Regulation of surface architecture by symbiotic bacteria mediates host colonization

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Edited by Rino Rappuoli, Novartis Vaccines, Siena, Italy, and approved January 10, 2008 (received for review October 4, 2007)

Microbes occupy countless ecological niches in nature. Sometimes these environments may be on or within another organism, as is the case in both microbial infections and symbiosis of mammals. Unlike pathogens that establish opportunistic infections, hundreds of human commensal bacterial species establish a lifelong cohabitation with their hosts. Although many virulence factors of infectious bacteria have been described, the molecular mechanisms used during beneficial host–symbiont colonization remain almost entirely unknown. The novel identification of multiple surface polysaccharides in the important human symbiont *Bacteroides fragilis* raised the critical question of how these molecules contribute to commensalism. To understand the function of the bacterial capsule during symbiotic colonization of mammals, we generated *B. fragilis* strains deleted in the global regulator of polysaccharide expression and isolated mutants with defects in capsule expression. Surprisingly, attempts to completely eliminate capsule production are not tolerated by the microorganism, which displays growth deficits and subsequent reversion to express capsular polysaccharides. We identify an alternative pathway by which *B. fragilis* is able to reestablish capsule production and modulate expression of surface structures. Most importantly, mutants expressing single, defined surface polysaccharides are defective for intestinal colonization compared with bacteria expressing a complete polysaccharide repertoire. Restoring the expression of multiple capsular polysaccharides rescues the inability of mutants to compete for commensalism. These findings suggest a model whereby display of multiple capsular polysaccharides provides essential functions for bacterial colonization during host–symbiont mutualism.

bacterial symbiosis | *Bacteroides fragilis* | capsular polysaccharide | intestinal microbiota

We live in a microbial world. Immediately upon birth, humans coordinately assemble a complex bacterial microbiota on almost all environmentally exposed surfaces (1). Although it has been appreciated for decades that humans harbor multitudes of commensal bacteria, recent studies have begun to reveal the extraordinary diversity and complexity of the ecosystem we provide to microorganisms. Advances in genomic technologies have demonstrated that we harbor dozens of bacterial species in our stomachs, hundreds on our skin and oral cavity, and thousands within our lower gastrointestinal tract (2–4). The magnitude of these interactions and the evolutionary forces that drive them must exert profound influences on the biology of both microbe and man. The gastrointestinal tract provides an excellent example of the complex interactions between the microbiota and the host (5). Bacteria dominate this biological niche, both numerically and in terms of diversity. Of the multitudes of bacterial species that colonize the mammalian gastrointestinal tract ($>10^{13}$ organisms from >1,000 different species), those of the genus *Bacteroides* are among the most numerically prominent in humans (6). For decades, bacteria have been known to perform the essential function of metabolizing complex carbohydrates subsequently used by their mammalian hosts; *Bacteroides* species have been shown to be essential for this function (7, 8). Analysis of the genome sequences of the human *Bacteroides* (*Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides distasonis*, and *Bacteroides fragilis*) reveals that this genus has evolved numerous glycosidases for carbohydrate degradation (9). *B. thetaiotaomicron* induces carbohydrate decorations of the intestinal epithelium to mediate the normal architectural development of host tissue (10). Furthermore, *B. fragilis* was first described to produce multiple surface capsular polysaccharides (11). It has been recently revealed that all studied *Bacteroides* contain numerous genomic loci for capsular polysaccharide production, a unique and distinguishing feature of this genus of bacteria. Studies show that various *Bacteroides* species share with their human host a mammalian-evolved biochemical pathway for the addition of sugar modifications to surface proteins and polysaccharides (12). Moreover, we have recently demonstrated that *B. fragilis* elaborates an important immunomodulatory polysaccharide that instructs the normal development of the host immune system (13). Thus, the *Bacteroides* have dedicated a significant proportion of their biology to the production and functions of capsular polysaccharides during coevolution with mammals.

Decades of research have assigned various functions to surface polysaccharides of pathogens, including biofilm production, tissue adherence, and antiphagocytic activity during immune evasion (14). Capsule production has been shown to be required for bacterial virulence in numerous animal models of disease, and polysaccharides are the key components of many vaccines developed to prevent pathogenic bacterial infections (15, 16). Conversely, the biologic significance of capsular polysaccharide production during beneficial host–bacterial commensalism has only recently been suggested (17). It is believed that the multiple capsular polysaccharides (and perhaps other surface structures) of the *Bacteroides* create systems for altering the physical properties of bacterial surfaces (9). Several reports have predicted that the expression of multiple capsular polysaccharides by *B. fragilis* provides functions that are critical for host–bacterial symbiosis (11, 18, 19). However, this notion currently remains without experimental corroboration. In the report contained herein, we examine the role of capsular polysaccharide production during the relationship between *B. fragilis* and its mammalian host. By generating bacterial mutants in the regulation of capsule expression, we find that production of at least one capsular polysaccharide is required for the viability of the microorganism. Most importantly, inhibiting the ability of *B. fragilis* to modulate its surface architecture renders it unable to compete for colonization of the gastrointestinal tract of animals. It appears that *Bacteroides* have invested heavily in the development of

