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Expression of a Uniquely Regulated Extracellular Polysaccharide Confers a Large-Capsule Phenotype to *Bacteroides fragilis*▽

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Bacteroides fragilis **synthesizes eight distinct capsular polysaccharides, more than any described bacterium outside the order *Bacteroidales*. Here, we show that this organism also produces a high-molecular-weight extracellular polysaccharide (EPS). Expression of the EPS results in the formation of a large polysaccharide layer around the bacteria which prevents them from forming a tight pellet upon centrifugation and from entering a Percoll density gradient. Like expression of the capsular polysaccharides, expression of the EPS is phase variable and dictated by DNA inversion of its promoter. EPS expression is regulated at one level by the DNA invertase Tsr19, which is encoded by a gene immediately upstream of the EPS locus and inverts the EPS promoter, causing an on or off phenotype. Expression of the EPS is also regulated at another level, which dictates the amount of EPS produced. By analyzing a panel of *tsr19* deletion mutants, we found that the number of inverted repeats (IRs) flanking the promoter is variable. Transcription into the EPS genes is greater in mutants with a single IR between the promoter and the downstream EPS genes than in mutants with more than one IR in this region, correlating with the synthesis of more EPS. By analyzing the relative orientations of the EPS promoter of bacteria obtained from human fecal samples, we showed that both DNA inversion and variation in the number of IRs are active processes of *B. fragilis* in the endogenous human intestinal ecosystem.**

The human colon houses a complex ecosystem containing trillions of microorganisms, including bacteria, archaea, eukaryotes, viruses, and bacteriophages. Members of this microbiota maintain relatively stable communities, in which they establish commensal and mutualistic relationships with the host and with each other. As this ecosystem comprises one of the densest populations of microorganisms known, its members live in very close association with each other. This close association highlights the importance of the surfaces of these organisms in the maintenance of mutualistic relationships in this environment.

Bacteria of the order *Bacteroidales* are abundant residents of this ecosystem, and several species in this order have been studied to determine their mutualistic properties (6, 10). We have found that the surface of one of these species, *Bacteroides fragilis*, is extremely adaptable and dynamic and that it has the ability to extensively alter its composition to an extent even greater than that described for most pathogenic microorganisms. This variability is conferred in part by the synthesis of eight distinct capsular polysaccharides (PSA to PSH), which are subject to a phase-variable on-off phenotype dictated by DNA inversion of the promoters of each of the polysaccharide biosynthesis loci (5). The inversions are mediated by a single global serine family site-specific recombinase designated Mpi (4). We recently extended our studies to analysis of other members of the *Bacteroidales* order and found that the synthesis of multiple phase-variable polysaccharides is a hallmark of the intestinal *Bacteroidales* (2). The ubiquity of this phenotype among these organisms strongly suggests that it confers a survival advantage during the life-long association of these organisms with the host and other members of the colonic microbiota.

Phase-variable expression of *B. fragilis* surface molecules is not limited to capsular polysaccharides. The expression of multiple surface proteins is also regulated by promoter inversions. We showed previously that three conserved tyrosine family site-specific recombinases (Tsr15, Tsr25, and Tsr26) act locally to bring about inversions of promoter regions immediately downstream of their genes (12). A *tsr15* mutant for which the downstream locus *aap* was locked on for constitutive transcription had an aggregative adherent phenotype, suggesting that the Aap proteins may be involved in adherence and biofilm formation in the intestine. Therefore, DNA inversion is a general mechanism for generating biologically relevant surface diversity and is mediated by members of two distinct families of site-specific recombinases.

We recently showed that the gene encoding the global polysaccharide promoter invertase, *mpi*, is adjacent to another gene, *tsr19*, whose product also has DNA invertase activity (9). Tsr19 is a tyrosine family site-specific recombinase that mediates inversion of a promoter in its immediate downstream region and a promoter located in a distant region of the chromosome. The invertible promoter that is just downstream of *tsr19* is necessary for transcription of the downstream gene BF2782; however, the distant promoter was not shown to be necessary for transcription of either of the adjacent operons. The three genes immediately downstream of *tsr19* (BF2782, BF2783, and BF2784) (Fig. 1A) are annotated as similar to genes encoding proteins involved in extracellular polysaccha-

