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Interstrain Variation of the Polysaccharide B Biosynthesis Locus of *Bacteroides fragilis*: Characterization of the Region from Strain 638R

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The sequence and analysis of the capsular polysaccharide biosynthesis locus, PS B2, of *Bacteroides fragilis* 638R are described, and the sequence is compared with that of the PS B1 biosynthesis locus of *B. fragilis* NCTC 9343. Two genes of the region, *wcgD* and *wcgC*, are shown by complementation to encode a UDP-*N*-acetylglucosamine 2-epimerase and a UDP-*N*-acetylmannosamine dehydrogenase, respectively.

Bacteroides fragilis is the anaerobe most frequently isolated from intra-abdominal abscesses. The capsular polysaccharide complex (CPC) of the prototype strain, NCTC 9343, is the major virulence factor for abscess formation, and purified CPC induces the formation of abscesses in animal models (8). The CPC of strain NCTC 9343 is composed of two distinct polysaccharides, polysaccharide A (PS A) and polysaccharide B (PS B) (10). The repeating units of PS A and PS B each contain both positively and negatively charged groups (19). The presence of both types of charged groups is required for the induction of abscesses, and other bacterial polysaccharides that have been chemically modified to contain these charged groups also induce abscess formation (17, 18).

Studies with monoclonal antibodies (MAb) have demonstrated that the capsular polysaccharides of *B. fragilis* are heterogeneous and that the majority of strains examined produce a CPC composed of two distinct polysaccharides (9, 11). It is not known whether other strains of *B. fragilis* synthesize capsular polysaccharides containing both positively and negatively charged groups.

The sequence of the PS B biosynthesis locus of strain NCTC 9343 has been reported (2). Here, we report the sequence of the PS B biosynthesis region of the most-studied *B. fragilis* strain, 638R, which produces a CPC that is immunologically distinct from the CPC of strain NCTC 9343. In this study, we determined the genetic relatedness of the PS B biosynthesis regions of two immunologically diverse strains of *B. fragilis* and revealed that the 638R PS B locus contains genes whose products are involved in conferring charged groups to the polysaccharide.

The PS B biosynthesis locus of strain 638R was located by transposon mutagenesis as described previously (2). Bacterial strains and plasmids used in this study are listed in Table 1. Mutant 2-42 did not react with a MAb specific to the CPC of strain 638R, and the *B. fragilis* DNA at the junction of mutant 2-42 was cloned, creating plasmid pLEC6.1 (2). This plasmid was used as a probe to select pMJC10 from a 638R cosmid gene bank constructed by using a previously described protocol (2). Cosmid clones pLEC17, pLEC18, and pLEC19 were selected by using a probe consisting of an internal portion of *orf7* of strain NCTC 9343, an open reading frame (ORF) found by PCR to be common to both strains (Fig. 1).

A total of 26,443 bp were sequenced, revealing 19 ORFs similar to products encoded by genes of other polysaccharide biosynthesis loci (Table 2). Comparison of the sequence of this region of the chromosome with that reported previously for strain NCTC 9343 showed that the genes involved in polysaccharide biosynthesis are distinct (Fig. 1). A region including 2,048 bp of DNA upstream of *rmlA* was sequenced from strain 638R and was found to be 99.1% identical to the region upstream of the PS B biosynthesis locus of strain NCTC 9343 (Fig. 1). The identity continues into *rmlA* and is 100% for 338 bp of *rmlA* before the two chromosomes begin to diverge. This complete divergence continues throughout the biosynthesis regions until just downstream of *orf5*, where the two chromosomes are 99.6% identical for the remainder of the DNA sequenced from both chromosomes (2,081 bp). To standardize the nomenclature, the polysaccharide that is synthesized from any *B. fragilis* strain by genes in this area of the chromosome is designated PS B. As these polysaccharides are genetically and immunologically distinct, an arabic numeral is placed after this designation; therefore, the polysaccharide synthesized by the *wcf* locus of NCTC 9343 is designated PS B1 and that synthesized by the 638R *wcg* locus is designated PS B2.

