Evidence for Differential Regulation of Genes in the Chondroitin Sulfate Utilization Pathway of *Bacteroides thetaiotaomicron*

VIVIAN HWA† AND ABIGAIL A. SALYERS*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 7 May 1991/Accepted 21 October 1991

Expression of the chondroitin sulfate utilization (*csu*) genes of *Bacteroides thetaiotaomicron* is regulated by chondroitin sulfate. We have now found, however, that the *csu* genes are not all regulated in the same way. In particular, the gene encoding β -glucuronidase (*csuE*) is expressed under two different conditions that do not lead to expression of other *csu* genes.

Bacteroides thetaiotaomicron, an obligate anaerobe found in high concentrations in the human colon, is notable for its ability to ferment a variety of polysaccharides (9). One of these polysaccharides is chondroitin sulfate (CS), a linear mucopolysaccharide consisting of repeating units of glucuronic acid linked to 4- or 6-sulfated *N*-acetylgalactosamine. The structures of the two isomers of CS and the enzymes involved in CS breakdown are shown in Fig. 1. Results of previous studies had shown that elevated levels of the enzymes shown in Fig. 1 were seen only when *B. thetaiotaomicron* was grown in medium containing one of the following substrates: CS, disaccharide subunits of CS (Δ Di-4S, Δ Di-6S, and Δ Di-OS), and hyaluronic acid, an unsulfated mucopolysaccharide which is similar in structure to CS (6). These results suggested that all of the *csu* genes would be regulated similarly.

Two of the genes, *csuB* and *csuC*, have now been cloned and appear to be part of the same operon (3, 4). Recently, we used a newly constructed transcriptional fusion vector, pMJF-3, to show that there is a CS-regulated promoter upstream of *csuC* (1). The reporter group in pMJF-3 is *Escherichia coli* β -glucuronidase (GUS [1]), and the clone tested (pVGA-3; Fig. 2) exhibited CS-regulated GUS expression in *B. thetaiotaomicron*. In subsequent testing of other clones of the *csuC* upstream region, we made the surprising observation that the presence of one of the clones (pVGA-1; Fig. 2) in *B. thetaiotaomicron* affected expression of *csuE*, a gene that is unlinked to the *csuCB* operon.

Effect of multiple copies of the *csuCB* promoter-operator region on expression of *csuE*. Normally, extracts from *B. thetaiotaomicron* grown on glucose have no detectable 4-sulfatase, 6-sulfatase, or β -glucuronidase activity (6). However, when *B. thetaiotaomicron* which carried pVGA-1 (Fig. 2) was grown on glucose, expression of *csuE* (β -glucuronidase) was detectable in cell extracts. No chondro-4-sulfatase or chondro-6-sulfatase activity, assayed as described previously (7), was detectable under these conditions.

To compare the levels of β -glucuronidase expression under different conditions, we incubated extract (20 μ g of total protein) with Δ Di-OS (30 μ g) in 50 mM potassium phosphate buffer (pH 7; final volume, 100 μ l) at 37°C. Portions of the incubation mixture were removed at various intervals and analyzed by descending paper chromatography

(7). The amount of substrate remaining at each point was determined by comparing the size and intensity of the Δ Di-OS spot with those of different amounts of authentic standard spotted on the same chromatogram. A comparison of the times at which 60% of Δ Di-OS was converted to monosaccharides (Table 1) revealed that β -glucuronidase activity in extracts from *B. thetaiotaomicron* (pVGA-1) grown on glucose was approximately 8% of that in extracts from *B. thetaiotaomicron* grown on CS.

β -Glucuronidase activity in the glucose-grown extracts was not due to expression of GUS from pVGA-1 because no GUS expression from pVGA-1 was detectable in glucose-grown *B. thetaiotaomicron* (Fig. 2). Moreover, pVGA-1B (GUS in the opposite orientation) had the same effect on *csuE* expression as pVGA-1. Finally, the 3.7-kbp *EcoRI*-*PvuII* segment containing the *csuC* upstream region was cloned into pVAL-1 (10), a plasmid that does not carry GUS, and introduced into *B. thetaiotaomicron*. The resulting clone (pVOH-18) had the same effect on *csuE* expression in glucose-grown cells as pVGA-1. The fact that no expression of GUS was seen in extracts from glucose-grown cells containing pVGA-1 suggests that the effect of pVGA-1 on *csuE* expression was probably due to titration of regulatory proteins rather than to expression of a gene on pVGA-1.