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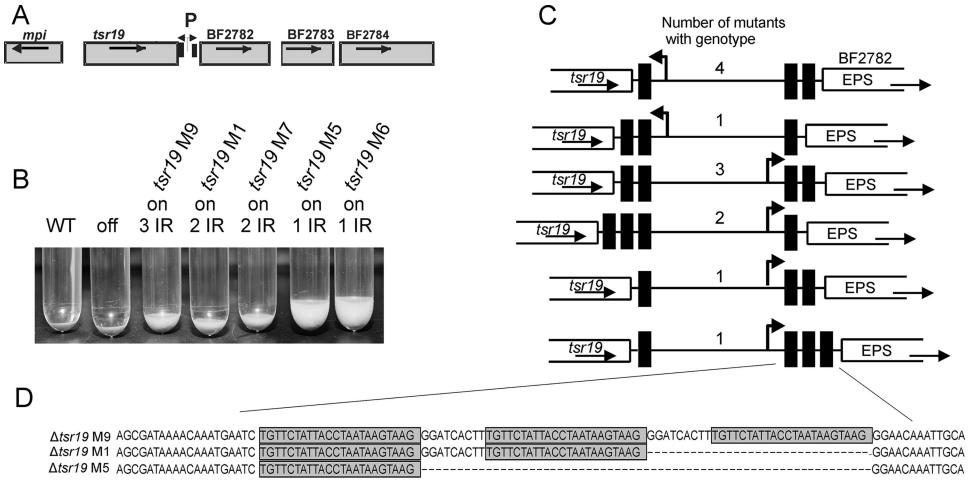

FIG. 1. Phenotype and genotype of *tsr19* deletion mutants. (A) Open reading frame map of the genetic region containing genes for the two DNA invertases, Mpi and Tsr19, and the downstream genes annotated as putatively involved in EPS production (BF2782 to BF2784). Black boxes represent IRs, and the arrow between them represents the invertible promoter. Arrows in genes indicate the direction of transcription. (B) Phenotypes of the *tsr19* deletion mutants grown in culture overnight and pelleted by high-speed centrifugation. On and off indicate the direction in which the EPS promoter is locked relative to BF2782. The numbers of IRs between the promoter and the EPS genes are indicated for the locked-on mutants. WT, wild type. (C) Genotypes of the Δ*tsr19* mutants, showing the variation in the number of IRs. The number of mutants with each genotype is indicated. The black boxes represent IRs, and the arrow between them indicates the direction in which the promoter is locked. (D) Sequences of the IR regions between the promoter and the downstream EPS genes for three *tsr19* mutants locked on for transcription of the downstream locus, Δ*tsr19* M9 (three IRs), Δ*tsr19* M1 (two IRs), and Δ*tsr19* M5 (one IR).

ride (EPS) biosynthesis. EPSs are typically distinguished from capsular polysaccharides in that they are more loosely associated with the bacterial cell, that they are usually very large molecules with molecular masses often greater than 500 kDa, and that they sometimes confer a mucoid colony phenotype to the bacteria. The product of the first gene, BF2782, is similar to enzymes that transfer a monosaccharide to the lipid carrier undecaprenyl phosphate, the first step in building a polysaccharide repeat unit. The product of the second gene, BF2783, is similar to undefined periplasmic products involved in EPS synthesis. The product of the third gene, BF2784, is similar to Wzc, a tyrosine kinase that provides the energetics for movement of polysaccharides across membranes (8).

To determine if this locus is actually involved in EPS synthesis, we analyzed *tsr19* mutants that are locked on and off for its transcription. We demonstrate that this locus is indeed involved in the synthesis of an EPS with an unusual mechanism of regulation that is active in the human colon.

MATERIALS AND METHODS

Bacterial growth conditions. *Escherichia coli* DH5α containing recombinant plasmids was grown on L broth or on L agar plates containing ampicillin (100 μg ml^{-1}) and/or kanamycin (50 μg ml^{-1}). *B. fragilis* NCTC9343 was the parental strain of all mutants. *B. fragilis* strains were grown anaerobically in basal medium or on brain heart infusion plates supplemented with hemin (50 μg ml^{-1}) and menadione (0.5 μg ml^{-1}); gentamicin (200 μg ml^{-1}) and erythromycin (5 μg ml^{-1}) were added where appropriate.