Like the NCTC 9343 *wcf* region, the ORFs of *wcg* are all transcribed from the same DNA strand, and most are tightly clustered; the exceptions are *rmlA* and *wzx*, which have a 321-bp gap between them. If transcription of the *wcf* and *wcg* regions were initiated upstream of *rmlA* (the first gene of each region), transcription would be driven by a promoter common to both loci.

Unlike the *wcf* region of NCTC 9343, which contains only genes whose products are similar to proteins involved in polysaccharide biosynthesis, the unique region of the 638R chromosome contains two additional ORFs flanking the 3′ end of the *wcg* region, which may not be involved in polysaccharide biosynthesis. OrfA6 is not similar to any sequences deposited in public databases. In addition, a region of 43 bp between *wcgP* and *orfA6* contains a 20-bp inverted repeat that may terminate transcription downstream of *wcgP*. OrfB6 is similar to an ORF of *Azotobacter chroococcum* that is not contained in a polysaccharide biosynthesis locus and whose function is unknown (3).

Structural analysis of the two capsular polysaccharides of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference and/or source
E. coli strains		
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ$^-$	1
AB1133	K-12 *thr-1 leuB6* Δ(*gpt-proA*)*66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 supE44*	7
21546	AB1133 with *rff*::Tn*10*-46	7
21566	AB1133 with *rff*::Tn*10*-66	7
B. fragilis strains		
NCTC 9343	Type strain, appendix abscess	5, ATCC[a]
638R (TM4000)	Clinical	12
2-42	638R PS B mutant with transposon insertion in *wcg* locus	2
Plasmids		
pHC79	Cosmid vector; Ap^r	4
pUC19	Cloning vector; Ap^r	New England Biolabs
pLEC6.1	900-bp *Eco*RI-*Hin*dIII fragment (*B. fragilis* DNA) cloned in pBluescript; Ap^r	2
pMJC10	638R gene bank clone including *orf3-wcgE* cloned in pHC79; Ap^r	This study
pLEC17	638R gene bank clone including *wcgM-orf7* cloned in pHC79; Ap^r	This study
pLEC18	638R gene bank clone including *wcgJ-orf7* cloned in pHC79; Ap^r	This study
pLEC19	638R gene bank clone including *wcgF-orf7* cloned in pHC79; Ap^r	This study
pWCGC	1,424-bp PCR amplicon containing *wcgC* cloned in pUC19; Ap^r	This study
pWCGD	1,518-bp PCR amplicon containing *wcgD* cloned in pUC19; Ap^r	This study
pWCGCD	2,711-bp PCR amplicon containing *wcgC-wcgD* cloned in pUC19; Ap^r	This study

[a] ATCC, American Type Culture Collection.

638R (PS A2 and PS B2) is currently under way in our laboratory. A brief discussion of the putative gene products synthesized by the *wcg* locus and a prediction of some of the monosaccharides likely to be present in the repeating unit of PS B2 follows.

The four genes downstream of *rmlA* may have been acquired from a common ancestor; the G+C content of all these ORFs is low (averaging 29.3%) and is different from those of the ORFs immediately upstream and downstream (38.1 and 34.6%, respectively). The first and last ORFs of the four-gene block likely encode a flippase and a polymerase, respectively. These products are not similar to the flippase and polymerase of the NCTC 9343 *wcf* locus. Wzx contains 13 putative transmembrane regions and is similar to various other products believed to function as flippases (Table 2). Wzy, the putative polymerase, has 11 potential transmembrane regions. Of the two ORFs between *wzx* and *wzy*, a putative function for only one of these products (WcgB) can be proposed by homology analysis. Because of its limited similarity to NodC and the presence of the conserved amino acids by hydrophobic cluster analysis (16), this gene is designated a putative glycosyltransferase.

Three clustered genes, *wcgJ* to *wcgL*, encode products that, along their entire lengths, are highly similar to products encoded by a cluster of genes from the *Staphylococcus aureus cap5* and *cap8* regions (*capE* to *capG*). These Cap proteins have been predicted to be involved in the formation of deoxynucleoside triphosphate-*N*-acetylfucosamine (6, 15). A fourth gene adjacent to this cluster, *wcgM*, encodes a product that is similar to another product encoded by the *S. aureus cap* region, CapL. The similarity of WcgM to putative glycosyltransferases and the detection of the mandatory conserved aspartic acid residues by hydrophobic cluster analysis suggest that this product functions as a glycosyltransferase.

