

Butanol Tolerance of *Clostridium beijerinckii* NCIMB 8052 Associated with Down-Regulation of *gldA* by Antisense RNA

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Abstract

Strain BR54 of *Clostridium beijerinckii* was derived from the wild type strain, NCIMB 8052, by mutagenesis with Tn1545 and selection for butanol tolerance. It harbours a single copy of Tn1545 in a 435 bp intergenic region separating two convergently transcribed genes, *accC* and *gldA*. The former encodes biotin carboxylase (E.C.6.3.4.14), a subunit of acetyl-CoA carboxylase and the latter encodes glycerol dehydrogenase (E.C.1.1.1.6). Since Tn1545 generates outwardly directed transcripts from its right end, we considered the possibility that the transposon inserted in strain BR54 might affect the expression of the adjacent *gldA* gene. RT-PCR experiments revealed that the mutant, but not the wild type, contains antisense RNA corresponding to the *gldA* gene. Correlated with this, the level of glycerol dehydrogenase activity in the mutant was only 25% of that in the wild type when bacteria were grown with either glucose or glycerol as the fermentable substrate. We conclude that transcripts emerging from the right end of the conjugative transposon, Tn1545, can reduce the expression of the adjacent *gldA* gene by the generation of antisense RNA and that this is associated with a butanol-tolerant phenotype.

Introduction

The solventogenic clostridia, *Clostridium acetobutylicum* and *C. beijerinckii*, are of interest because of the potential economic importance of their fermentation end products, butanol, acetone and hydrogen (reviewed by Minton and Clarke, 1989; Sebald, 1992; Woods, 1993). These organisms could once again be used for the production of useful chemicals from renewable biomass, if the efficiency of product recovery were increased and strain performance improved (Morris, 1993). Solventogenesis and the concomitant formation of endospores in these organisms

are generally thought to be limited by the accumulation of toxic fermentation end products in the culture. Butanol is the most toxic of these (Ballongue *et al.*, 1987; Izard *et al.*, 1989), and it is produced in greater amounts than either acetone or ethanol (Jones and Woods, 1986; Rogers and Gottschalk, 1993).

Butanol-tolerant *C. acetobutylicum* mutants, both spontaneous and nitrosoguanidine-generated, have been selected by growth in butanol-containing media. Some butanol-tolerant strains produced more solvent than the parental cells (Hermann *et al.*, 1985; Pinazo *et al.*, 1993; Quratulain *et al.*, 1995). Other mutant strains produced only minimal amounts of solvent and one sporulated poorly (Baer *et al.*, 1987). In contrast, a hyper-amyolytic mutant of *C. beijerinckii* NCIMB 8052, strain BA101, could also be butanol-tolerant, as it produces significantly higher levels of solvents than the wild type (Formanek *et al.*, 1997). The molecular basis of butanol tolerance has not yet been established.

In this report we describe the isolation of butanol-tolerant transposon-insertion mutants of *C. beijerinckii* NCIMB 8052 and present evidence suggesting that butanol-tolerance is associated with reduced activity of the enzyme, glycerol dehydrogenase. We also present evidence that antisense RNA decreases the expression of the *gldA* gene, which encodes glycerol dehydrogenase. The relevant transcripts originate in the transposon, which is inserted downstream of the gene in the mutant strains.

Results

Butanol Tolerance of Wild Type *C. beijerinckii* NCIMB 8052 and Strain BR54

A number of transposon-insertion mutants from independent matings were isolated as colonies on agar plates consisting of medium T.5 supplemented with erythromycin and 1.2% w/v butanol, and purified by re-streaking for single colonies on similar plates. The butanol tolerance of strains BR54 and BR71, which were derived in separate matings, was tested in liquid culture (Figure 1). The wild type cells were able to grow in the presence of up to 0.4% 1-butanol; in other experiments these cells grew with 0.6% 1-butanol. In contrast, strains BR71 and BR54 were able to grow in the presence of up to 1-1.1% 1-butanol. In other respects the mutant phenotype did not appear to differ from that of the wild type. In the absence of added alcohol the wild type and strain BR54 grew equally rapidly (culture doubling times, 38 and 39.6 min) in medium T.5 (data not shown), and produced comparable amounts of the fermentation end products, butanol, acetone, butyrate, acetate, and ethanol (data not shown). The cells did not produce detectable amounts of 1,3-propanediol (data not shown).