***tsr19* deletion mutants.** *tsr19* deletion mutants were constructed so that 733 bp of the 918-bp gene was removed by allelic replacement. The DNA segment upstream of the region to be deleted was PCR amplified using primers TACC ATGGTGCTTCAGAGAAGGTGACATACAT and TCGGATCCATTTGCC ATAGTTCGTGAGTCTCT, and the DNA segment downstream of the region to be deleted was PCR amplified with primers CTGGATCCACTTTCTGTTC CTTGAAACCATTC and ATCCATGGTATTGGCTGTGCTGTAACG (restriction sites are underlined). The two PCR products were digested with BamHI and NcoI and cloned by three-way ligation into the BglII site of the *Bacteroides* conjugal suicide vector pJST55 (11). *E. coli* DH5α was transformed with the ligation mixture, and resistant colonies were screened for proper orientation of the left and right flanking DNA. The resulting plasmid was conjugally transferred into *B. fragilis* NCTC9343, and cointegrates were selected on the basis of Em^r encoded by pJST55. The cointegrate strain was passaged, plated on nonselective medium, and replica plated on medium containing erythromycin. Em^s colonies were screened by PCR to select colonies that had the mutant genotype.

Analysis of IR number. We sequenced PCR amplicons of the invertible promoter regions downstream of *tsr19* and upstream of BF3223 from 12 *tsr19* deletion mutants to determine the number of inverted repeats (IRs) present. The invertible region downstream of *tsr19* was PCR amplified with primers CAGA GAAGGTGACATACATT and ATACAATCCTTTTAATGACT, and the latter primer was also used for sequencing of the amplicon. The invertible region between BF3223 and BF3224 was PCR amplified with primers AACAGTATC GCCTGCCTTTAATAC and TCAATGACACTTGTTTCATGTTTG, and this amplicon was sequenced with primer ATGAGTGACTAATTGGGTGCTACA.

***xylE* transcriptional fusion clones.** Reporter plasmid pLEC23 was used to create transcriptional fusion clones (4). DNA containing the EPS promoter in the on orientation and 1, 2, and 3 IRs were amplified with forward primer TAGGATCCGATACTTCAACTGGTTATAATCCAC and reverse primer GT GGATCCCTTTTAGAAATGTTTGCGATAGTTG from Δ*tsr19* M5, M1, and M9, respectively, and cloned into pLEC23 in the proper orientation to transcribe the downstream *xylE* gene. The plasmids were mobilized into *B. fragilis* NCTC9343, and XylE activity was quantified as previously described (13). XylE assays were performed in triplicate, and the results were expressed as means ± standard deviations.

Percoll density gradient centrifugation. Percoll density gradient centrifugation was performed as described by Patrick and Reid (7). In short, a 9:1 stock solution of Percoll (Sigma) was prepared with 1.5 M NaCl (pH 7). Solutions containing 20, 40, 60, and 80% Percoll in 0.15 M NaCl were prepared from the stock, and 2 ml of each solution was layered in an open-top polyclear centrifuge tube (Seton, Los Gatos, CA) with the 80% Percoll solution at the bottom and the 20% Percoll solution at the top. Overnight broth cultures (2.5 ml) were added to the top of the 20% Percoll layer, and the gradient was centrifuged at 2,600 × *g* for 20 min.

Light microscopic visualization of EPS layer. The staining method used for visualization of the EPS material was a variation of a standard protocol (7). In short, 10 μl from an overnight culture was placed on a slide with an equal volume of 6% (wt/vol) glucose in distilled H_2O, and 10 μl of India ink was added. The mixture was spread over the slide to obtain a thin film and allowed to air dry. The dried film was then fixed with methanol and air dried again before addition of

the counterstain, basic fuchsin (0.25% [wt/vol] in 10% ethanol). The slide was washed with distilled H_2O, air dried, and visualized using an Axioskop 2 Plus light microscope (Zeiss) with a ×100 oil immersion objective.

EPS-specific antiserum and Western immunoblot analysis. To obtain antibodies specific to the EPS, a standard protocol for adsorbing antiserum in our lab was used (1). For generation of EPS-specific antiserum, whole bacterial cells of a *tsr19* mutant locked on for transcription of the downstream locus with a single IR between the promoter and BF2782 were formalin fixed and used as the immunogen for generation of antiserum at Lampire Biologicals (Pipersville, PA), using their express protocol. This antiserum was adsorbed with Δ*tsr19* M3 to remove the antibodies to all surface molecules except the EPS. The resulting adsorbed antiserum was specific to the EPS.