WcgC and WcgD are similar over their entire length to two products of the *Escherichia coli wec* region (formerly *rff*) and the *S. aureus cap* region. In *E. coli*, these products are necessary for the synthesis of enterobacterial common antigen (ECA) and have been shown to convert UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-*N*-acetylmannosaminuronic acid (UDP-ManNAcA) in a two-step process (7). WcgD is a homolog of WecB (RffE) and CapP. WecB is a UDP-GlcNAc 2-epimerase that converts UDP-GlcNAc to UDP-*N*-acetylmannosamine (UDP-ManNAc) (7, 14). WcgC is a homolog of CapO and WecC (RffD). WecC has been demonstrated to have dehydrogenase activity and catalyzes the conversion of UDP-ManNAc to UDP-ManNAcA (7), one of the biosynthesis precursors of ECA. The synthesis of ECA is restored to an *E. coli wecB wecC* mutant by *cap5O* and *cap5P* in *trans* (6).

Due to the requirement of a negatively charged group for abscess induction, the involvement of WcgC and WcgD in the production of UDP-ManNAcA (a negatively charged monosaccharide) from UDP-GlcNAc was investigated. *wcgC* was amplified as a 1,424-bp product with primers wcgC-F (5′ CATAGAATTCGGCTCACCCTTTATATCCTATAC) and wcgC-R (5′ CAGGGGATCCTTTTAATGGCTTCGGGAC). *wcgD* was amplified as a 1,518-bp product with primers wcgD-F (5′-CGAGGAATTCCTGACATTTTGGTTGTAGAGC) and wcgD-R (5′ CCAGGGATCCGGAACAAAAACAAAAGGACC). In addition, *wcgC* and *wcgD* were amplified as a single 2,711-bp product with primers wcgC-F and wcgD-R (Fig. 1). The PCRs were performed by using HiFi PCR Supermix (Life Technologies, Gaithersburg, Md.) with 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min. The reaction products were run over a nucleotide removal column, digested with *Eco*RI and *Bam*HI, gel purified, and cloned into *Eco*RI-*Bam*HI-digested pUC19. This orientation allows for utilization of the vector-based promoter, while translation is initiated from within the cloned *B. fragilis* DNA. The ligations were first transformed into DH5α, and the correct clone from each ligation was selected (pWCGC [*wcgC* only], pWCGD [*wcgD* only], or pWCGCD [*wcgC* and *wcgD*]). The *E. coli* ECA mutants