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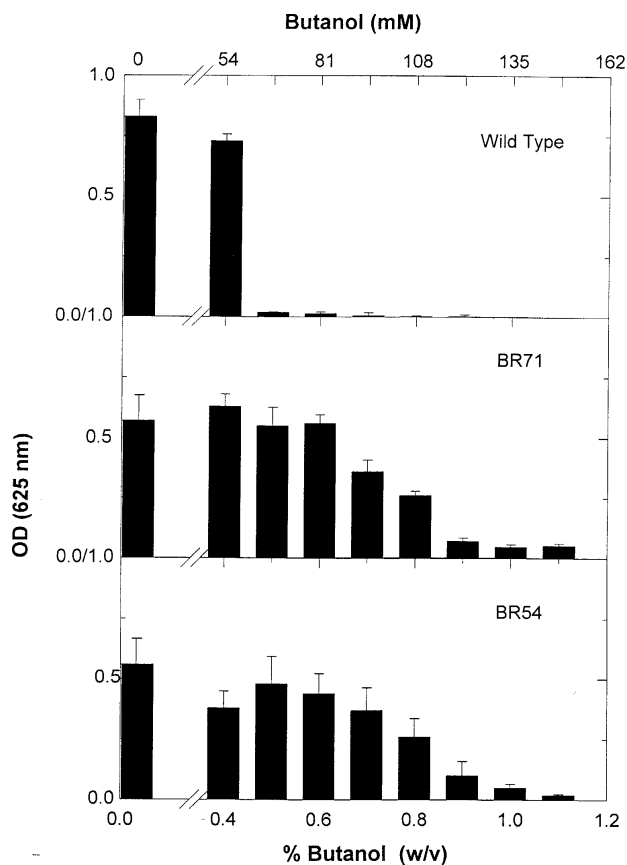


Figure 1. Butanol tolerance of *C. beijerinckii* NCIMB 8052 and Strain BR54. Inoculated cultures were activated, supplemented with 1-butanol, and the OD (625 nm) was determined at intervals. The values shown are those obtained at the end of growth after 2 d at 37°C. The values are the means of the ODs (625 nm) \pm SEM of 3 replicate experiments.

Characterization of the Tn1545 Insertion in Strain BR54

DNA from strains BR54 and BR71 was digested with *Hind*III and probed with plasmid pAT187 (Trieu-Cuot *et al.*, 1987), which contains an *aphA-3* gene very similar to that of Tn1545 (Caillaud *et al.*, 1987). In both cases, the probe hybridized with one 7.3-kb fragment, indicating that a single copy of Tn1545 had inserted into the chromosome of these

independently isolated strains, leading to an identical butanol-tolerant phenotype. We concluded that the butanol-tolerant phenotype is the result of the Tn1545 insertion. Strain BR54 was selected for further studies.

To investigate the transposon insertion site we cloned junction fragments containing the transposon ends and adjacent chromosomal DNA segments, and sequenced them. Two primers, RER3 and LER1 (Figure 2), were then designed and used to obtain the corresponding, uninterrupted DNA segment from wild type cells by PCR amplification. The sequence of this DNA segment precisely matched that of the chromosomal segments within the junction fragments previously amplified from strain BR54.

Tn1545 is Located in an Intergenic Region Downstream from the *gldA* Gene

The 2298 bp fragment between the *Hind*III recognition sites contains one complete open reading frame (ORF) and one partial ORF, separated by a 435 bp intergenic sequence into which Tn1545 is inserted in strain BR54 (Figure 2). The deduced amino acid sequence of the complete ORF bears a strong similarity to *gldA*, the gene encoding glycerol dehydrogenase in a number of bacteria (Figure 3). Similarity was highest (47-51% identity, 62-70% similarity) to the *dhaB* gene of *Citrobacter freundii* (GenBank accession no. P45511), and the *gldA* genes of *Escherichia coli* (P32665), *Pseudomonas putida* (P50173), and *Bacillus subtilis* (P32816), followed by a large number of alcohol dehydrogenases. These dehydrogenases belong to the Type III iron-activated family of ADH enzymes (Reid and Fewson, 1994). They share two signature motifs, ADH_IRON_1 and ADH_IRON_2 (PROSITE motifs PS00913 and PS00060). The truncated ORF was only 633 bp in length, but its corresponding amino acid sequence was sufficiently similar to those of other genes in the DNA sequence database to permit unambiguous identification as *accC*. This gene encodes biotin carboxylase (E.C. 6.3.4.14), a subunit of acetyl-CoA carboxylase. The genes most closely related to the *C. beijerinckii accC* gene (56 to 65% identity and 71 to 82% similarity) were those of several prokaryotes (GenBank accession numbers), *Bacillus subtilis* (D84432), *Methanobacterium thermoautotrophicum* (AE000942), *Methanococcus jannaschii* (Q58626), *Archaeoglobus fulgidus* (AE001090), as well as *Anabaena* (Q06862) and *Synechocystis sp.* (D64001).

