Orientations of the \emph{Bacteroides fragilis} capsular polysaccharide biosynthesis loci promoters during symbiosis and infection

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Running title: \emph{B. fragilis} PS loci promoter orientations \textit{in vivo}

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ABSTRACT

Orientations of the seven invertible polysaccharide biosynthesis loci promoters of *B. fragilis* were quantified from bacteria grown *in vitro*, from feces of monoassociated and complex colonized mice, and from *B. fragilis*-induced murine abscesses. Bacteria grown *in vivo* have greater variability in orientations of polysaccharide locus promoters than culture grown organisms.

Intestinal Bacteroidales species are prominent members of the gut microbiota but can become opportunistic pathogens following spillage of intestinal contents into the abdominal cavity with bacteria frequently isolated from blood and intra-abdominal abscesses (8, 16). The synthesis of a large number of capsular polysaccharides (PS) that undergo phase variation is a relatively conserved property within intestinal Bacteroidales species (3). The model intestinal Bacteroidales species used to study PS synthesis and regulation is *Bacteroides fragilis*. The prototype strain NCTC9343 synthesizes eight distinct capsular polysaccharides, designated PSA-PSH (7). Previous studies have shown that PS production is necessary for gut colonization (2, 11). In addition, antigen-specific immune responses to PSA can stimulate immune system development in germ-free mice and prevent intestinal inflammation in animal models of colitis (12, 13). Furthermore, PSA production is required for *B. fragilis* to initiate abscess formation in experimental models (5, 18).

Genes encoding the enzymes necessary for the synthesis of the individual PSs are organized as distinct operons, each with a single upstream promoter, referred to hereafter as *ps* promoters. Seven of the eight *ps* promoters are flanked by 19 to 25 basepair inverted repeat sequences (IRs) (7). The DNA segments between the IRs range in size from 168 to 193.
basepairs and undergo reversible inversions (7). A serine family site-specific recombinase, Mpi, mediates the inversions of each of the seven promoter regions, thereby placing them in the correct (on) or incorrect (off) orientation for transcription of the downstream polysaccharide biosynthesis genes (6).

Despite the importance of PS synthesis for intestinal colonization and abscess formation, little is known about ps promoter orientations in vivo. This study builds upon our current knowledge of PS regulation by examining the orientations of all seven invertible ps promoters from bacteria grown in culture and from in vivo sites representing symbiosis and disease.

A previously described PCR digestion technique was used to quantify the percentage of B. fragilis that had each ps promoter oriented on or off (7) (Table S1 and Fig. 1A, S1A). Chromosomal DNA was isolated from B. fragilis grown under various conditions and PCRs were performed using primers that annealed outside the IRs flanking each invertible promoter region such that there was no bias in the PCR for either promoter orientation (Fig. S1B). Each PCR product was digested with a restriction enzyme that cut asymmetrically between the IRs resulting in four differently sized fragments, two from a promoter in the on orientation and two from a promoter in the off orientation (Fig. 1A, S1A). The percent of bacteria that had a ps promoter in the on orientation was calculated by measuring the intensity of each of the resulting DNA fragments following ethidium bromide staining in agarose gels and dividing the intensity of the fragments from the promoters in the on orientation by the intensity of the fragments from the promoters in both the on and off orientations using Image J software (available at rsbweb.nih.gov/ij/).

To study B. fragilis ps promoter orientations independent of host factors and other microbial species, ps promoter orientations from three independent exponential phase broth
cultures were calculated. While the *psa* promoter was in the on orientation from greater than 80% of the bacteria from each of the broth cultures, none of other *ps* promoters, with the exception of *pse*, were observed in the on orientation in greater than 8% of bacteria (Figs. 1, 2).