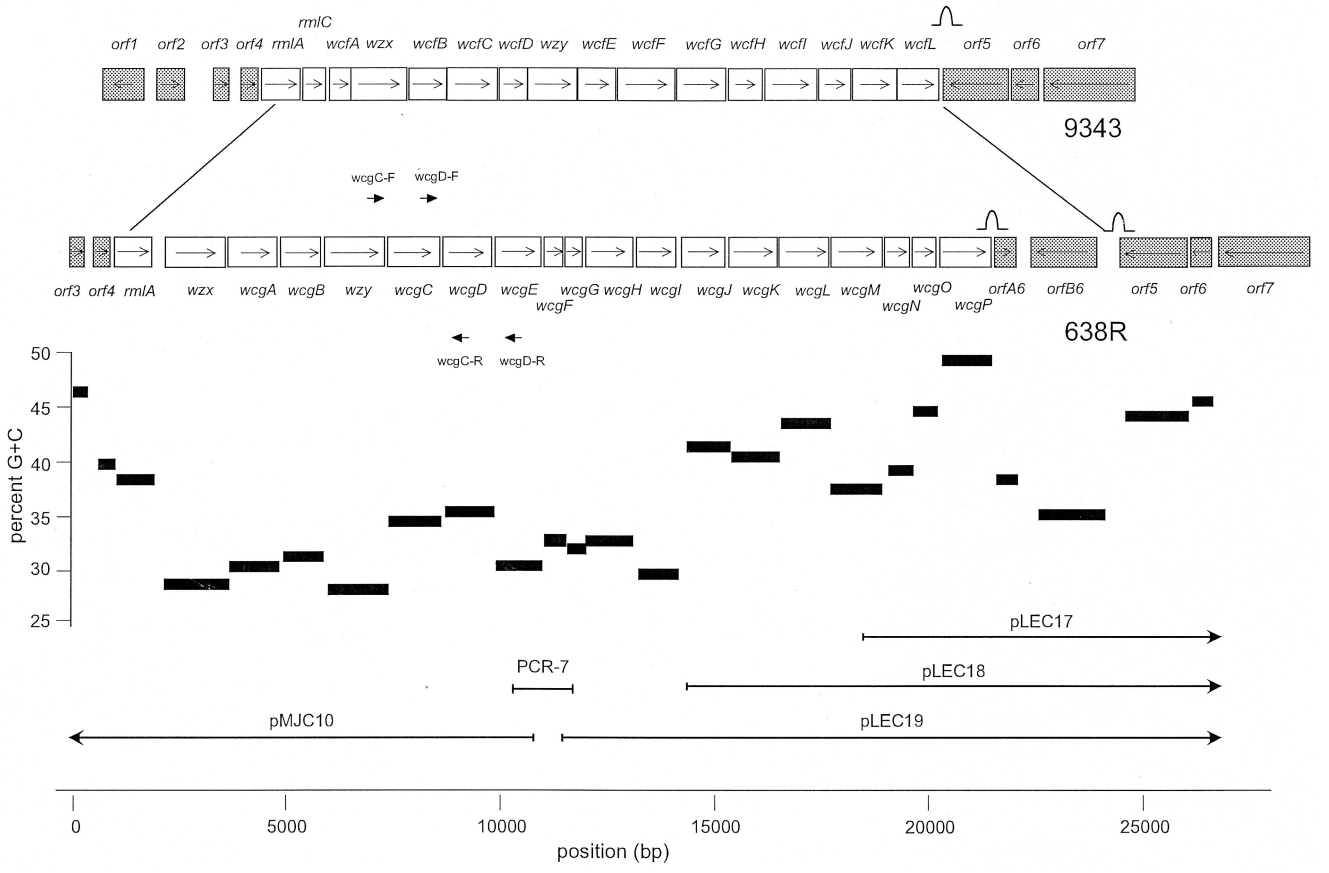

FIG. 1. Comparison of the PS B1 and PS B2 biosynthesis regions of *B. fragilis* NCTC 9343 and 638R. The direction of transcription of each ORF (boxes) is designated with an arrow. The ORFs that do not demonstrate homology with genes involved in polysaccharide biosynthesis are shaded. The area between the diagonal lines indicates where the two chromosomes diverge. Two stem-loop regions that may serve to terminate transcription in the 638R region are indicated, as are the locations of primers used to amplify *wcgC* and *wcgD* for complementation studies. The average G+C content of each ORF of the 638R region is indicated by a black bar. Cosmid clones and a PCR product used as sequencing templates are shown.

21546 (*wecC*) and 21566 (*wecB wecC*) (7) were transformed with pUC19, pWCGC, pWCGD, or pWCGCD, and the synthesis of ECA was monitored by using the ECA-specific MAb 898 (13).

Figure 2 is a Western blot of the complementation results. Both pWCGC and pWCGCD were able to complement 21546 (*wecC*); however, pWCGD was not. These results demonstrate that *wcgC* encodes a dehydrogenase involved in the formation of UDP-ManNAcA.

wcgC alone was not able to complement 21566 (*wecB wecC*). The presence of *wcgD* in 21566 allowed a very slight amount of ECA to be produced (Fig. 2, lane 8). This result is consistent with that obtained by Kiser and Lee, using *capP* (*wecB* homolog) for complementation of this mutant (6). These authors attribute this ECA expression to low levels of dehydrogenase activity in 21566 that was not detected until high levels of a substrate, UDP-ManNAc, were introduced. When both *wcgC* and *wcgD* (pWCGCD) were supplied to mutant 21566, ECA synthesis was restored (Fig. 2, lane 9). As with complementation of mutant 21566 by *cap5O* and *cap5P*, ECA was overexpressed compared to the level of expression found in the wild type and likely reflects the presence of these genes in multiple copies. As WcgC is a functional homolog of WecC, this finding shows that WcgD is a UDP-GlcNAc 2-epimerase and can replace WecB in the synthesis of ECA in *E. coli*. These observations strongly suggest that the repeating unit of PS B2 contains ManNAcA (a negatively charged monosaccharide).