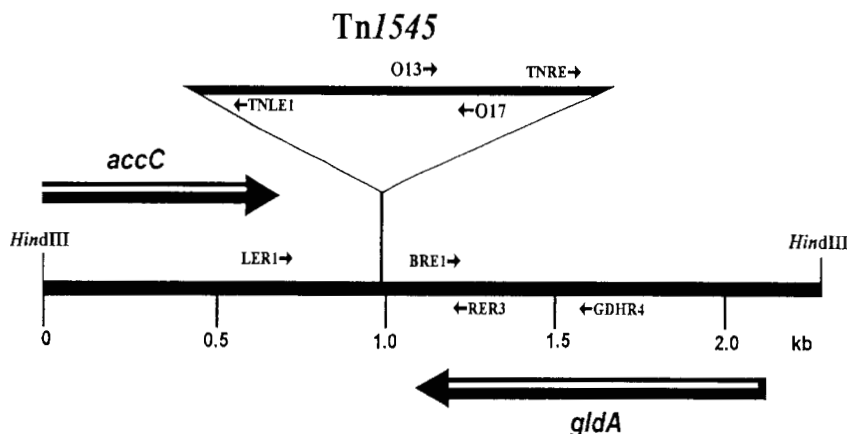


Figure 2. Diagram of the chromosomal region of *C. beijerinckii* NCIMB 8052 disrupted by Tn1545 insertion in strain BR54. The 2298 bp *Hind*III chromosomal DNA fragment and Tn1545 are shown as thick lines. The positions of some of the PCR primers used are shown in relation to the *gldA* and *accC* genes (double lines with arrowheads) and to the Tn1545 insertion site at nt 995. Numerical values are in units of kilobase pairs. Tn1545 is not drawn to scale.

GLDA_CBEIJMI	APSKYIQNG	ELKNIAEYVS	VLGEKALCLI	SESGLRKRVK	42
GLDA_CITFR	...MLKVIQ	SPAKYLQGP	ASTLFGQYAK	NLADSEFFVIA	DDFVVKLAGE	46
GLDA_PSEPU	...MDRAIQ	SPGKYVQGD	ALQRLGDYLK	PLADSWLVIA	DKFVLGFAED	46
GLDA_ECOLI	...MDRIIQ	SPGKYIQGD	VINRLGEYK	PLAERWLTVG	DKFVLGFAQS	46
GLDA_BACST	..MAAERVFI	SPAKYVQGN	VITKIANYLE	GIGNKTVVIA	DEIVVKIAGH	48
*	..*	..*	..*	
GLDA_CBEIJ	TIDKSFETKN	IGVKYDLFNG	ECSITEVNRI	IKICDENQLT	VLIGIGGGKI	92
GLDA_CITFR	KVLNGLHSHD	ISCHAERFNG	ECSHIEINRL	IAILKQHGCR	GVVIGGGKT	96
GLDA_PSEPU	TIRQSLSKAG	LAMDIVAFNG	ECSQGEVDRL	CQLATQNGRS	AIVGIGGGKT	96
GLDA_ECOLI	TVEKSFKDAG	LVVEIAPFNG	ECSQNEIDRL	RGIAETAQCG	AIVGIGGGKT	96
GLDA_BACST	TIVNELKKG	IAAEVVFSG	EASRNEVERI	ANIARKAEAA	IVIGVGGGKT	98
*	..*	..*	..*	
GLDA_CBEIJ	IDTIKAVGYY	ANLPVVIVPT	IAATDAPCSA	LSVLYTDDGV	FDKYLFLKQN	142
GLDA_CITFR	LDTAKAIGYY	QKLPVVIVPT	IASTDAPCSA	LSVIYTEAGE	FEEYLIYPKN	146
GLDA_PSEPU	LDTAKAVAFF	QKVPVAVAPT	IASTDAPCSA	LSVLYTDEGE	FDRYLMPLPN	146
GLDA_ECOLI	LDTAKALAHF	MGVPAIAPT	IASTDAPCSA	LSVIYDEGE	FDRYLLLPNN	146
GLDA_BACST	LDTAKAVADE	LDAYIVIVPT	AASDAPCSA	LSVIYSDDG	FESYRFYKKN	148
	..*	..*	..*	..*	..*	
	-----	ADH_IRON_1	-----			
GLDA_CBEIJ	PNIVLVDTGI	IAKAPVRLV	SGMGDALATY	FEARACEKSN	AGTCSIFGTT	192
GLDA_CITFR	PDMVMDTAI	IAKAPVRLV	AGMGDALSTW	FEAKACYDAR	ATMA.GGQS	195
GLDA_PSEPU	PALVVVDTAI	VARAPARLLA	AGIGDALATW	FEARAASRSS	AATMA.GGPA	195
GLDA_ECOLI	PNMVIVDTKI	VAGAPARLLA	AGIGDALATW	FEARACSRSG	ATTMA.GGKC	195
GLDA_BACST	PDLVLVDTKI	IANAPRLLA	SGIADALATW	VEARSVIKSG	GKTMA.GGIP	197
	..*	..*	..*	..*	..*	
	-----		-----			
GLDA_CBEIJ	TITQAALARL	CYDTLITEGY	KAKLAVEEGV	CTKSVEKIVE	ANTLLSGLGF	242
GLDA_CITFR	TVAALSLARL	CYDTLLAEGE	KARFAAQAGV	VTDALERIVE	ANTYLSGLGF	245
GLDA_PSEPU	TQTALNLARF	CYDTLLEEGE	KAMLAVQAQV	VTPALERIVE	ANTYLSGVGF	245
GLDA_ECOLI	TQAALALAE	CYNTLLEEGE	KAMLAAEQHV	VTPALERVIE	ANTYLSGVGF	245
GLDA_BACST	TIAAEIAEK	CEQTLFKYK	LAYESVKAKV	VTPALEAVVE	ANTLLSGLGF	247
	..*	..*	..*	..*	..*	
	-----	ADH_IRON_2	-----			
GLDA_CBEIJ	ESGGIAARHA	IHNGLTVLP.	ACHHMYHGEK	VAFGTLAQLV	LENAPMDEIE	291
GLDA_CITFR	ESSGLAGAHA	IHNGF TILE.	ECHHLYHGEK	VAFGTLAQLV	LQNSPMEEIE	294
GLDA_PSEPU	ESGGVAAAHA	VHNGLTAVA.	ETHHFYHGEK	VAFGVLVQLA	LENASNAEMQ	294
GLDA_ECOLI	ESGGIAAAHA	VHNGLTAIP.	DAHYYHGEK	VAFGTLTQLV	LENAPVEEIE	294
GLDA_BACST	ESGGIAAAHA	IHNGF TALEG	EIHHLTHGEK	VAFGTLVQLA	LEHSSQEEIE	297
	..*	..*	..*	..*	..*	
GLDA_CBEIJ	EVLDFSTRVG	LPVTLKQLGI	NEIKPEEIE	VAKAATSKED	TAHNMPFEVT	341
GLDA_CITFR	TVLNFQCKVG	LPVTLAEMGV	KDDIDGKIMA	VAKATCAEGE	TIHNMPFSVT	344
GLDA_PSEPU	EVMSLCHAVG	LPITLAQLDI	TEDIPTKMRA	VAEALACAPGE	TIHNMPGGVT	344
GLDA_ECOLI	TVAALSHAVG	LPITLAQLDI	KEDVPAKMRI	VAEAACAEGE	TIHNMPGGAT	344
GLDA_BACST	RYIELYLSLD	LPVTLEDIKL	KDASREDILK	VAKAATAEGE	TIHNA.FNVT	346
	..*	..*	..*	..*	..*	
GLDA_CBEIJ	PEDVDCYFN	S.....	352		
GLDA_CITFR	PESVHAAILT	ADLLGQQWLA	R...	365		
GLDA_PSEPU	VEQVYGALLV	ADQLGQHFL	F...	365		
GLDA_ECOLI	PDQVYAALLV	ADQYQRF.	363		
GLDA_BACST	ADDVADAI	FAADQYAKAYKE	KHRK	370		
	..*	..*	..*	..*	..*	