To study the effects of host factors on *ps* promoter orientations during intestinal colonization, we examined bacteria from fresh fecal samples of nine-week-old Swiss-Webster gnotobiotic mice that were monocolonized with *B. fragilis* from birth via fecal-oral transmission from the monocolonized mother. Using this model, monocolonization with *B. fragilis* is stably maintained over time at a level of approximately $3 \times 10^{10}$ CFU/gram (4). Samples were collected from nine male mice from two different litters (Fig S2) and examined individually. Bacterial DNA was isolated using the QiaAmp DNA Stool Mini Kit (Qiagen). Similar to the broth cultures, the *psa* promoter was in the on orientation in a majority of *B. fragilis* from the feces of each of the monocolonized mice (Fig. 2). The *psb* and *psh* promoter orientations were variable between different monocolonized mice; in some mice, the *psb* and *psh* promoters were on in greater than 90% of bacteria, whereas in other mice, less than 25% of the bacteria had these promoters oriented on (Figs 2, S3). In all cases, the percentages of bacteria with these two *ps* promoters oriented on were greater than from bacteria grown *in vitro*. Analysis of the other four *ps* promoters revealed consistent trends between all mice of both litters with a very small percentage of the bacteria with these promoters oriented on (Figs 2, S3). In comparison to the *in vitro* grown bacteria, which demonstrate has very little variability in the percentage of the population with a particular *ps* promoter oriented on, i.e. tight clustering between samples, analysis of bacteria nine weeks after monoaosociation demonstrated more variability in *ps* promoter orientations. We applied Levene’s test of variance homogeneity (9) and rejected the
null hypothesis of equivalent variances between in vitro and monocolonized bacteria and obtained significant values for the PSB (p = 0.026) and PSE (p < 0.001) promoters.

The experiments in monocolonized mice provided insights into the influence of host factors on ps promoter orientations. However, as no other microbes are present, it is a simplified model of the intestinal environment. To examine the effects of a complex microbiota on ps promoter orientations, the mice in the two litters, henceforth referred to as litter A and litter B, were split immediately following the promoter orientation analysis described above. Half of the mice of each litter were removed from the isolator and placed in a cage with an eight to ten-week old specific-pathogen-free (SPF) Swiss-Webster mouse. After twenty-four hours, the SPF mouse was removed from the cage. Fecal samples were collected from the pups two weeks after they had been moved into the SPF facility and from monocolonized littermates that remained in the gnotobiotic isolator for the duration of the experiment (Fig. S2).

The greatest differences were observed when comparing promoter orientations from B. fragilis from the monocolonized and complex colonized mice from the two litters (Figs 2, S4). Within litter A, a larger percentage of B. fragilis from the complex colonized mice had the psa psd and psg promoters in the on orientation when compared to the B. fragilis from the monocolonized mice. The largest increase was observed in the psg promoter which was in the on orientation in only 3.3+/−0.5% of the bacteria from the monocolonized mice but 40.5+/−32.8% of those from the complex colonized mice of litter A. In contrast, within litter B, there was a decrease in the percentage of the bacteria with the psa and psh promoters in the on orientation and no increase in the psd and psg promoters in the complex colonized mice relative to the two monocolonized mice. Furthermore, with the exception of the psb promoter in one of the two mice, no other ps promoters were in the on orientation in greater than 11% of the bacteria from
either of the two mice of litter B (Fig. 2, S4). To assess differential clustering of promoter orientations between monocolonized and complex colonized states, we used extended generalized estimating equations (20) to estimate the intralitter correlation coefficients for proportion ps promoters in on orientation in each of the two states. There was no evidence of ps promoter clustering by litter from bacteria of monocolonized mice (intralitter correlation = -0.036, p = 0.67). However, ps promoter orientations from bacteria from the complex microbiota demonstrated high intralitter clustering (correlation = 0.65, p = 0.022).

These data suggest that the presence of a complex microbiota influences B. fragilis ps promoter orientations. The significant differences observed between the two litters under complex colonization may be due to differences in the composition of the microbiota. The complex microbiontas were acquired by co-habitation with SPF mice. The two litters of mice were born 45 days apart and were housed with unrelated SPF mice. A study has shown that kinship is a contributing factor to the composition of the mouse microbiota, and that unrelated mice of the same strain and housed in the same animal facility have significant compositional differences in their microbiontas at the genus level (10). Hence, there may have been significant differences in the microbiontas acquired by these two litters of mice, which may have altered host-B. fragilis or microbe-B. fragilis interactions in a way that affected ps promoter orientations.

As B. fragilis is both an intestinal symbiont and an opportunistic pathogen, we sought to determine if ps promoter orientations are different from bacteria in symbiotic and pathogenic states. Therefore, analyses were performed with B. fragilis from murine intra-peritoneal abscesses. The abscess model was performed similar to the model previously described (19). Briefly, six to eight-week-old male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) were injected intra-peritoneally with B. fragilis (1x10^8 CFU/mouse) mixed with sterile rat cecal...
contents (1:1 v/v, 0.2 ml total volume), which mimics the spillage of colonic contents into the peritoneal cavity (14, 17). Seven days later, intraabdominal abscesses were excised and processed for bacterial DNA isolation. One abscess was collected from each of seven mice. Five abscesses were isolated from the liver and two from the mesentery.

