in *Bam*HI site of pBR322	This work
pBAK5	Ap^r *budR*, 1,800-bp *Sau*3A fragment from chromosomal DNA of *K. terrigena* cloned in *Bam*HI site of pBR322	This work
pBAK14	Ap^r *budR*, 1,800-bp *Hin*dIII fragment from pBAK1 cloned in *Sma*I site of pUC19, same orientation as *budR* and *lacZ*	This work
pBAK16	Like pBAK14, inverse orientation of the insert	This work
Phages		
λRS45	*lacZ lacY*$^+$ *lacA*$^+$ *imm*^{21} *ind*$^+$	37
λBTK142	λRS45 Φ(*budA′-lacZ*$^+$)	This work
λBTL142	λRS45 Φ(*budA′-′lacZ*)	This work
λRBT1	λRS45 Φ(*budR*, *budA′-lacZ*$^+$)	This work

[a] DSM, Deutsche Sammlung für Mikroorganismen.

some of strain MC4100 by transformation of the plasmid and by infection with λRS45 (37). The lysates obtained were used to transduce MC4100 to kanamycin resistance. Lysates were then generated from selected clones by UV irradiation (36) and used to infect again MC4100 cells; one of the stable lysogens, strain BTL142, was used for the expression experiments.

Generation of a gene library from *K. terrigena* chromosomal DNA and cloning of *budR*. *K. terrigena* DNA was subjected to partial digestion with endonuclease *Sau*3A (0.02 U/μg of DNA). Fragments of between 3 and 10 kb were separated electrophoretically and ligated into the *Bam*HI site of the linearized and dephosphorylated vector pBR322. Strain JM109 was transformed with the ligation mixture. About 50% of the transformants carried a plasmid with an insert. The plasmids were isolated and used to transform *E. coli* BTL142.

Transformants conferring a Lac$^+$ phenotype on strain BTL142 were chosen, and the plasmid (pBAK1) of one of them was further investigated. The region carrying *budR* was isolated from it as an 1,800-bp *Hin*dIII fragment and, after filling in the protruding ends, was cloned into the *Sma*I site of plasmid pUC19, resulting in plasmid pBAK14 (orientation of *budR* identical to that of *lacZ*) and plasmid pBAK16 (orientation opposite that of *lacZ*). Suitable subclones for sequencing were generated by unidirectional shortening of the inserts of pBAK14 and pBAK16 with exonuclease III, starting from the *Asp*718 site of the multiple cloning site of pUC19. *Sac*I restriction protected the vector against simultaneous degradation (13). The sequence of the subclones was determined by the chain termination method (28) with T7 DNA polymerase (Pharmacia). GC compressions were resolved by including dITP instead of dGTP in the reaction mixture. The universal primers were used in the sequence reactions.

Construction of *budA′-lacZ* fusions carrying the complete sequence of *budR* and the intergenic region between *budR* and *budA*. By using oligonucleotide 2 (see above) and oligonucleotide 3, 5′-GAAATGCCAAGATCTGGGCCGC CCGGG-3′, a 226-bp fragment of pBAK14 was amplified by symmetrical PCR. Oligonucleotide 3 carries the authentic sequence of the noncoding strand of *budR* from positions 79/80 to 106/107 relative to the transcription start sites of *budR* and contains a unique *Bst*XI site. Restriction with *Bam*HI and *Bst*XI yielded a 205-bp fragment which was cloned into *Bam*HI- and *Bst*XI-digested pBAK14, resulting in plasmid pBU2. The correct sequence of the insert was verified by DNA sequencing.

pBU2 was digested with *Eco*RI and *Bam*HI, and a 1,222-bp fragment was

isolated. Cloning this fragment into *Bam*HI- and *Eco*RI-digested plasmid pRS551 yielded plasmid pRBT1 (*budR*, intergenic region, and *budA′-lacZ* transcriptional fusion). Sequencing confirmed the correct joints of insertion.

Introduction of *budR* and the *budA′-lacZ* fusions into *E. coli* MC4100 and into *E. coli* mutants carrying defects in global regulatory genes. *lacZ* fusions were introduced into the *E. coli* chromosome by λ infection, with plasmid pRBT1 used for generating the λ lysate λRBT1 (36). Chromosomal integration of the *lacZ* fusions into *E. coli* MC4100 yielded strain BK1. Strains carrying such deficiencies in global regulators are listed in Table 1. Construction of the *hip* strain BK16 was carried out via P1 transduction of BK1 with P1::MC252 by the method of Miller (22).

Primer extension analysis of transcripts. RNA was isolated by the method of Aiba et al. (1) from cells of a culture grown to an optical density at 600 nm (OD_{600}) of 0.6. The primer extension analysis was performed as described previously (30) with 20 μg of RNA. Oligonucleotide 1 (see above) served for determination of the transcriptional start site of *budA* in *E. coli* FM420 and *K. terrigena* DSM2687, both carrying plasmid pBTK142. Oligonucleotide 3 (see above) was used for determination of the transcriptional start site of *budR* on plasmid pRBT1 present in *E. coli* MC4100 and *K. terrigena* DSM2687. Cells were grown in rich medium under inducing and noninducing conditions.

β-Galactosidase assays. Cultures were grown for about three to four generations to the exponential growth phase and used to determine the β-galactosidase activity. Units given are those defined by Miller (22). All experiments were performed with both the transcriptional and the translational fusions to confirm the identity of the regulatory pattern. The values are the averages of three independent experiments, each one conducted in triplicate. The maintenance of the pH was checked at the end of the experiment. The variation observed was ±0.3 pH units.