Figure 3. Multiple amino acid sequence alignment of bacterial glycerol dehydrogenases. The sequences of four gene products most closely related (database accession numbers in parentheses) to the predicted product of *gldA* of *C. beijerinckii* NCIMB 8052 are shown. The order from top to bottom is: *C. beijerinckii* NCIMB 8052 (AF136281); *Citrobacter freundii* (P45511); *Escherichia coli* (P32665), *Pseudomonas putida* (P50173), *Bacillus stearothermophilus* (M65289). BLAST searches are based on the method of Altschul *et al.* (1997). Gene products were aligned with the MACAW (Multiple Alignment Construction and Analysis Workbench) software (Schuler *et al.*, 1991) and the Multalin program (Corpet, 1988). Residues conserved in at least 4 gene products are in boldface. Residues conserved or conservatively substituted, in all 5 gene products, are marked with an asterisk and a dot, respectively. Iron-containing alcohol dehydrogenase signature motifs ADH_IRON_1 (PROSITE motif PS00913) and ADH_IRON_2 (PS00060) are indicated.

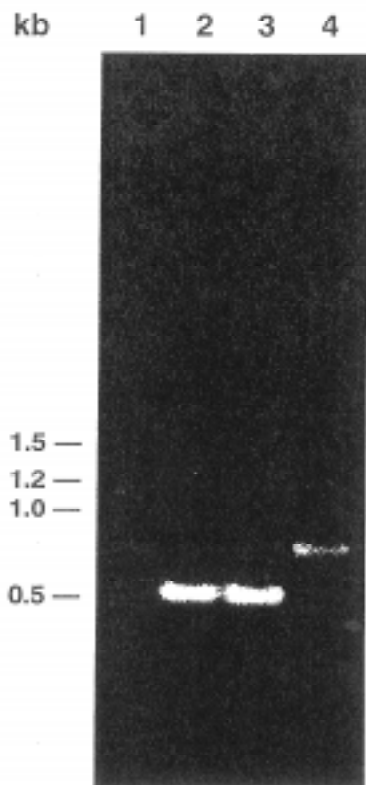


Figure 4. Reverse transcriptase PCR assays. Total RNA extracted from the wild type (lanes 1 and 3) and from strain BR54 (lanes 2 and 4) was employed for RT-PCR using the primer pairs, TNRE and GDHR4 (lanes 1 and 4), and BRE1 and GDHR4 (lanes 2 and 3). The lines at the left indicate the sizes of marker DNA fragments.