For Western immunoblot analysis, bacteria were boiled in LDS sample buffer and subjected to electrophoresis using NuPAGE 4 to 12% gradient sodium dodecyl sulfate-polyacrylamide gels with morpholineethanesulfonic acid (MES) buffer (Invitrogen, Carlsbad, CA). The contents of the gels were transferred to polyvinylidene difluoride membranes, probed first with the EPS-specific antiserum, and then probed with an alkaline phosphatase-labeled anti-rabbit immunoglobulin G secondary antibody. The membranes were subsequently developed with a 5-bromo-4-chloro-3-indolylphosphate (BCIP)—nitroblue tetrazolium (NBT) substrate (KPL, Gaithersburg, MD).

Quantitative EPS promoter orientations for in vitro and in vivo samples. Mouse studies were approved by the Harvard Medical Area Standing Committee on animals. Gnotobiotic mice (Swiss Webster males that were 3 to 5 weeks old) were obtained from Taconic (Germantown, NY), were housed in germfree isolators, and were fed an autoclaved rodent chow diet (Zeigler Bros., Inc., Gardners, PA). Mice were monoassociated with wild-type *B. fragilis* NCTC9343 as described previously (3), and fresh stool samples were collected 3 to 4 weeks after colonization. Human fecal samples were obtained as secondary use material from a study of healthy pregnant women in which informed consent was obtained from all subjects. The samples were renumbered so that no identifiers linked the samples to specific individuals. Approval for experimentation with these samples was obtained from the Partners Human Research Committee. Mouse and human fecal samples were suspended in phosphate-buffered saline, particulate material was allowed to sediment for 5 min, and 200 μl of the nonsedimented material was pelleted and used for chromosomal extraction with an ExtractMaster fecal DNA extraction kit (Epicenter, Madison, WI).

The proportions of bacteria with the EPS promoter in the on and off orientations from in vitro-grown samples and from mouse and human fecal samples were determined by PCR amplification and restriction digestion using a protocol similar to that described previously (5). For each DNA sample, a DNA segment spanning the IRs and promoter region was PCR amplified with primers AGCA CAGCCAATACCTACCTCTAC and TAACCAGGTAGCATAAGTTGA GCA. The amplified DNA segments were digested with HpaI, separated by electrophoresis, and visualized by ethidium bromide staining.

RESULTS

Characterization of *tsr19* deletion mutants. We previously created *tsr19* deletion mutants in order to study the genotype that resulted from deletion of this gene. Deletion of *tsr19* resulted in an inability of two promoters, one located in the immediate downstream region, to invert (9). We reported two such mutants, one which had this promoter locked on for transcription of the downstream operon (Δ*tsr19* M1) and one which had this downstream promoter locked in the off orientation (Δ*tsr19* M3). In this study, we sought to study the phenotype resulting from constitutive expression of the downstream operon.

We expanded our panel of *tsr19* deletion mutants and created and analyzed a total of 12 mutants, 7 which had the invertible promoter locked on to constitutively drive transcription of the downstream genes and 5 which had the promoter locked off. We noted an unusual phenotype in the locked-on mutants that was not previously observed in our genotypic studies of Δ*tsr19* M1. The locked-on mutants did not form a tight pellet upon centrifugation but rather produced a large buoyant layer at the bottom of the tube (Fig. 1B). Some locked-on mutants, such as Δ*tsr19* M5, formed a large amount of this layer, while others, such as Δ*tsr19* M1, made less. We assumed that the locked-on mutants constitutively expressed an EPS that prevented the bacteria from forming a tight pellet. What was not understood, however, is why this layer was much larger in some locked-on mutants than in others. The amount of the nonpelleting layer was consistent for a given *tsr19* deletion mutant and therefore was likely to have a genetic basis.

To understand the phenotypic differences between the locked-on mutants that were initially thought to be genetically identical, we sequenced the invertible promoter region of each of the 12 *tsr19* deletion mutants. The sequence data demonstrated that neither the locked-on mutants nor the locked-off mutants were genotypically identical to each other; rather, the number of IRs flanking the promoter in these mutants was variable (Fig. 1C). Each mutant had a total of either three or four IRs flanking the promoter region; some mutants had multiple IRs on the *tsr19* side, some had multiple IRs on the BF2782 side, and some had two IRs on each side. A 9-bp sequence (GGATCACTT) was present between all adjacent IRs (Fig. 1D). The locked-on mutants that produced the largest amount of nonpelleting material had only a single IR between the promoter and the first EPS gene, compared to the locked-on mutants with two or three IRs in this region. These data suggest that the variation in the number of IRs provides an additional level of regulation of the putative EPS locus, secondary to promoter inversion.