Computer analysis of sequence. Comparison of the amino acid sequence derived from *budR* and related genes from *E. coli* and *Bacillus subtilis* was performed with the GeneWorks program, version 2.2, from IntelliGenetics, Inc.

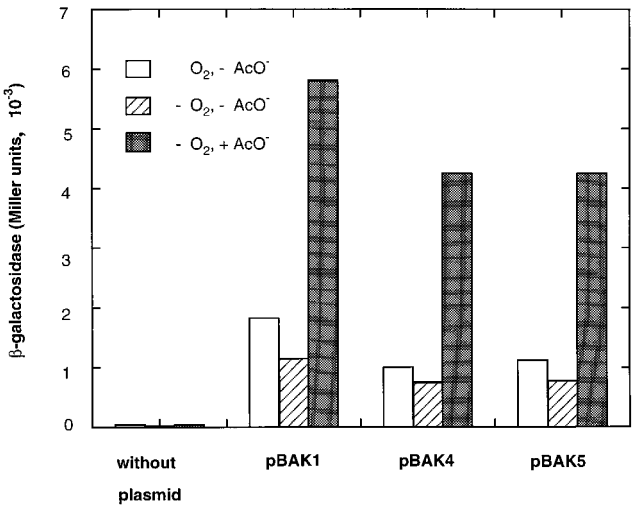


FIG. 1. Activation of *budA′-lacZ* expression by plasmids from a gene library from *K. terrigena* DNA transformed into *E. coli* BK142. Transformants were grown in TGYEP medium at pH 6.5 under aerobic conditions (open bars) and under anaerobic conditions in the absence (hatched bars) and presence (shaded bars) of 40 mM sodium acetate (AcO$^-$).

RESULTS

Construction of transcriptional and translational *budA′-lacZ* fusions and their expression in *K. terrigena* and *E. coli*. The cloning and the nucleotide sequence of the *budABC* operon from *K. terrigena* and of a 178-bp segment in the 5′ untranslated region have been reported by Blomqvist and coworkers (7). It was shown that transcription of the operon starts at an A 36 bp upstream of a GTG codon considered the translational start site (see Fig. 2).

To search for *cis*-active regulatory elements in the 5′ nontranslated region, the DNA segment between positions −142 and +68 with respect to the transcriptional start site of *budA* (see Fig. 2) was amplified by symmetrical PCR from chromosomal DNA of *K. terrigena* and inserted into plasmid pUC19 by means of *Bam*HI linkers, yielding plasmid pBU1. The transcriptional fusion (carried by plasmid pBTK142) and the translational fusion (carried by plasmid pBTL142) between *budA* and *lacZ* were then constructed by cloning the *Bam*HI insert of pBU1 into plasmids pRS551 and pRS552, respectively. In pBTL142, the 10 5′-terminal codons of *budA* are fused to the ninth codon of *lacZ*.

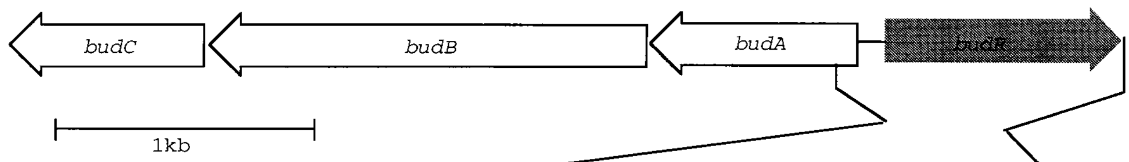
Plasmids pBTK142 and pBTL142 were transformed into *K. terrigena* KT14, which is a *lac* mutant, and into *E. coli* FM420. The transformants were cultivated under different physiological conditions and analyzed for β-galactosidase formation. In *K. terrigena*, β-galactosidase synthesis was induced by anaerobiosis, low pH, and the presence of acetate (see Fig. 5A), whereas the *E. coli* transformants showed a basal expression level of 400 to 600 Miller units but no significant variation of this level when they were grown under these conditions (data not shown). The regulatory pattern of β-galactosidase formation in *K. terrigena* qualitatively resembled the expression of the *bud* operon genes and related genes in other organisms investigated previously (7, 21, 39). In all experiments, the translational fusion was expressed at about a 20% lower level but with an identical regulatory pattern. The results of these expression studies allow the conclusions that (i) the 5′ nontranslated region of the fusions harbors all the *cis* elements required for the characteristic control, (ii) *E. coli* appears to lack the regulatory gene(s) or transacting component(s) required for the specific control of *budABC* operon transcription, and (iii) the 3′ translated region of *budA* has no major effect on the regulatory pattern. Whether *E. coli* possesses any analogous component, probably another LysR protein which is able to cross-talk with the *Klebsiella* promoter region under certain physiological conditions, however, cannot be completely ruled out.

To test whether the unregulated, basal expression is from some plasmid-derived or fortuitous promoter recognized by *E. coli* RNA polymerase, the start site of transcription of *budA′-lacZ* on plasmid pBTL142 was determined in *E. coli* and compared with the start site obtained in *K. terrigena* carrying the same plasmid under both *bud*-inducing and noninducing conditions. It was found that the transcription start sites of both organisms are identical to that determined for *budABC* operon transcription in *K. terrigena* (7), supporting the notion that the indigenous *budA* promoter is used in the heterologous host.