Tn916 and its cognate element, Tn1545, contain outwardly directed promoters at their right ends, but not their left ends (Celli and Trieu-Cuot, 1998; Clewell *et al.*, 1988). Tn1545 is located in the intergenic region separating the convergently transcribed *accC* and *gldA* genes in strain BR54 with the left end adjacent to *accC* and the right end adjacent to *gldA* (Figure 2). It is unlikely that Tn1545 would affect *accC* expression and further work therefore focused on *gldA*.

Generation of Antisense RNA

We considered the possibility that the transposon inserted in strain BR54 might interfere with the expression of *gldA* located adjacent to its right end. This hypothesis was tested using antisense RNA-specific primers for RT-PCR amplification (Figure 2). With the antisense RNA-specific primer, GDHR4, the RNA prepared from strain BR54 yielded a cDNA, which was then amplified to give a PCR product of approximately 750 bp (Figure 4). A 764 bp product was expected from the distance between the priming sites in strain BR54 DNA (Figure 2). RNA prepared from the wild type did not yield a product. In the control experiment the primer pairs designed from sequences within the *gldA* coding sequence resulted in RT-PCR products of the expected size (533 bp) with both the wild type and the strain BR54 RNA preparations.

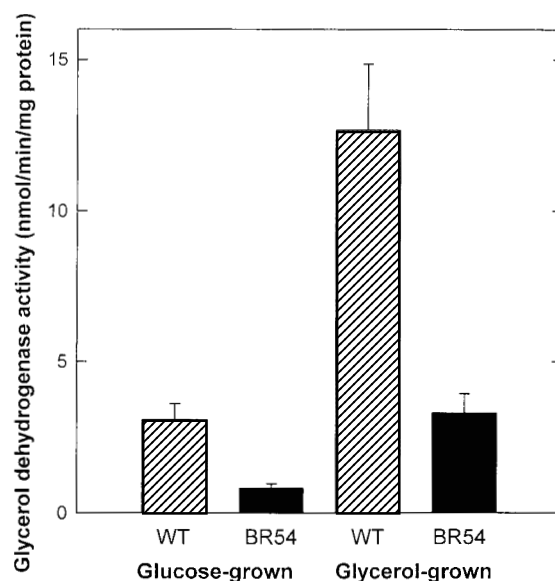


Figure 5. Glycerol dehydrogenase activity of cell-free extracts of wild type *C. beijerinckii* NCIMB 8052 (WT) and strain BR54. Cells were grown with glucose or glycerol, as indicated on the abscissa, and extracts prepared as described in the text. The means and standard errors of the means of 11-13 replicate glycerol dehydrogenase assays carried out in 5-8 experiments are shown.

Expression of *gldA* in the Wild Type and Mutant Strain

We postulated that the generation of antisense RNA specific for the *gldA* gene could result in decreased gene expression. To test this hypothesis we assayed cell-free extracts of strain BR54 and the wild type for glycerol dehydrogenase activity (Figure 5). Extracts of glucose-grown cells of the wild type and strain BR54 had glycerol dehydrogenase activities with mean values of 3.07 and 0.81 nmol/min/mg protein, respectively. The glycerol dehydrogenase activity in strain BR54 was reduced to ca. 25% of the activity seen in the wild type. The same 75% reduction in the mutant strain was found in cells grown with glycerol as the fermentable substrate. *C. beijerinckii* NCIMB 8052 is classified as glycerol-negative (Woolley, 1988), and the cells grew very poorly indeed with glycerol as fermentable substrate, reaching an $OD_{625\text{ nm}}$ of only 0.2-0.3 after 24 h incubation. In contrast, cultures with glucose as fermentable substrate, had a doubling time of 39 min and reached an $OD_{625\text{ nm}} > 1$ after 24 h incubation. Nevertheless, glycerol-grown cells had a higher glycerol dehydrogenase activity than the glucose grown cells. Thus, in wild type extracts, the glycerol dehydrogenase activity ranged from 7 to 28 nmol/min/mg protein (mU/mg protein), with a mean value of 12.7 nmol/min/mg protein. The mutants had a mean activity of 3.3 nmol/min/mg protein, equivalent to 26% of the wild type activity. The mutant extracts were significantly lower in activity than those of the parental cells in each of the 10 independent experiments carried out ($P < 0.001$) in both glucose and glycerol-grown cultures.