We inferred from the sequence analysis of the *tsr19* deletion mutants that the number of IRs flanking the EPS promoter is constantly changing in the wild-type population. In the *tsr19* deletion mutants, the number of IRs is fixed for a given mutant, representing the state of the wild-type organism when *tsr19* was deleted from a particular bacterial cell. Therefore, the gain or loss of an IR must rely on Tsr19 and likely occurs during inversion. As Tsr19 mediates inversion of a second promoter located in a distant region of the chromosome between the divergently transcribed genes BF3223 and BF3224, we sequenced this second promoter region from all 12 *tsr19* deletion mutants to determine if variation in the number of IRs also occurs in this region. In 4 of these 12 mutants this second promoter was locked in one orientation, and in 8 mutants the promoter was locked in the other orientation; however, for each mutant there was only a single IR on each side of the promoter. Therefore, the amplification and deletion of IRs that occur during inversion of the EPS promoter appear not to occur in this distant promoter region.

Number of IRs affects transcription into BF2782. Based on the phenotype, we hypothesized that an increase in the number of IRs between the promoter and BF2782 decreases transcription into this gene. To test this hypothesis, we made *xylE* transcriptional fusion constructs by cloning DNA between the promoter in the on orientation and BF2782 from Δ*tsr19* mutants with one, two, or three downstream IRs (Fig. 2). These constructs were mobilized into the wild type, and the resulting XylE activity was quantified. As shown in Fig. 2, the XylE activity of the clone with only one IR between the promoter and BF2782 was much greater than the activities of clones containing two or three IRs. We previously showed that when the promoter is cloned in the off orientation, no XylE activity is produced (9). These results are in accordance with

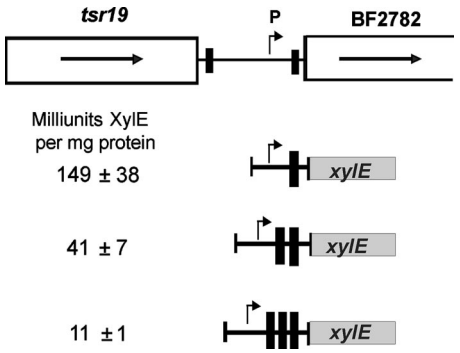

FIG. 2. Number of IRs regulates transcription into BF2782: diagrams of the *xylE* transcriptional fusion clones that were created for analysis of transcription into BF2782. The diagram at the top shows the corresponding chromosomal region. Black boxes represent IRs; P is the promoter. Each clone includes DNA containing the promoter in the on orientation, one, two, or three IRs, and DNA up to 6 bp before the start codon of BF2782. The corresponding XylE activity resulting when each of the clones was placed in *trans* in the wild-type background is indicated.

the phenotypic observations and demonstrate that the presence of multiple IRs between the promoter and downstream gene decreases the level of transcript produced, resulting in a reduction in the amount of putative EPS material produced. This is the first report of this type of regulatory mechanism in *B. fragilis* and demonstrates that there are at least two levels of transcriptional regulation of the EPS genes, DNA inversion of the promoter and number of IRs between the promoter and the downstream genes, both of which are dependent upon Tsr19.

EPS expression accounts for the previously reported large capsule phenotype. A phenotypic study published 25 years ago showed that a very small percentage of wild-type cells grown in vitro produce large capsules whose width is greater than that of the underlying bacterial cells (7). Although *B. fragilis* NCTC9343 produces multiple capsular polysaccharides, all of which undergo phase variation, these molecules form relatively small capsules, and their phase-variable expression does not account for the observed large capsule phenotype. Based on the phenotype of the *tsr19* mutants that are locked on for transcription of the putative EPS locus, we hypothesized that the phase-variable expression of this molecule could account for the large capsule phenotype. Bacterial cells producing large capsules remained at the 20% Percoll-broth interface in a Percoll density gradient and thus migrated differently than the remainder of the cells, which segregated between the 40% Percoll-60% Percoll and 60% Percoll-80% Percoll interfaces (7). To determine if expression of the putative EPS locus resulted in a phenotype consistent with that of bacteria producing large capsules, we analyzed the wild-type strain, Δ*tsr19* M5 (EPS locked on, one IR), and Δ*tsr19* M3 (EPS locked off) using Percoll density gradients. This analysis showed that bacteria constitutively transcribing the putative EPS locus were unable to enter the gradient and remained at the 20% Percoll-broth interface (Fig. 3A, tube 2), like the bacteria producing large capsules. This is in contrast to the migration of wild-type bacteria (tube 1) and Δ*tsr19* M3 cells (tube 3), which