Cloning and nucleotide sequence of *budR*. The heterologous system was used to clone the gene coding for the putative activator protein. First, the *budA′-lacZ* fusion of plasmid pBTK142 was integrated into the *E. coli* chromosome via phage λRS45, resulting in strain BK142. Under inducing conditions (pH 6.5, 40 mM acetate in the medium), strain BK142 displayed a level of β-galactosidase activity of 70 Miller units, giving rise to light blue colonies on TGYEP plates containing X-Gal.

Cells of strain BK142 were transformed with the gene library of *K. terrigena*. On X-Gal plates, dark blue colonies came up with a frequency of 10^{-6}. After retransformation, three stable clones were purified and analyzed by determining their β-galactosidase activity under inducing and noninducing conditions. They showed the regulatory pattern expected for specific activation of the *budA′-lacZ* fusion (Fig. 1). Their plasmids (pBAK1, pBAK4, and pBAK5) were isolated and analyzed by restriction. The inserts were of different lengths; all three inserts, however, covered one *Ssp*I-*Hin*dIII DNA segment which was identical (data not shown).

A *Hin*dIII fragment (one *Hin*dIII site originating from pBR322) was isolated, and its nucleotide sequence was deter-




```
                                        budA <----
                                           AATCCACTTCCTCGTTCAACAA
60    TC CGA GAG GAC CGT CCA CGT AAG TCC TAT TAC TAA GTG TTAGGTGAAGGAGCAAGTTGTT
       l   s   e   q   c   t   c   e   p   y   h   n   v
               +1
120   ATATAAGAAAGATTAAATAAATATTGACCCGATTCAGCTCTCAGTTCCAATATAGAATCC
      TATATTCTTTCTAATTTATTTATAACTGGGCTAAGTCGAGAGTCAAGGTTATATCTTAGG
                              budR ---->
180   ATGCTGGTTTGAGACGTTTACGAT ATG GAA CTT CGC TAT TTA CGT TAT TTT GTC GCC GTT
      TACGACCAAACTCTGCAAATGCTA
        +1                     M   E   L   R   Y   L   R   Y   F   V   A   V

240   GCC GAG GCG CGG AAC TTC ACC CGG GCG GCC CAC GAT CTT GGC ATT TCT CAA CCG CCA CTA
      A   E   A   R   N   F   T   R   A   A   H   D   L   G   I   S   Q   P   P   L

300   AGT CAG CAA ATT CAG CGA CTT GAG CGA GAA ATA GGG ACT CCG CTG CTG CGT CGT TTG ACG
      S   Q   Q   I   Q   R   L   E   R   E   I   G   T   P   L   L   R   R   L   T

360   CGG GGG GTT GAG CTG ACG GAG GCC GGA GAG TCG TTC TAC GTC GAC GCG TGT CAG ATC CTC
      R   G   V   E   L   T   E   A   G   E   S   F   Y   V   D   A   C   Q   I   L

420   GCC TTA AGC GAT GCG GCG CTG GAA AAA ACC AAG GGG ATT GCG CGG GGC ATG AAC GGT AGC
      A   L   S   D   A   A   L   E   K   T   K   G   I   A   R   G   M   N   G   S

480   CTG GTG CCG GGG ATC ACC AGT TCA GCT GCT TTT CAT TCG CAG ATT TTC TCT TTG CTG TAC
      L   V   P   G   I   T   S   S   A   A   F   H   S   Q   I   F   S   L   L   Y

540   CAG TTT CAG CAG CGC TAT CCG GCG GTG GCT CTG CGC CAG GTC GAA GGC AAT ATG GCG ACG
      Q   F   Q   Q   R   Y   P   A   V   A   L   R   Q   V   E   G   N   M   A   T

600   CTG ATG CAT GCC CTG GGC GAG GCG GAG CTG GAT ATC GCC TTT GTG CGC CTG CCG TGT GAA
      L   M   H   A   L   G   E   A   E   L   D   I   A   F   V   R   L   P   C   E

660   AGC AGC AAG GCG TTT AAT TTG CGC ATT ATT GCC GAG GAG CCG ATG GTT ATC GCG CTG CAT
      S   S   K   A   F   N   L   R   I   I   A   E   E   P   M   V   I   A   L   H

720   CGC TCG CAC CCG CTC TCC GGG GAA AGT GCG CTC TCT CTG GCG CAG CTG AGC GAC GCG GTG
      R   S   H   P   L   S   G   E   S   A   L   S   L   A   Q   L   S   D   A   V

780   CCG GTT ATT TTC CCG CCG GAG GTG GCG CCG GGC CTC TAC GAG CAG GTT TAT GAT GGC TGT
      P   V   I   F   P   P   E   V   A   P   G   L   Y   E   Q   V   Y   D   G   C

840   CGG CGT GCC GGG GTC GAT ATG AGC CGC GCC AGG CAA TCT TCA CAG ATC TCG TCT TCT ATT
      R   R   A   G   V   D   M   S   R   A   R   Q   S   S   Q   I   S   S   S   I

900   AGC ATG GTG GAC GCG GGC TTC GGC TTT GCG CTG GTG CCT CAG TCG ATG ACC TGT ATC TGC
      S   M   V   D   A   G   F   G   F   A   L   V   P   Q   S   M   T   C   I   C

960   CTT CCC AAC GTC ACA TGG CAT CCC TTG CAG GAC GCG TCG CTG AAG ACG GAG ATC GCC ATC
      L   P   N   V   T   W   H   P   L   Q   D   A   S   L   K   T   E   I   A   I

1020  GCG TGG CGG CGT TTT GAA CGT TCG CGG ACG GTA AAG CGT TTT CTG GAG ATG TTT TAGGCG
      A   W   R   R   F   E   R   S   R   T   V   K   R   F   L   E   M   F
```