The *wcg* locus contains two genes, *wcgH* and *wcgP*, whose products display significant similarity to a variety of aminotransferases. WcgH and WcgP may each transfer a free amino group (positive charge) to the repeating unit of PS B2. Since both positively and negatively charged groups are integral to the biological activity of the polysaccharides, the enzymatic activity of these putative aminotransferases will be investigated in future studies.

In addition to *wcgB* and *wcgM*, two other genes, *wcgE* and *wcgI*, may also encode glycosyltransferases. WcgE is similar to putative glycosyltransferases, and both WcgE and WcgI contain the conserved aspartate residues detected by hydrophobic cluster analysis. WcgN is similar to many products that transfer galactose to an undecaprenol phosphate carrier as the first step in subunit assembly.

The data gathered by homology and complementation analyses suggest that the subunit of PS B2 contains five sugars, including one that is likely to be ManNAcA and one that may be similar to *N*-acetylfucosamine. The demonstration of dehydrogenase activity and of two genes whose products are similar to various aminotransferases further suggests that the repeating unit of PS B2 may contain both positively and negatively charged groups. Elucidation of the structure of PS B2 will

TABLE 2. Identity of the products encoded by the *wcg* region with sequences in the GenBank database

ORF	G+C content (%)	Size (amino acids)	Organism and gene	Gene product	% Identity/similarity[a]	Accession no.
rmlA	38.1	294	*B. fragilis* NCTC 9343, *rmlA*	Putative glucose 1-phosphate thymidyltransferase	92/96 (1–294)	AF048749
			Shigella flexneri, *rmlA*	Glucose 1-phosphate thymidyltransferase	67/80 (2–289)	D55213
wzx	28.8	509	*E. coli* 0111, *wzx*	Putative flippase	18/37 (21–351)	U13629
			Yersinia enterocolitica O:3, *trsA*	Putative flippase	21/38 (13–359)	S51260
wcgA	30.3	369	*Vibrio cholerae* O139, *wbfL*	Unknown	31/45 (262–348)	Y07786
wcgB	31.3	315	*E. coli*, *kfiA*	Unknown	24/47 (65–174)	X77617
			Rhizobium tropici, *nodC*	*N*-acetylglucosaminyltransferase	20/38 (67–167)	X98514
wzy	28.3	478	*Streptococcus pneumoniae*, *cap1H*	Unknown	24/52 (392–468)	Z83335
wcgC	34.6	408	*E. coli*, *wecC* (formerly *rffD*)	UDP-*N*-acetylmannosamine dehydrogenase	49/66 (6–400)	P27829
			S. aureus, cap5/8 O	UDP-*N*-acetylmannosamine dehydrogenase	41/60 (1–400)	U73374 U81973
wcgD	35.7	384	*E. coli*, *wecB* (formerly *rffE*)	UDP-*N*-acetylglucosamine-2-epimerase	55/71 (3–382)	P27828
			S. aureus, *cap5/8 P*	UDP-*N*-acetylglucosamine-2-epimerase	47/64 (1–378)	U73374 U81973
wcgE	30.4	363	*Synechocystis* sp., *icsA*	Putative glycosyltransferase	27/45 (178–334)	BA17441
			S. aureus, *capJ*	Unknown	25/50 (213–338)	P39859
wcgF	32.9	143	*B. fragilis*, *wcgG*	Unknown	49/66 (9–136)	AF125164
			Vibrio anguillarum, *orf15x4*	Unknown	40/58 (6–131)	AF025396
wcgG	32.2	141	*B. fragilis*, *wcgF*	Unknown	49/66 (12–139)	AF125164
			V. anguillarum, *orf15x4*	Unknown	43/55 (23–133)	AF025396
			S. pneumoniae, *cp13-1*	Unknown	33/61 (55–96)	S21550
wcgH	32.9	366	*V. anguillarum*, *orf41x4*	Putative aminotransferase	55/77 (1–365)	AF025396
			Streptomyces antibioticus, *oleN2*	Putative transaminase	41/61 (2–362)	AF055579
wcgI	29.7	331	*Leptospira borgpetersenii*, *orfH18*	Unknown	35/54 (1–300)	AF078135
			Bacillus subtilis, ORF	Unknown	33/52 (4–239)	D86418
wcgJ	41.4	341	*L. borgpetersenii*, *orfH1*	Unknown	66/84 (3–339)	AF078135
			S. aureus, *cap8E*	Unknown	63/80 (3–337)	U81973
wcgK	40.5	383	*Acholeplasma laidlawii*, ORF	Putative nucleotide-binding protein	66/84 (1–383)	U81973
			S. aureus, *cap8F*	Unknown	55/68 (1–383)	U73374
wcgL	43.7	394	*S. aureus*, *cap8G*	Unknown	57/71 (17–393)	U73374
			L. borgpetersenii, *orfH11*	Unknown	46/64 (19–394)	AF078135
			Methanobacterium thermoautotrophicum, ORF	Putative UDP-*N*-acetylglucosamine 2-epimerase	34/54 (18–305)	AAB85335
wcgM	37.6	402	*L. borgpetersenii*, *orfH12*	Unknown	26/47 (22–402)	AF078135
			S. aureus, *cap8L*	Unknown	24/45 (55–391)	U73374
			E. coli, *wcaI*	Putative glycosyltransferase	20/41 (1–343)	P32057
wcgN	39.2	202	*Campylobacter jejuni*, *orfC*	Putative UDP-galactose phosphate transferase	61/68 (41–202)	AF001498
			Streptococcus agalactiae, *cpsD*	Putative UDP-galactose phosphate transferase	42/55 (39–186)	Q04664
wcgO	44.8	194	*Caulobacter crescentus*, *lpsB*	Putative UDP-*N*-acetylglucosamine acetyltransferase	43/59 (52–190)	AF062345
			C. jejuni, *orfD*	Putative acetyltransferase	27/53 (1–185)	AF001498
wcgP	49.2		*C. jejuni*, *orfF*	Putative aminotransferase	45/60 (24–395)	AF001497
			M. thermoautotrophicum, ORF	Putative perosamine synthetase	37/53 (32–395)	AE000818
orfA6	38.3	167	None			
orfB6	35.2	519	*A. chroococcum*, ORF	Unknown	35/54 (7–519)	P24423