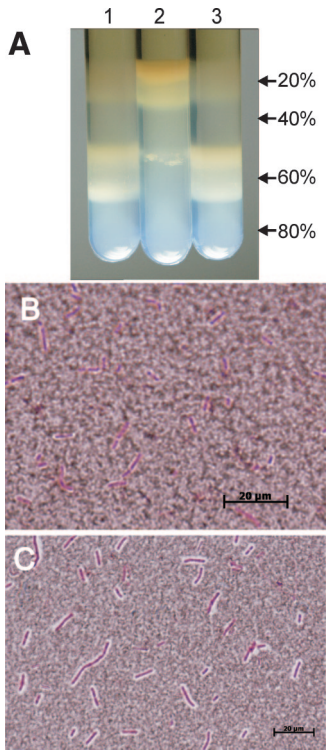

FIG. 3. EPS locus confers a large capsule phenotype to the bacteria. (A) Percoll density gradient analysis of sedimentation of the wild type (tube 1), Δ*tsr19* M5 (tube 2), and Δ*tsr19* M3 (tube 3). The arrows on the right indicate the different concentrations of Percoll. (B and C) Light microscopy images of negatively stained overnight grown broth cultures of the wild type (B) and Δ*tsr19* M5 (C), showing small capsules surrounding the wild type and large capsules surrounding Δ*tsr19* M5.

settled between the 40% Percoll-60% Percoll and 60% Percoll-80% Percoll interfaces.

To conclusively determine if Δ*tsr19* M5 cells produce large capsules, light microscopy analysis was performed. Using a standard negative staining technique to visualize the size of a bacterial capsule, we observed that Δ*tsr19* M5 cells were surrounded by a much larger polysaccharide layer than wild-type cells (Fig. 3B and 3C). The size of the small capsules surrounding the wild-type cells is consistent with the size of the polysaccharide layer produced by the capsular polysaccharides. These combined data demonstrate that the putative EPS locus in fact encodes products involved in the synthesis of an extracellular polysaccharide, which confers a large capsule phenotype to the bacteria and prevents them from forming a tight pellet upon centrifugation. The expression of this molecule is phase variable, explaining why only a small percentage of wild-type bacteria grown in vitro have this phenotype. These data provide a molecular rationale for 25 years of phenotypic observation of variations in *B. fragilis* capsule size.

EPS is a high-molecular-weight molecule. EPSs of various bacteria are very-high-molecular-weight molecules and often have molecular masses greater than 500 kDa. In contrast, the sizes of the capsular polysaccharides of *B. fragilis* are heterogeneous; most molecular masses range from as little as 60 kDa to more than 500 kDa for a single capsular polysaccharide (5).

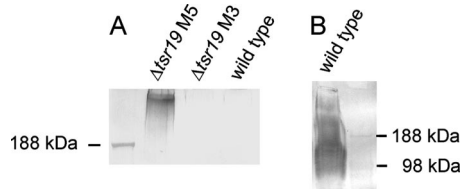

FIG. 4. Analysis of EPS size. (A) Western immunoblot analysis of whole-cell lysates demonstrating that the locked-on mutant (Δ*tsr19* M5) synthesizes a very-high-molecular-weight EPS not produced by the locked-off mutant (Δ*tsr19* M3) or the wild type. (B) Western immunoblot analysis of a whole-cell lysate of wild-type bacteria showing heterogeneous capsular polysaccharide H as a representative of the capsular polysaccharides. The sizes of molecular mass standards are indicated on the right.

To determine the molecular size of the *B. fragilis* EPS, we made specific antiserum to this molecule by immunizing rabbits with a *tsr19* locked-on mutant, constitutively expressing EPS, and adsorbing the antiserum with a *tsr19* locked-off mutant. Western immunoblot analysis using this EPS-specific antiserum demonstrated that the EPS is a very-high-molecular-weight molecule with a molecular mass of more than 500 kDa (Fig. 4A), which is much larger than the average size of the capsular polysaccharides of *B. fragilis* (Fig. 4B).