The region needed for expression of *csuE* in glucose-grown cells was larger than the region needed to give CS-regulated GUS expression. pVGA-3, which exhibited CS-regulated GUS activity similar to that of pVGA-1 (Fig. 2), did not have this effect. A unidirectional exonuclease III deletion of pVOH-18, starting at the *PvuII* site and continuing toward the *EcoRI* site, produced a clone that contained about 400 bp downstream of the *PstI* site at which the fusion junction occurs in pVGA-3. This clone (pVOH-18A) had the same effect on *csuE* expression in glucose-grown *B. thetaiotaomicron* as pVGA-1 or pVOH-18. Thus, one end of the region needed for derepression of *csuE* was located within this 400-bp region. Since pVGA-2 did not derepress *csuE* expression in glucose-grown cells (Fig. 2 and Table 1), the other end of the region must be located between the *EcoRI* and *ClaI* sites.

Effect of heparin on *csuE* expression. Extracts from *B. thetaiotaomicron* grown on heparin also exhibited low but easily detectable Δ Di-OS hydrolyzing activity (Table 1). This was surprising because heparin did not induce expression of *csuA*, *csuB*, *csuC*, or *csuD*, i.e., extracts from *B. thetaiotaomicron* grown on heparin had no detectable chondro-4-sulfatase or chondro-6-sulfatase activity and the level of

* Corresponding author.

† Present address: Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201-3098.

FIG. 1. Enzymes involved in the breakdown of the two isomers of CS to monosaccharides in *B. thetaiotaomicron*. Gene designations for the enzymes are given in parentheses. HNAC, N-acetyl.

chondroitin lyase activity did not rise above the low basal level detected under noninducing conditions.

To confirm that the $\Delta\text{Di-OS}$ hydrolyzing activity induced by heparin and derepressed by pVGA-1 in glucose-grown cells was due to the *csuE* gene product and not another enzyme that recognized $\Delta\text{Di-OS}$, we compared the pIs of these activities with the pI of the activity in extracts from *B. thetaiotaomicron* grown on CS. Isoelectric focusing of cell

TABLE 1. Effect of multiple copies of the *csuCB* upstream region and heparin on expression of *csuE* in *B. thetaiotaomicron*

Plasmid	Growth substrate	% of $\Delta\text{Di-OS}$ converted to monosaccharides at the following incubation times (min) ^a :							
		0	5	10	20	40	60	90	120
None	Glucose	—	—	—	—	—	—	—	0
	CS	0	60	100	—	—	—	—	—
	Heparin	0	—	—	20	40	60	80	>90
pVGA-1	Glucose	0	—	—	20	40	60	80	>90
	Heparin	0	20	60	80	100	—	—	—
pVGA-1B, pVOH-18, ^b pVOH-18A ^b	Glucose	—	—	—	—	—	—	—	>90
pVGA-2, pVGA-3	Glucose	—	—	—	—	—	—	—	0

^a Cell extracts from bacteria were grown on the indicated substrates and incubated as described in the text. At the indicated intervals, portions of the incubation mixture were removed and analyzed by descending paper chromatography to determine the percent of $\Delta\text{Di-OS}$ that had been converted into monosaccharides. — indicates that no sample was taken at this time point.

^b pVOH-18 is the 3.7-kbp *EcoRI-PvuII* segment of pVGA-1 cloned instead into pVAL-1 (10), a vector that does not carry GUS. pVOH-18A is a unidirectional exonuclease III deletion of pVOH-18 that is equivalent to the DNA cloned in pVGA-3 plus 400 bp downstream of the *PstI* site.

extracts was done as described previously (2), and slices of the gel were assayed for $\Delta\text{Di-OS}$ hydrolyzing activity. In all three cases, the pI of this activity was 4.4.

Although heparin partially induced *csuE* expression, *csuE* was not essential for growth on heparin. A transposon-generated mutant of *B. thetaiotaomicron* that cannot grow on CS (46-4) had been isolated previously (8). This mutant grew normally on heparin. However, no $\Delta\text{Di-OS}$ hydrolyzing activity was detectable in extracts from the heparin-grown cells. *csuE* was still intact in this mutant because multiple copies of the *csuCB* upstream region caused the same derepression of *csuE* in glucose-grown 46-4 as seen with the wild type (data not shown).