FIG. 2. Organization of the *budABC* operon and the *budR* gene in *K. terrigena* and nucleotide sequence of *budR* and the nontranslated region between *budR* and *budA*. The derived amino acid sequences are given in capital letters for BudR and lowercase letters for BudA (divergent orientation). Transcriptional start sites of *budA* and *budR* are indicated by arrows designated +1. Potential Shine-Dalgarno sequences are underlined. Boldface letters indicate a potential Fnr binding site. The sequence has been deposited in the EMBL database under accession number Z48600.

mined (Fig. 2). Analysis of the sequence revealed an open reading frame of 870 bp, corresponding to a protein of 290 amino acids and a molecular weight of 32,124. The gene was designated *budR*; it is located in an orientation divergent from that of the *budABC* operon. The translational start sites of *budA* and *budR* are separated by an intergenic region spanning 106 bp (Fig. 2, top).

Derived properties of BudR. BudR has significant sequence similarity with regulatory proteins of the LysR family (14, 32). Representative examples are compared with BudR in Fig. 3. This family of regulators has the following common properties (14, 33): (i) they are typically 32 to 35 kDa; (ii) most of them act as transcriptional activators and, for this purpose, require an inducer molecule; (iii) they have considerable sequence similarity in the N-terminal region, namely, a helix-turn-helix motif (24, 32), which acts as a DNA-binding region; and (iv)

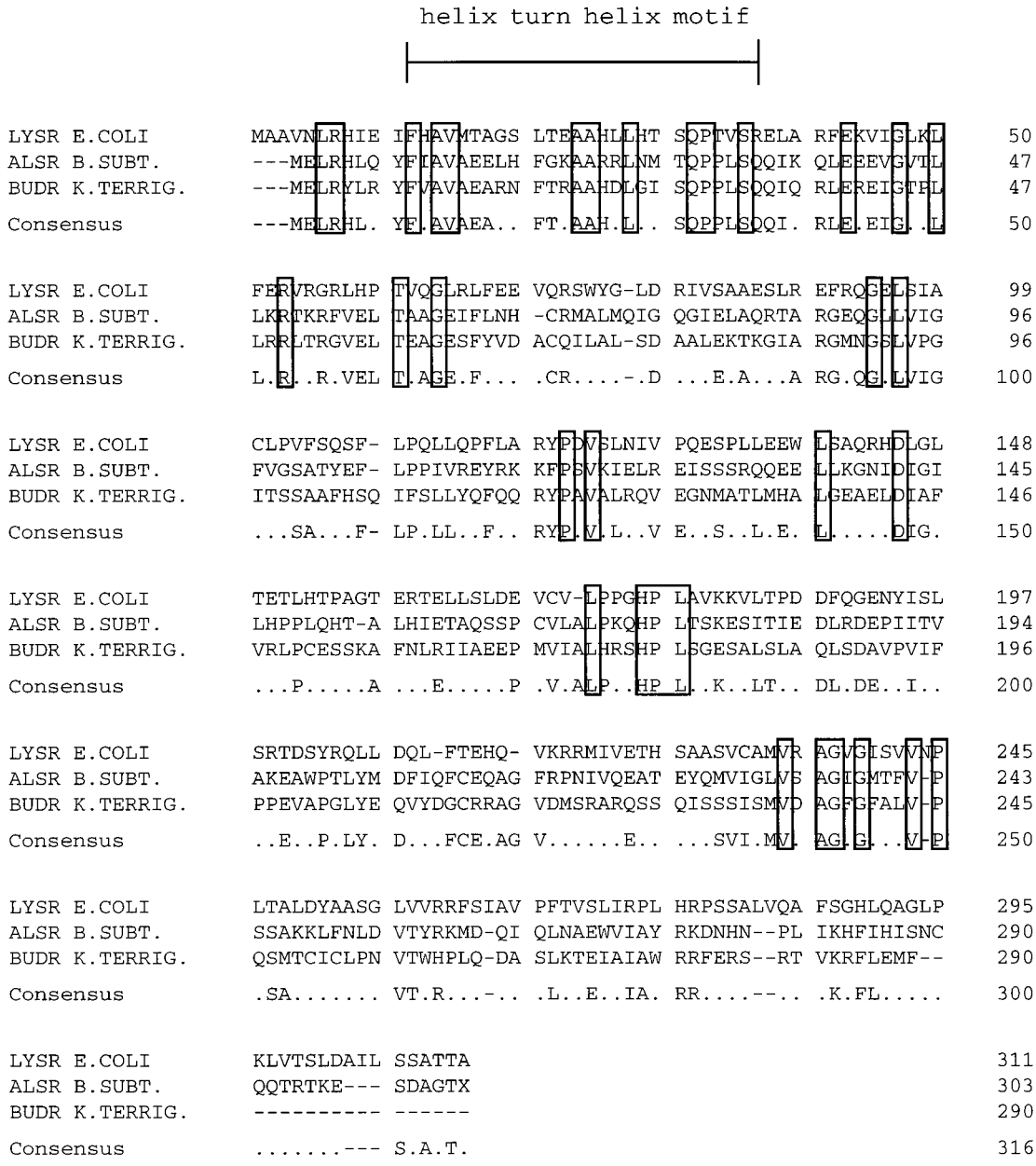


FIG. 3. Alignment of the amino acid sequences of LysR (*E. coli*), AlsR (*B. subtilis*), and BudR (*K. terrigena*). Identical residues are boxed. BudR has 23 and 30% identity with LysR and AlsR, respectively.