Discussion

Insertion of transposons into bacterial chromosomes usually results in the loss or impairment of function if the inserted DNA has disrupted a gene. However, insertion at a site upstream from a transcriptional start site but downstream from a promoter region can result in gene expression if the transposon provides an alternate promoter. This is the case for the conjugative transposons, Tn916, and its cognate element, Tn1545, which have the same sequence for about 250 bp at either end, and virtually identical structures within their right ends (reviewed by Rice, 1998). Tn1545 and Tn916 have outwardly directed promoters in their right ends (Celli and Trieu-Cuot, 1998; Clewell *et al.*, 1988) which may be responsible for the hyper-hemolytic phenotype frequently observed in *Enterococcus faecalis* in which Tn916 has inserted proximally to the hemolysin determinant in plasmid pAD1 (Ike *et al.*, 1992). Detailed analysis of Tn916 promoters, carried out by Celli and Trieu-Cuot (1998), demonstrated that transcripts originating from the right end of this element extend through the transposon attachment site in the circular intermediate formed during transposition. This activates the *tra* determinants carried by the other arm of the transposon. A similar effect was also seen in Tn1545 mutants of *C. beijerinckii* NCIMB 8052 selected for reduced tendency to degenerate caused by reduced growth rates (Evans *et al.*, 1998). Tn1545 was inserted into the *fms* gene, truncating 23 amino acid residues from the N terminus of its product, peptide deformylase. Nevertheless, a functional enzyme was present in the mutant, and its transcription was apparently initiated from promoter(s) within the right end of the transposon (Evans *et al.*, 1998).

Transposons Tn916 and Tn1545 prefer to insert into A+T-rich regions of clostridial DNA, i.e., intergenic regions (Woolley *et al.*, 1989). In this investigation we have shown that Tn1545 can also affect expression of an adjacent gene from a downstream location, probably through the production of antisense RNA.

The regulatory role of natural antisense RNA in gene expression in prokaryotes has been recognized for many years. Some of the best-known examples of antisense RNA-regulated systems concern processes associated with accessory elements (e.g. bacteriophage development, plasmid copy number control and IS10 transposition). However, a role for antisense RNA in the regulation of chromosomal gene expression in bacteria is also well established (reviewed by Coleman *et al.*, 1984; Delilhas, 1995; Inouye, 1988; Simons, 1988; Simons and Kleckner, 1988; Wagner and Simons, 1994). Antisense RNA usually exerts a negative effect on gene expression. Naturally occurring antisense RNA can be synthesized from promoters adjacent to the gene so that both strands of the same DNA segment are transcribed, giving the sense and antisense RNA molecules with complete complementarity. Antisense RNA may also be transcribed from DNA at a separate site from its target gene, in which case the two RNA molecules may have only partial complementarity. There are numerous examples of small, <200 nt *trans*-acting RNA molecules regulating gene expression (Delilhas, 1995). Glutamine synthetase regulation in *Clostridium acetobutylicum* P262 involves a 43 nt antisense RNA transcribed from a promoter located 227 bp downstream from the 3' end of the *glnA* structural gene. This antisense

RNA is complementary to the 5' end of *glnA* overlapping the ribosomal binding site and the transcriptional start site. The antisense RNA thus inhibits expression of the mRNA generated from the 5' promoters of *glnA* by hybridizing with it (Fierro-Monti *et al.*, 1992).

Since one or more transcripts emerge from the right end of Tn1545 (Evans *et al.*, 1998; Trieu-Cuot *et al.*, 1991), it seemed likely that they would extend into adjacent sequences, even if these encompassed a convergently transcribed gene. In strain BR54 of *C. beijerinckii*, Tn1545 is positioned and oriented such that it would produce a counter-transcript of *gldA*. We have demonstrated the existence of antisense RNA corresponding to this counter-transcript in strain BR54 by reverse transcriptase-effected synthesis of the predicted PCR product when strand-specific oligonucleotides were used as primers. The similarity of the ends of Tn1545 and Tn916 suggests that one (or more) of the counterparts of the several promoters identified in Tn916 (Celli and Trieu-Cuot, 1998) directs the synthesis of antisense RNA by Tn1545 in strain BR54. The biological relevance of this antisense RNA was confirmed by measurements of the activity of glycerol dehydrogenase, which was decreased by 75% in the mutant, as compared with the wild type strain. Moreover, the slightly increased glycerol dehydrogenase activity that was associated with growth in glycerol-supplemented medium, was also 75% lower in strain BR54 as compared with the wild type. To our knowledge, this is the first documented evidence of antisense regulation of gene expression associated with insertion the conjugative transposon, Tn1545.

Glycerol dehydrogenase activity is readily seen in cell-free preparations from a number of bacterial species grown anaerobically on glycerol. Specific activities ranging from 1.4 to 4.5 $\mu\text{mol}/\text{min}/\text{mg}$ protein have been reported for crude cell extracts from a variety of organisms, including *C. butyricum* (Abbad-Andaloussi *et al.*, 1996), *C. pasteurianum* (Luers *et al.*, 1997), *Citrobacter freundii* (Daniel *et al.*, 1995; Luers *et al.*, 1997), and *Klebsiella aerogenes* (Ruch *et al.*, 1974). In contrast, the activity measured in *C. beijerinckii* NCIMB 8052 extracts was approximately 3 nmol/min/mg protein, which is three orders of magnitude lower. A low enzyme activity is consistent with the inability of *C. beijerinckii* NCIMB 8052 to grow significantly on glycerol (Woolley, 1998). The level of glycerol dehydrogenase activity in *C. beijerinckii* NCIMB 8052 was similar to that of wild type *E. coli*, which is also unable to grow on glycerol if the *glp* glycerol dissimilation system is not present (Jin *et al.*, 1983). *E. coli gldA* promoter mutants have been isolated (Jin *et al.*, 1983; Truninger and Boos, 1994) that are able to grow on this substrate. In *C. beijerinckii* NCIMB 8052 no mutants able to grow on glycerol were isolated from the wild type upon prolonged incubation or repeated transfer to fresh glycerol-containing media (H.L. and E.R.K., unpublished data). Unlike *E. coli* (Jin *et al.*, 1983; Trieu-Cuot *et al.*, 1987; Truninger and Boos, 1994), the *gldA* gene of *C. beijerinckii* NCIMB 8052 was not induced by hydroxyacetone. Like the enzyme of *E. coli*, the role of the clostridial glycerol dehydrogenase is not clear. One phenotypic consequence of lowering its level of expression in *C. beijerinckii* is reduced sensitivity of the cells to butanol. The underlying basis of the biotechnologically important phenotype is currently under investigation.