FIG. 2. GUS-specific activities in extracts of *B. thetaiotaomicron* which carried different GUS fusions. A partial restriction map of the region which carried the *csuCB* operon is shown above the clones, together with the location and direction of transcription of *csuC* and *csuB*. The dashed line next to *csuC* indicates uncertainty about the location of the beginning of this gene. Thick black arrows indicate the direction of transcription of the GUS gene with respect to the cloned fragment. Cloning and other DNA manipulations were done by standard procedures (5). Fusions were introduced into *B. thetaiotaomicron* 5482 by conjugation (10) and were assayed for GUS activity as described previously (1). One unit of GUS activity is defined as an increase in A_{415} of 0.01 at 37°C. Abbreviations: R, *EcoRI*; C, *ClaI*; P, *PstI*; Pv, *PvuII*; RV, *EcoRV*; Glc, glucose; GUS, *E. coli* β -glucuronidase (*uidA*); β -Gase, $\Delta\text{Di-OS}$ hydrolyzing activity (*csuE*). Symbols: +, *csuE* expression; —, no *csuE* expression.

Synergistic effect of heparin and pVGA-1 on *csuE* expression. Although the level of *csuE* expression produced separately by pVGA-1 (in glucose-grown bacteria) and by heparin was low, expression of *csuE* could be increased considerably by combining the two conditions. The level of *csuE* expression in extracts from heparin-grown *B. thetaiotaomicron* which carried pVGA-1 rose to 50% of that in extracts from CS-grown *B. thetaiotaomicron* (Table 1). Despite the high level of *csuE* expression, there was still no detectable chondro-4-sulfatase or chondro-6-sulfatase activity and chondroitin lyase activity remained at the basal level. Thus, *csuE* is clearly regulated differently from the other CS-inducible genes. Moreover, our finding that the combined effect of pVGA-1 and heparin produced a higher level of *csuE* expression (50% of fully induced) than the additive effects of the two conditions separately (16% of fully induced) suggests that the low level of *csuE* expression seen with glucose-grown bacteria which carried pVGA-1 was not due simply to the relatively low copy number of this plasmid (5 to 10 copies per cell [1]) but may instead reflect the participation of more than one regulatory protein involved in the regulation of *csuE*.

This work was supported by grant AI 17876 from the National Institutes of Health.

REFERENCES

1. Feldhaus, M. J., V. Hwa, Q. Cheng, and A. A. Salyers. 1991. Use of an *Escherichia coli* β -glucuronidase gene as a reporter gene for investigation of *Bacteroides* promoters. *J. Bacteriol.* **173**:4540-4543.
2. Gherardini, F., M. Babcock, and A. A. Salyers. 1985. Purification and characterization of two α -galactosidases associated with catabolism of guar gum and other α -galactosides by *Bacteroides ovatus*. *J. Bacteriol.* **161**:500-506.
3. Guthrie, E. P., and A. A. Salyers. 1985. Cloning and expression in *Escherichia coli* of a gene coding for a chondroitin lyase from *Bacteroides thetaiotaomicron*. *J. Bacteriol.* **164**:510-515.
4. Guthrie, E. P., and A. A. Salyers. 1987. Evidence that the *Bacteroides thetaiotaomicron* chondroitin lyase gene is adjacent to the chondro-4-sulfatase gene and may be part of the same operon. *J. Bacteriol.* **169**:1192-1199.
5. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. Salyers, A. A., and S. F. Kotarski. 1980. Induction of chondroitin sulfate lyase activity in *Bacteroides thetaiotaomicron*. *J. Bacteriol.* **143**:781-788.
7. Salyers, A. A., and M. O'Brien. 1980. Cellular location of enzymes involved in chondroitin sulfate breakdown by *Bacteroides thetaiotaomicron*. *J. Bacteriol.* **143**:772-780.
8. Salyers, A. A., M. Pajean, and R. E. McCarthy. 1988. Importance of mucopolysaccharides as substrates for *Bacteroides thetaiotaomicron* growing in intestinal tracts of germfree mice. *Appl. Environ. Microbiol.* **54**:1970-1976.
9. Salyers, A. A., J. R. Vercellotti, S. E. H. West, and T. D. Wilkins. 1977. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. *Appl. Environ. Microbiol.* **33**:319-322.
10. Valentine, P. J., N. B. Shoemaker, and A. A. Salyers. 1988. Mobilization of *Bacteroides* plasmids by *Bacteroides* conjugal elements. *J. Bacteriol.* **170**:1319-1324.