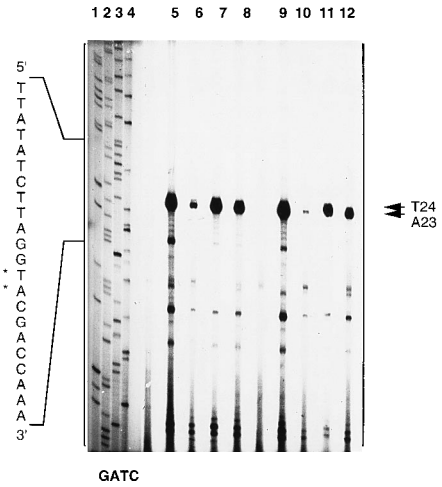


FIG. 4. Primer extension analysis of transcripts from the *budR* gene. The first four lanes give the sequence analysis. In lanes 5 to 8, transcripts from *E. coli* MC4100/pRBT1 and in lanes 9 to 12, transcripts from *K. terrigena*/pRBT1 were separated. Lanes 5 and 9, aerobic growth in TGYEP medium, pH 6.5; lanes 6 and 10, aerobic growth in the presence of 40 mM sodium acetate; lanes 7 and 11, anaerobic growth in TGYEP medium, pH 6.5; lanes 8 and 12, anaerobic growth in the presence of 40 mM acetate. Asterisks denote the 5′ ends of the transcripts on the DNA sequence. Arrows indicate the positions of the transcripts.

their genes are located in the vicinity and in inverse orientation to the structural genes which they regulate. All these characteristics also hold for BudR.

Determination of the transcription start site of *budR*. Most of the LysR regulatory genes autoregulate their own synthesis via a mechanism involving overlap of promoters between the structural gene and the regulated unit (for a review, see reference 32). To test whether BudR autoregulates its formation and in order to determine the transcriptional start site, primer extension analyses were performed (Fig. 4). Although there are many background bands, the results show that transcription commences at two positions, T24 and A23, and both sites seem to be used equally under noninduced conditions. Induction appears to shift initiation to position A23. Both the start sites and the quantity of the transcript are identical in *K. terrigena* and *E. coli*. Furthermore, there is much less transcript of *budR* under inducing conditions than in the noninduced state, which supports the notion that BudR strongly autoregulates its own expression. The high level of *budR* transcripts under aerobic conditions (Fig. 4, lane 5 and lane 9) may arise from a higher copy number of plasmid pRBT1 in the aerobic cells.

Effect of growth conditions on expression of the *bud* operon. To analyze whether the 5′ untranslated region carried by the *budA′-lacZ* fusions harbors all the *cis*-acting motifs relevant for regulation and whether BudR is the main control component acting in *trans*, plasmid pBTK142 was transformed into *K. terrigena* KT14 (Fig. 5A) and *E. coli* BK1 (Fig. 5B), and the transformants were tested for β-galactosidase formation under different growth conditions. Strain BK1 carries a chromosomally encoded *budR* gene as well as the *budR-budA* intergenic region and the *budA′-lacZ* fusion originating from plasmid pRBT1, which was integrated into the λ attachment site. *K. terrigena* KT14 possesses an intact *budR/budABC* genetic system.

The expression pattern obtained was independent of the host; activation of expression of the *budA′-lacZ* fusion is stimulated by low pH and especially by the presence of acetate in

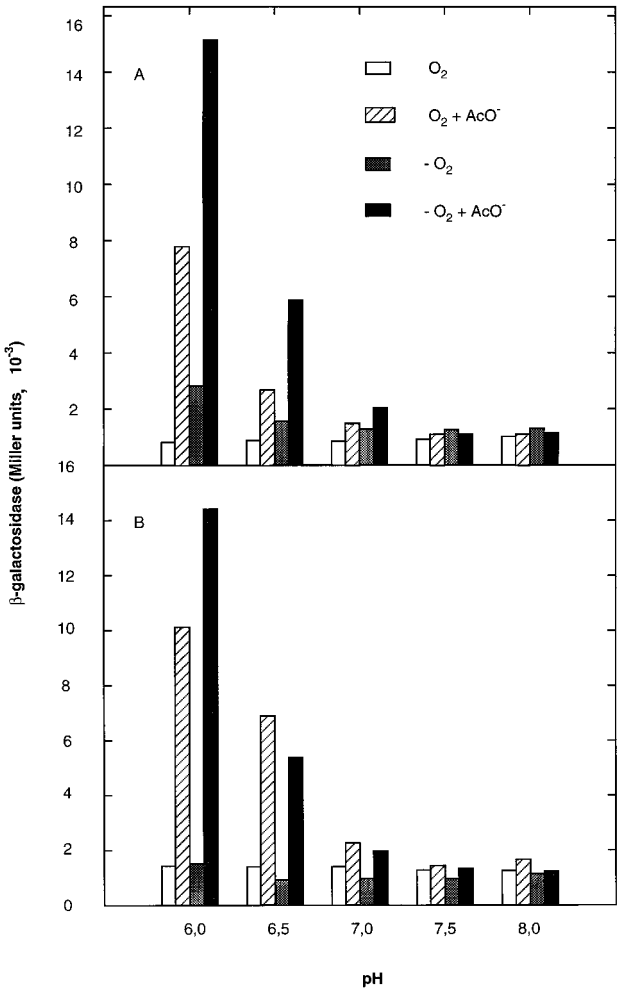


FIG. 5. Expression of the *budA′-lacZ* fusion carried by plasmid pBTK142 in *K. terrigena* KT14 (A) and in *E. coli* BK1 (B). Growth was in TGYEP medium adjusted to different initial pHs. When added, the concentration of acetate (AcO^-) (adjusted to the respective pH) was 40 mM.