The three ps promoters that were oriented on in the greatest percentage of bacteria from abscesses were the psa, psb and psh promoters; the same promoters that were most frequently oriented on from bacteria in the feces of monocolonized mice (Figs. 2, S5). Both the abscess and monocolonization models are devoid of other microbes in the compartment studied and reflect the interaction of B. fragilis alone with the host. Clinical abscesses, however, are usually polymicrobial. It is possible that within abscesses, the ps promoter orientations would vary if other bacteria were present. PSA has been demonstrated to have a role in the induction of intraabdominal abscesses in this model system (5, 18). This is the first study to demonstrate that the psa promoter is actually oriented on in a majority of the bacteria in abscesses, supporting that this abscess-inducing polysaccharide may be relevant to the disease process.

Overall, there are relatively consistent patterns of ps promoter orientations between different growth environments although there is more ps promoter orientation diversity between mice in the polymicrobial environment. The psa promoter is the only ps promoter that is oriented on in the majority of bacteria from all sites analyzed. In the in vitro grown bacteria, only two ps promoters, psa and pse, are present in the on orientation in more than 5% of the bacteria. Bacteria present in various in vivo environments tended to have other ps promoters, most frequently psb and psh, present in the on orientation.

The inversions of each of the seven ps promoters are mediated by the same DNA invertase, Mpi, which binds the IRs that flank each of the promoter regions. The ps promoters
that are most frequently observed in the on orientation and demonstrate the most variability in terms of orientations in the environments examined, \textit{psa, psb, pse,} and \textit{psh}, are flanked by identical IRs (Fig. S6). The IRs surrounding the \textit{psd, psf,} and \textit{psg} promoters share the same 10-bp IR consensus sequence, but differ from each other and the other four IRs outside this region (7). In addition, DNA sequences extending beyond the IRs are likely also recognized by the recombinase (15). Differences in the sequences of these IRs and the surrounding regions may influence the binding affinity of Mpi and lead to certain promoters such as \textit{psd, psf} and \textit{psg} oriented off in most of the bacteria.

The experiments outlined in this paper provide evidence that there are as yet undefined factors that influence \textit{ps} promoter orientations, and hence, PS synthesis in \textit{B. fragilis}. Potential factors influencing \textit{ps} promoter orientation include environmental cues or selection against bacteria synthesizing a particular PS. PS synthesis is controlled by multiple levels of regulation, with on orientation of a \textit{ps} promoter being a necessary, yet incomplete requirement for expression of the respective polysaccharide (1). Future investigations are necessary to reveal factors that influence \textit{ps} promoter orientations in different \textit{in vivo} environments.

REFERENCES


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FIGURE LEGENDS

FIG. 1. ps promoter orientations from B. fragilis grown in vitro. A. Schematic diagram of the PCR digestion method used to determine psa promoter orientation. All PCR programs had an initial incubation of 2 minutes at 94°C followed by 30 cycles of 94°C, 30 s, 59°C, 30 s, 72°C, 90 s and a final 5 minute incubation at 72°C. The DNA fragments were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. B. Ethidium bromide-stained agarose gels demonstrating the fragments resulting from PCR digestion analysis of bacteria from three independent broth cultures. Each lane represents the data from one independent broth culture.

FIG. 2. B. fragilis ps promoter orientations from bacteria grown in broth cultures (triangles), from fecal samples of monoclonized mice at nine weeks of age (week 9 mono, diamonds), from fecal samples of the same mice two weeks later that remained in the gnotobiotic isolator (week
11 mono, circles), or that were removed from the isolator at nine weeks and acquired a complex flora (week 11 complex, squares), and from murine intra-peritoneal abscesses (Xs). Each data point represents one independent broth culture, mouse, or abscess. Pertaining to the fecal samples, open symbols indicate the mice were from litter A and closed symbols indicate the mice were from litter B. Fecal samples from the mice of litter B that remained in the isolator for the duration of the experiment were not analyzed at the first time point. Horizontal bar represents the median.
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Volume 192, no. 21, pages 5832–5836, 2010. Supplemental material: In Table S1, the restriction enzymes for the PCR digestions of the following four PS locus promoters should be as follows: for the *psd* promoter, *DraI*; for the *psf* promoter, *EarI*; for the *psg* promoter, *PacI*; and for the *psh* promoter, *SfcI*. Revised supplemental material is posted at http://jb.asm.org/content/192/21/5832/suppl/DC1.

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