Analysis of EPS expression potential in vitro and in vivo. All of the previous phenotypic analyses have shown that wild-type bacteria grown in vitro have characteristics similar to those of the EPS locked-off mutants rather than to those of the locked-on mutants. Wild-type bacteria form a tight pellet upon centrifugation, have only a small capsule layer rather than a large capsule layer, and segregate in a Percoll density gradient like mutants lacking EPS. Also, the large capsule phenotype reported by Patrick and Reid occurred in less than 1% of a wild-type population grown in vitro (7). Based on these observations, we predicted that the EPS promoter must be in the off orientation in the great majority of cultured wild-type bacteria. In order to quantitatively determine the relative orientation of the EPS promoter from wild-type bacteria isolated from different sources, the PCR-digestion technique illustrated in Fig. 5A was used. This assay allows quantitative determination of promoter orientation based on the intensity of the resulting PCR digestion fragments representing the promoter on and off bands. We analyzed three separate wild-type populations grown on different days in vitro and found that in nearly all of the bacteria from all three samples the EPS promoter was oriented off, with no detectable fragments corresponding to on promoters (Fig. 5B).

We next analyzed the orientation of this promoter in bacteria grown in vivo. We began by analyzing bacteria present in the fecal samples of four different gnotobiotic mice that had been colonized with the wild type for 3 to 4 weeks. As shown in Fig. 5B, in approximately 50% of the bacteria from the mouse 1 and 2 fecal samples the EPS promoters were oriented on. Mice 3 and 4 yielded samples with bacteria whose EPS promoter was largely oriented off; however, these samples revealed an interesting result. The presence in these samples of two small restriction fragments that were different sizes was very obvious. Based on our previous finding regarding variation in IR number, we predicted that these multiple smaller fragments were due to variations in the number of IRs upstream of BF2782. The two small restriction fragments obtained from the sample from mouse 4 were separately excised from the gel and sequenced. As predicted, the larger fragment contained two copies of the IR upstream of BF2782, whereas the smaller fragment contained only one IR. These data demonstrate that the variation in the number of IRs flanking the EPS promoter is a natural process in *B. fragilis* and occurs in vivo.

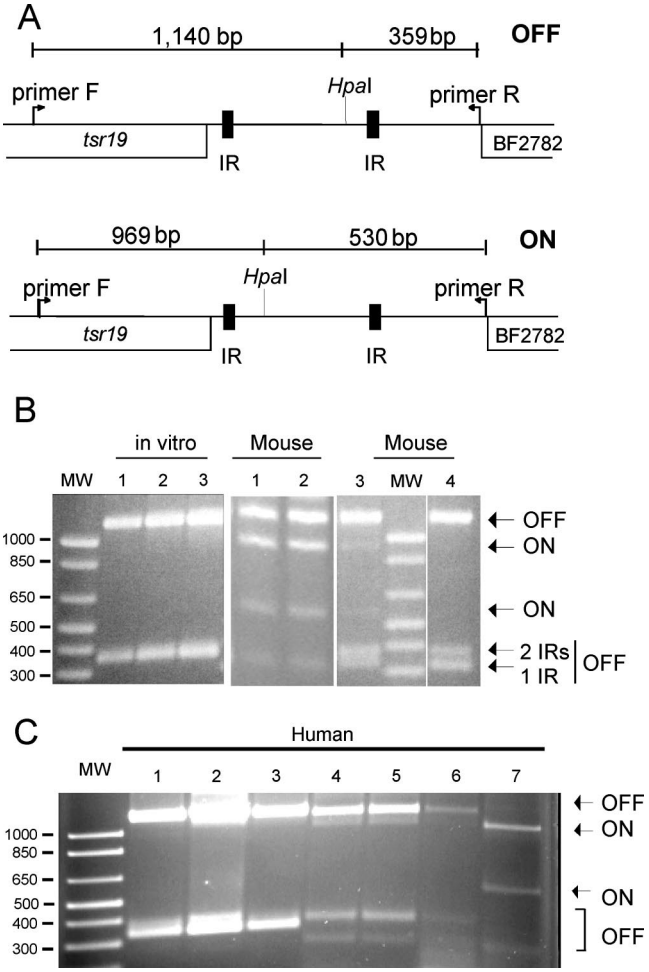

FIG. 5. EPS promoter orientations of in vitro and in vivo samples. (A) Schematic diagram of the quantitative PCR-digestion assay used to determine EPS promoter orientation. (B) Ethidium bromide-stained agarose gels of the fragments resulting from PCR-digestion analysis of bacteria grown in culture or isolated from feces of mice monoassociated with the *B. fragilis* wild-type strain. The number of IRs determined to be present in each fragment is indicated. (C) PCR-digestion analysis of the *B. fragilis* EPS region from human fecal samples showing differences in promoter orientation and the number of IRs flanking the promoter. The sizes of molecular weight standards (MW) (in base pairs) are indicated on the left in panels B and C.