the medium, under both aerobic and anaerobic conditions. In *K. terrigena*, anaerobiosis, however, provides an approximately 100% increase above the aerobic expression level, a fact which was not observed in *E. coli*, in which there was even a slight decrease in β-galactosidase activity under anaerobic conditions in the absence of external acetate. Apart from this, however, the regulatory pattern reflects the pattern of transcription and parallels precisely that of synthesis of the enzymes of the butanediol pathway (7). There is convincing evidence, therefore, that the 5′ nontranslated region carries the complete set of motifs active in regulation.

Since acetate appeared to be the prime compound activating transcription mediated by BudR, it was tested whether this is specific or whether other compounds can substitute for acetate. Figure 6 shows that acetate appears to be the physiological inducer, corroborating previous studies in which the level of enzymes of the pathway was measured in cells grown under different conditions (for a review, see reference 21).

Involvement of genes that function in regulation of anaerobiosis. For *E. coli*, a number of genes whose products have some function in the regulation of aerobic and anaerobic metabolism have been identified (see Table 2) (for a review, see

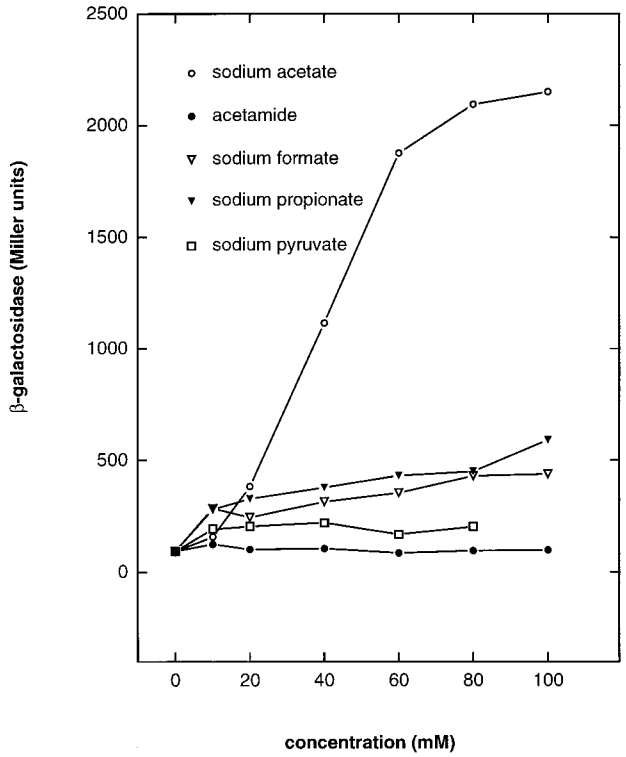


FIG. 6. Effect of organic compounds on the expression of the *budA′-lacZ* fusion from *E. coli* BK1. Anaerobic growth was in TGYEP medium at pH 6.5.

reference 17). To test their involvement in *budABC* operon expression, strains which had the chromosomally located *budR* gene together with the intergenic region and the *budA′-lacZ* fusion of BK1 and single mutations in one of the global regulatory genes were constructed. These strains were analyzed for β-galactosidase formation under anaerobic conditions. Table 2 shows that none of the following genes, when mutated, had a significant influence on the expression: *fhlA*, *arcA*, *ntrA*, *katF*, and *hip*. A deletion of the *fnr* gene, on the other hand, led to an approximately threefold increase in expression, and the presence of the *fnr* gene in *trans* on plasmid pUFR-1 reduced expression by about 50%.

DISCUSSION

During mixed-acid fermentation, enterobacteria degrade carbohydrates into a mixture of acids, alcohols, and CO_2 and H_2. The ratio at which these end products are formed depends on two biochemical constraints: (i) the need to maintain redox balance and (ii) the requirement for maintaining the pH of the medium in a physiological range. Thus, at different pHs, different relative amounts of end products are produced, with the proportion of neutral compounds increasing with decreasing pH of the medium (40).

The biochemical and genetic mechanisms involved in these adjustments are largely unknown. Detailed information is available only for the expression of the formate hydrogenlyase system from *E. coli*. It is regulated by the intracellular concentration of formate and by the level of two regulatory proteins, FhlA and HycA. FhlA activates transcription from σ^{54}-dependent promoters, and HycA counterbalances this activation in an as yet unknown manner. At neutral pH, formic acid is excreted into the medium, thus saving this high-energy compound for energy conservation under anaerobic respiratory conditions. Induction of the formate hydrogenlyase system at low pH appears to be a consequence of the increase in the internal pool of formate. Since the energy of formic acid is conserved in the form of the molecular hydrogen produced and since no redox changes are involved, the main physiological purpose of the formate hydrogenlyase reaction can be seen in pH homeostasis (6, 26, 29, 35).