Experimental Procedures

Growth of Bacteria

The methods used for growing *Clostridium beijerinckii* NCIMB 8052 cells and assaying their fermentation end products have been described previously (Kashket and Cao, 1993). The cells were routinely grown in medium T.5, which is a modified (Kashket and Cao, 1993) medium TYA (Ogata and Hongo, 1973) containing 0.5% (w/v) glucose; erythromycin (Em) was added at 25 µg/ml for strain BR54. Fermentation end products were assayed by GC as described (Kashket and Cao, 1993). For 1,3-propanediol assays the chromatographic conditions were modified so that the column temperature was 200°C and the detector, 250°C (Forsberg, 1987). Strains BR54 and BR71 were isolated from two separate banks of Tn1545 insertions in *C. beijerinckii* NCIMB 8052 (wild type) using previously published procedures (Kashket and Cao, 1993; Woolley *et al.*, 1989).

Butanol Tolerance Assays

Thick-walled glass tubes containing 5 ml medium T.5 were inoculated with 100 µl sporulated cultures, sealed with rubber stoppers and crimped with aluminum seals, activated by heating 10 min at 70°C, and then replaced within the anaerobic hood. 1-butanol was added to the cultures to give the desired concentration, the tubes were resealed and incubated at 37°C.

Cloning and Nucleotide Sequence Determination of Clostridial DNA Flanking Tn1545

Chromosomal DNA was isolated from strain BR54, digested with *Hind*III and ligated to *Hind*III-cut pBluescript KS+ (Stratagene, La Jolla, CA), as described previously (Evans *et al.*, 1998). Ligation-mediated PCR amplification was used to isolate the clostridial DNA adjacent to the right and left ends of Tn1545. PCR amplification of the right end junction DNA was carried out using as the primers, TnRE (5' CACAACA GGAAGATTTCATAG 3'), specific for the right end of Tn1545 (Trieu-Cuot *et al.*, 1991), and KS, specific for the pBluescript vector (Stratagene). Rare ligation products where the appropriate vector end had ligated with the appropriate Tn1545 junction fragment end were amplified, giving a fragment of approximately 1.4 kb. This fragment was digested with *Hind*III and ligated to *Hind*III/*Hinc*II-cut pBluescript. The inserted DNA was manually sequenced on both strands, using the dideoxy chain termination method of Sanger *et al.*, (1977) and Sequenase 2.0 (USB Corp., Cleveland, OH). For the Tn1545 left end junction DNA, the primers TNLE1 and KS were used. Primer TNLE1 (5'-ATGAAAGCTTGGATTAATCGTCGTATCAAAG-3') is primer TNLE (Caillaud and Courvalin, 1987; Trieu-Cuot *et al.*, 1991) modified to include a *Hind*III restriction enzyme recognition site near its 5' end. The ca. 1.0 kb fragment obtained, was digested with *Hind*III and ligated into *Hind*III-cut pBluescript; 630 bp of the inserted DNA were sequenced.

Nucleotide Sequence Determination of the Corresponding DNA Segment from the Wild Type Strain

Two primers derived from the known sequences of clostridial DNA flanking the Tn1545 insertion site were used to amplify the corresponding DNA segment from the wild type strain. The positions and orientations of the primers in relation to the DNA fragment under investigation are shown in Figure 3. The primers were RER3 (5' GCACTCGAGTTCCTGTTACT TAAACAATT3'), which incorporates an *Xho*I site near its 5' end, and LER1 (5' TACTCTAGAATAAATCTTATGATTTAAT3'), which has an added *Xba*I site near its 5' end. The resulting ca. 0.6 kb fragment was cloned into pBluescript KS+ and sequenced on both strands.

RT-PCR

RNA was extracted from wild type *C. beijerinckii* NCIMB 8052 and strain BR54 using the RNeasy kit (Qiagen, Valencia, CA). The RNA was digested with RNase-free DNase I (Life Technologies, Grand Island, NY) and re-purified using RNeasy columns (Qiagen). The strand-specific primers GDHR4 (5' AGCTCCAGTTCGTCTTTTAGTATCT3'), which is specific for antisense RNA, and BRE1 (5' GCAGTATCTCTTTTGAAGTAGCAGCC3'), which is specific for sense RNA, were used for first strand cDNA synthesis using SuperScript II AMV Reverse Transcriptase (Life Technologies). PCR was conducted using the primer pair TNRE and GDHR4. In other reaction tubes the primer pair, GDHR4 and BRE1, was used.