Due to the fact that most humans are colonized with relatively high numbers of *B. fragilis* and because the EPS genetic region is conserved in *B. fragilis*, we reasoned that it should be possible to directly analyze the orientation of the EPS promoter from *B. fragilis* present in human fecal samples. For this analysis, DNA was harvested directly from fecal samples ob-

tained from seven healthy adults, and the PCR-digestion assay was performed. These analyses revealed that in more than 90% of *B. fragilis* cells from one of the samples (sample 7) the EPS promoter was oriented on (Fig. 5C). In the *B. fragilis* cells from the other six samples the EPS promoters were oriented off; however, we were able to observe the same level of variation in the number of IRs that was seen in the mouse experiments. In fact, we were able to detect restriction fragments containing one, two, and three IRs on the BF2782 side of the promoter in these human samples. Most of the bacteria from samples 4 and 5 produced fragments containing one or three IRs upstream of BF2782, whereas the bacteria from samples 1, 2, and 3 produced fragments containing mainly two IRs. Sequence analysis of the lower restriction fragment resulting from human sample 7 demonstrated that *B. fragilis* from this individual contained a single IR between the on promoter and BF2782, suggesting that these bacteria were expressing a large amount of EPS. These data conclusively demonstrate that the variation in the number of IRs flanking the EPS promoter is a natural occurrence in the wild-type population in the endogenous human intestinal ecosystem.

DISCUSSION

The synthesis of eight capsular polysaccharides by a bacterium has not been reported outside the intestinal *Bacteroidales*. The genetic machinery that is dedicated to the synthesis and regulation of these multiple polysaccharides is tremendous. Here we demonstrate that *B. fragilis* synthesizes yet another polysaccharide, an EPS. This EPS is distinguished from the capsular polysaccharides because it is an extremely high-molecular-weight molecule and forms a large polysaccharide layer surrounding the bacteria, preventing them from forming a tight pellet upon centrifugation and from entering a Percoll density gradient. The phase-variable synthesis of this molecule provides a genetic basis for the large capsule that was described surrounding a small proportion of *B. fragilis* cells grown in vitro (7).

The small number of genes in the EPS locus suggests that the EPS may have a very simple structure. Alternatively, it is possible that other genes outside this locus are also involved in EPS synthesis; if so, the EPS may be a more complex molecule. Elucidation of the structure of the EPS should aid in determining if other products are likely to be involved in its synthesis and may help to identify putative gene products.

The capsular polysaccharides of *B. fragilis* are regulated by invertible promoters, as is the EPS; however, the DNA invertases that mediate these events belong to two distinct families of site-specific recombinases. In addition, the EPS has a second level of regulation not found in the regulation of the capsular polysaccharides, a variable number of IRs. This is the first study to show that the number of IRs has a role in gene regulation in *B. fragilis*. The variation in the number of IRs requires the tyrosine family site-specific recombinase Tsr19. Site-specific recombinases mediate recombination events that differ from general recombination in that they recognize short, specific sequences that are present as direct repeats or IRs. It is likely that the number of IRs on each side of the promoter changes following inversion depending upon which IR in a series of adjacent IRs (or direct repeats with respect to each other) is used for recombination. For example, if the region upstream of the promoter contains a single IR and the region downstream of the promoter contains three IRs, the number of IRs on each side following inversion depends upon which of the three downstream IRs is the target for recombination. If the most internal of the three downstream IRs is the site of inversion, the number of IRs on each side does not change. If, however, the most downstream of the three IRs is the site of recombination, the two IRs internal to it are part of the invertible element and flip to the other side. The data indicate that the absolute number of IRs is also not constant. For example, some mutants contained a total of three IRs, while others contained four IRs. It is possible that adjacent IRs which are direct repeats of each other could recombine with each other, leading to the loss of a repetitive element.

Because we were able to detect differences in the number of IRs flanking the EPS promoter in bacteria present in human fecal samples, this regulatory mechanism likely has relevant biological consequences. The synthesis of a large polysaccharide could be advantageous for the organism under certain in vivo conditions as this polysaccharide may mask other surface antigens or provide a protective barrier. However, the constitutive synthesis of such a copiously produced molecule may be energetically unfavorable, especially when the concentration of carbon sources in the ecosystem is low. Therefore, phase-variable regulation of this molecule coupled with alterations in the amount produced may ensure that there is always a population of organisms that are properly equipped to survive in a changing ecosystem.

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