Not much is known about how the formation of neutral products is adjusted to the formation of acidic compounds in enterobacteria (11). To address this question, we have chosen *K. terrigena*, which, besides ethanol, produces butanediol. The production of butanediol might be an alternative mechanism to prevent acidification of the medium. Previous work had also shown that there is a link between the formation of the butanediol pathway and acidification and that the presence of acetate in the medium is important for induction. Whether it is acetate itself or a biochemically related product is not yet resolved (for a review, see reference 21).

In this communication, we have identified the regulatory protein, termed BudR, responsible for activation of the expres-

TABLE 2. Effects of mutations in global regulatory genes on expression of the *budA′-lacZ* fusion carried by strain BK1[a]

Strain	Mutation	Plasmid	β-Galactosidase activity (Miller units)	
			Uninduced	Induced with 40 mM acetate
BK1	None	None	100 ± 9	725 ± 48
BK11	Δ*fhlA*	None	102 ± 35	949 ± 121
		pSA32 (*fhlA*$^+$)	95 ± 10	968 ± 95
BK12	Δ*fnr*	None	308 ± 13	2,968 ± 414
		pUFR-1 (*fnr*$^+$)	64 ± 12	635 ± 29
BK13	*arcA2 zjj*::Tn*10*	None	117 ± 18	935 ± 45
		pUAA-1 (*arcA*$^+$)	104 ± 14	1,426 ± 50
BK14	*ntrA208*::Tn*10*	None	138 ± 22	1,201 ± 140
		pBN61 (*ntrA*$^+$)	156 ± 32	1,020 ± 119
BK15	*rpoS359*::Tn*10*	None	98 ± 15	945 ± 135
		pRH320 (*katF*$^+$)	83 ± 10	1,035 ± 71
BK16	Δ*3*(*himD*)::Cam^r	None	99 ± 19	1,674 ± 100
		p$P_L$hip.himA-5 (*hip*$^+$)	177 ± 9	1,049 ± 125

[a] Cells were grown in TGYEP medium at pH 6.5 under anaerobic conditions. In order to confirm the results, all measurements were also done with the translational fusions. In all cases, similar values were obtained.

sion of the genes of the butanediol operon. Like AlsR in *B. subtilis*, which regulates acetoin formation under stationary-phase growth conditions (25), BudR belongs to the LysR family of transcriptional activators (14, 32). Its gene, *budR*, is divergently oriented and separated by an intergenic region of 106 bp from the *budABC* operon, encoding the enzymes of the butanediol pathway.

Transfer of *budR* and of the *budABC* operon with the 5′-flanking sequence to the heterologous host *E. coli* confers on this organism the expression characteristics of *K. terrigena*. The dependence of expression on the presence of acetate in the heterologous system indicates that acetate or a follow-up metabolite of it is indeed a ligand of BudR. Purification of BudR and acetate binding experiments should resolve this issue. It is also open whether anaerobiosis and low pH affect expression indirectly via the intracellular level of acetate. The missing induction of the *budA′-lacZ* fusion in *E. coli* compared with that in *K. terrigena* in the absence of external acetate under anaerobic versus aerobic conditions (see Fig. 1 and 5B) may reflect different levels of intracellular acetate. Further evidence for the critical role of acetate is that it can also induce the *budABC* operon substantially under aerobic conditions. This resembles the regulation of the formate hydrogenlyase system by FhlA and formate (26), in which the effect of anaerobiosis resides in the generation of the inducer formate. Thus, in both cases, oxygen does not directly influence expression but acts, upon withdrawal, by adjusting the pool of an inducing metabolite. If this turns out to be true, it is an elegant example for the integration of reactions serving pH homeostasis, the maintenance of the redox balance, and the maximization of energy conservation.

Finally, an intriguing observation is that expression of the *bud* operon under anaerobic conditions responds to the level of the Fnr protein in a reciprocal manner. The relatively moderate effect may reflect the presence of only a half-site of the Fnr binding motif (reference 12 and citations therein). This site overlaps the promoter region of the *budR* gene, and repression could therefore result from competition of Fnr with the binding of RNA polymerase. It is important to note that when the *fnr* mutation was complemented by the Fnr-overproducing plasmid pUFR-1, expression of the *budA′-lacZ* fusion was decreased again. This may indicate that Fnr and BudR compete with each other for binding to their overlapping DNA targets. As to the physiological function of the modulation by Fnr, we can only speculate. One possibility is that Fnr represses expression of the butanediol pathway until the pH decreases below a critical level, under which conditions BudR functions as an *bud*-specific activator. Thus, Fnr may act as a counterbalance to induction by acetate/BudR, a control strategy followed in many other systems.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm "Molekulare Analyse von Regulationsnetzwerken in Bakterien") and by the Consortium für Elektrochemische Industrie GmbH.