Sequencing the Right End of Tn1545

Based on the transcriptional analysis of Tn916 (Celli and Trieu-Cuot, 1998), the primers, O₁₃ (5' CGGAATTCGGTACGTCCACCAATGTGG3'), which also contained an added *Eco*RI site at the 5' end, and O₁₇ (5' CGGGATCCGAGGAAAGGGAATCATAGG3'), which also contained an added *Bam*HI site at the 5' end, were used to amplify a region containing orf7 from BR54 DNA. The fragment obtained was cloned into pBluescript and sequenced using the primers, O₁₃ and O₁₇. The nucleotide sequence of the insert was identical to that of the corresponding segment of Tn916 (Celli and Trieu-Cuot, 1998).

Preparation of Cell-Free Extracts

Inoculum cultures were prepared from activated spores and grown overnight at 37°C as described previously (Kashket and Cao, 1993). They were used to inoculate 150 ml medium T.5 containing 0.5% glucose and the cells were grown until mid-exponential phase (OD_{625 nm} = 0.6-0.7). Cells were also grown with 0.5% glycerol instead of glucose, reaching an OD_{625 nm} = 0.2-0.3 in 24 h. The cells were centrifuged at 4°C at 10,000 x g in a Servall centrifuge (Kendo Laboratory Products, Newtown, CT) for 15 min in closed Oak Ridge centrifuge tubes (Nalge Nunc International, Rochester, NY). The tubes were returned to the anaerobic hood (Bactron II, Sheldon Manufacturing Inc., Cornelius, OR), the cells washed with 30 ml 10 mM Tris buffer, pH 8, and re-centrifuged. The pellets were kept for at least 1 h on ice within the anaerobic hood to ensure anaerobiosis, then suspended in 500 µl 10 mM Tris buffer, pH 9. The cells were disrupted by agitation in three 60-s bursts with 0.1 mm glass beads in a Mini-BeadBeater (BioSpec Products Inc., Bartlesville, OK). The beads were allowed to settle and the supernatant fluid centrifuged in an Eppendorf microcentrifuge for 30 s at 14,000 x g to remove intact cells and cell debris. The supernatant fluid (500 µl) was eluted through a Sephadex G-25 (Bio-Rad Laboratories, Hercules, CA) equilibrated in 10 mM Tris, pH 9.5, in a 2-ml column within a 3-ml syringe. The protein fraction was collected (1.5 ml) and assayed for glycerol dehydrogenase activity. The recovery of protein was 90%.

Glycerol Dehydrogenase Assay

The assay, based on the methods of Nachlas *et al.* (1960) and Ruch *et al.* (1974), measures the reduction of a colorless tetrazolium salt to a magenta formazan. The reaction mixture consisted of (final concentrations): 100 mM KHCO₃ buffer, pH 9.5; 30 mM (NH₄)₂SO₄; 250 µg/ml gelatin; 100 mM glycerol where indicated; 2 mM NAD; 1 mM 2-*p*-iodo-3-*p*-nitrophenyl-5-phenyl-2H-tetrazolium chloride (INT, Sigma); 0.065 mM N-methylidibenzopyrazine methyl sulfate (phenazine methosulfate, PMS, from Sigma). The reaction was started by the addition of enzyme preparation to the pre-warmed reaction mixture and was carried out at 37°C. Samples (1.0 ml) of the reaction mixture were removed at intervals and added to 120 µl 0.7 N HCl. The formazan concentration was assessed from the absorbance at 540 nm. A standard curve was constructed using a commercial glycerol dehydrogenase (Worthington Biochem. Corp., Freehold, NJ). Protein concentrations were estimated with the Bradford (1976) reaction using a commercial reagent (Bio-Rad) and bovine serum albumin as the standard. The rates reported are those for the glycerol-specific enzymatic activity after subtraction of the reduction of INT in the absence of glycerol. All the manipulations, except centrifugation in the Servall centrifuge, were carried out within the anaerobic hood.

Nucleotide Sequence Accession Number

The nucleotide sequence of the 2299 bp *C. beijerinckii* NCIMB 8052 chromosomal DNA fragment, which consists of *gldA*, the intergenic region and part of the *accC* gene, has been deposited in the GenBank database under accession no. AF136281.

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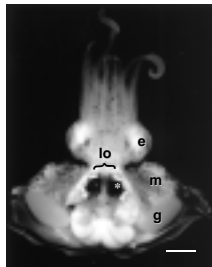
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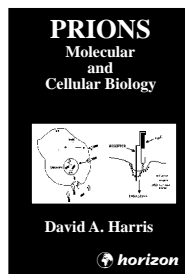
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