[a] The identity and similarity percentages are based on the stated amino acid region (in parentheses) of the *wcg* product.

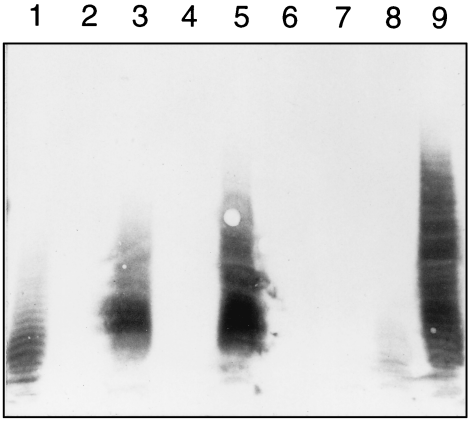

FIG. 2. Western immunoblot analysis of *E. coli* AB1133 and ECA mutants 21546 and 21566 containing *B. fragilis wcg* DNA in *trans*. Bacterial cultures were grown to an optical density at 600 nm of 0.8, the bacteria were pelleted by centrifugation and lysed with 1× loading buffer, and volumes of bacteria equivalent to 40 μl of culture were added to each well of the sodium dodecyl sulfate–12% polyacrylamide gel, transferred to a nylon membrane, and probed with MAb 898 (ECA specific). Lanes: 1, AB1133; 2, 21546 (pUC19); 3, 21546(pWCGC); 4, 21546(pWCGD); 5, 21546(pWCGCD); 6, 21566(pUC19); 7, 21566(pWCGC); 8, 21566(pWCGD); 9, 21566(pWCGCD).

provide a foundation from which to continue enzymatic analysis of various gene products of the *wcg* locus.

Nucleotide sequence accession number. The sequence of the PS BZ biosynthesis locus of strain 638R, described herein, has been deposited in GenBank under accession no. AF125164.

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