We are greatly indebted to R. Hengge-Aronis and G. Sawers for providing strains and plasmids used in this study.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidmann, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. Greene Publishing Associates and Wiley-Interscience, New York.
3. **Begg, Y. A., J. N. Whyte, and B. A. Haddock.** 1977. The identification of mutants of *Escherichia coli* deficient in formate dehydrogenase and nitrate reductase activities using dye indicator plates. FEMS Microbiol. Lett. **2:**47–50.
4. **Birkmann, A.** 1989. Charakterisierung eines DNA-Elements zur anaeroben Expression eines Gärungsenzyms in *Escherichia coli*. Ph.D. dissertation, Ludwig-Maximilians-Universität, Munich, Germany.
5. **Birkmann, A., R. G. Sawers, and A. Böck.** 1987. Involvement of the *ntrA* gene product in the anaerobic metabolism of *Escherichia coli*. Mol. Gen. Genet. **210:**535–542.
6. **Birkmann, A., F. Zinoni, R. G. Sawers, and A. Böck.** 1987. Factors affecting transcriptional regulation of the formate-hydrogen-lyase pathway of *Escherichia coli*. Arch. Microbiol. **148:**44–51.
7. **Blomqvist, K., M. Nikkola, P. Lehtovaara, M. L. Suihko, U. Airaksinen, K. B. Straby, J. K. C. Knowles, and M. E. Penttilä.** 1993. Characterization of the genes of the 2,3-butanediol operons from *Klebsiella terrigena* and *Enterobacter aerogenes*. J. Bacteriol. **175:**1392–1404.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
9. **Brown, T. D. K., C. R. S. Pereira, and F. C. Stromer.** 1972. Studies of the acetate-kinase-phosphotransacetylase and the butanediol-forming systems in *Aerobacter aerogenes*. J. Bacteriol. **112:**1106–1111.
10. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76:**4530–4533.
11. **Chen, Y. M., and E. C. C. Lin.** 1991. Regulation of the *adhE* gene, which encodes ethanol dehydrogenase in *Escherichia coli*. J. Bacteriol. **173:**8009–8013.
12. **Green, J., and J. R. Guest.** 1994. Regulation of transcription at the *ndh* promoter of *Escherichia coli* by FNR and novel factors. Mol. Microbiol. **12:**433–444.
13. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
14. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activators. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
15. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
16. **Iuchi, S., D. Furlong, and E. C. C. Lin.** 1989. Differentiation of *arcA*, *arcB*, and *cpxA* mutant phenotypes of *Escherichia coli* by sex pilus formation and enzyme regulation. J. Bacteriol. **171:**2889–2893.
17. **Iuchi, S., and E. C. C. Lin.** 1993. Adaptation of *Escherichia coli* to redox environments by gene expression. Mol. Microbiol. **9:**9–15.
18. **Johansen, L., K. Bryn, and F. C. Stormer.** 1975. Physiological and biochemical role of the butanediol pathway in *Aerobacter* (*Enterobacter*) *aerogenes*. J. Bacteriol. **123:**1124–1130.
19. **Kleckner, N., D. F. Barker, D. G. Ross, and P. Botstein.** 1978. Properties of the translocatable tetracycline resistance element Tn*10* in *Escherichia coli* and bacteriophage lambda. Genetics **90:**427–450.
20. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of the stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:**49–59.
21. **Magee, R. J., and N. Kosaric.** 1987. The microbial production of 2,3-butanediol. Adv. Appl. Microbiol. **32:**89–161.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nash, H. A., C. A. Robertson, E. Flamm, R. A. Weisberg, and H. I. Miller.** 1987. Overproduction of *Escherichia coli* integration host factor, a protein with nonidentical subunits. J. Bacteriol. **169:**4124–4127.
24. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53:**293–321.
25. **Renna, M. C., N. Najimudin, L. R. Winik, and S. A. Zahler.** 1993. Regulation of the *Bacillus subtilis alsS*, *alsD*, and *alsR* genes involved in post-exponential-phase production of acetoin. J. Bacteriol. **175:**3863–3875.
26. **Rossmann, R., G. Sawers, and A. Böck.** 1991. Mechanism of regulation of the formate-hydrogen-lyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. Mol. Microbiol. **5:**2807–2814.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Sauter, M., R. Böhm, and A. Böck.** 1992. Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli*. Mol. Microbiol. **6:**1523–1532.
30. **Sawers, G., and A. Böck.** 1989. Novel transcriptional control of the pyruvate-formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. J. Bacteriol. **171:**2485–2498.
31. **Sawers, G., and B. Suppmann.** 1992. Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. J. Bacteriol. **174:**3474–3478.
32. **Schell, M.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.

33. **Schlaman, H. R. M., R. J. H. Okker, and B. J. J. Lugtenberg.** 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. **174:**5177–5182.
34. **Schlensog, V.** 1993. Genetische Analyse, Struktur und Funktion des Formiat-Hydrogen-Lyase-Aktivatorproteins (FHLA) von *Escherichia coli*. Ph.D. dissertation, Ludwig-Maximilians-Universität, Munich, Germany.
35. **Schlensog, V., and A. Böck.** 1990. Identification and sequence analysis of the gene encoding the transcriptional activator of the formate-hydrogen-lyase system of *E. coli*. Mol. Microbiol. **4:**1319–1327.
36. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
38. **Stromer, F. C.** 1968. Evidence for induction of the 2,3-butanediol-forming enzymes in *Aerobacter aerogenes*. FEBS Lett. **2:**36–38.
39. **Stromer, F. C.** 1968. The pH 6 acetolactate-forming enzyme from *Aerobacter aerogenes*. I. Kinetic studies. J. Biol. Chem. **243:**3735–3739.
40. **Wood, W. A.** 1961. Fermentation of carbohydrates and related compounds, p. 59–149. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria: a treatise on structure and function, vol. 2. Academic Press, Inc., New York.
41. **Yanisch-Perron, G., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
42. **Zinoni, F., A. Birkmann, T. C. Stadtman, and A. Böck.** 1986. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**4650–4654.