Author contributions: C.H.L. and S.M.L. contributed equally to this work; C.H.L., S.M.L., D.L.K., and S.K.M. designed research; C.H.L., S.M.L., and J.M.V. performed research; C.H.L., S.M.L., D.L.K., and S.K.M. analyzed data; and S.M.L. and S.K.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709266105/DC1.

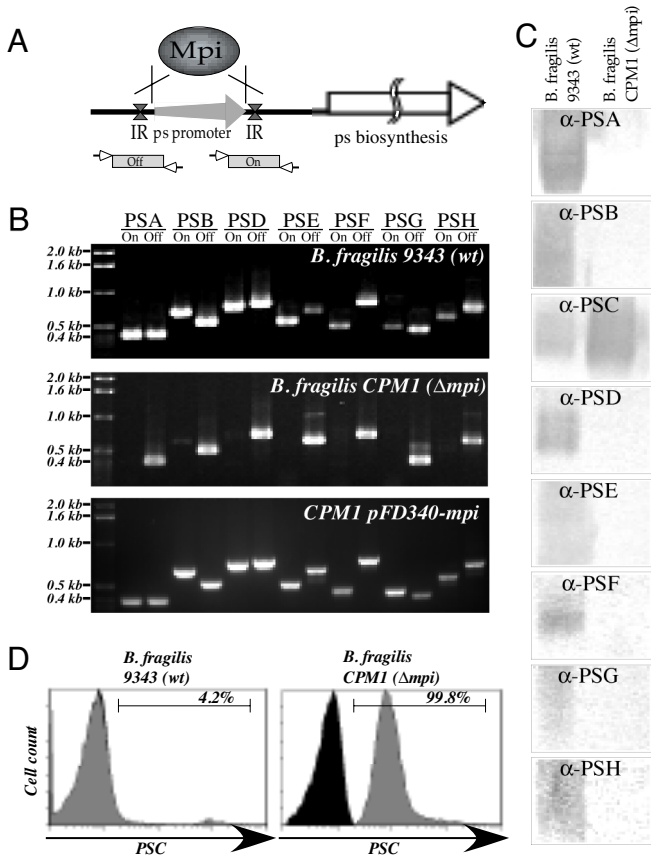

Fig. 1. Deletion of the global regulator of polysaccharide promoter inversion in *B. fragilis*. (*A*) Schematic representing promoters flanked by inverted repeats (IR) of polysaccharide biosynthesis loci. Mpi induces recombination at the IR sites to invert promoters. Boxes below represent PCR products distinguishing ''on'' and ''off'' orientations. (*B*) PCR products generated from chromosomal DNA of wild-type *B. fragilis* 9343, an isolated Δ*mpi* mutant (CPM1), and CPM1 strain complemented with the *mpi* gene (CPM1 pFD340-*mpi*). Unlike wild-type, all Mpi-controlled capsular polysaccharide (CPS) promoters are locked ''off'' in CPM1. Genetic rescue of *mpi* restores phase variation in CPM1. (*C*) Immunoblot analysis demonstrates that expression of PSC is not controlled by Mpi. Note the absence of all Mpi-regulated CPS and the overexpression of PSC. (*D*) Flow cytometry analysis of surface CPS demonstrates that all CPM1 cells express PSC (99.8%) compared with 4.2% in wild-type cultured cells. Black histograms represent the control antibody; gray histograms (α-PSC) show PSC on the surface of *B. fragilis* CPM1.

dynamic molecular mechanisms to establish and maintain residence within the mammalian ecosystem. These results provide the foundation for understanding our evolutionary cohabitation with the microbial world around and within us.

Results

Capsular Polysaccharide Expression Is Critical for *B. fragilis*. The eight known capsular polysaccharide (CPS) biosynthesis loci of *B. fragilis* are scattered throughout the genome as distinct polycistronic operons of 11–22 genes (PSA–PSH) (18, 20). Mpi (multiple promoter invertase), a member of the serine site-specific DNA recombinase family, acts as a global transcriptional regulator of polysaccharide expression through inverting promoters between the ''on'' and ''off'' orientations (Fig. 1*A*) (20). We introduced a genomic mutation in the *mpi* gene by homologous recombination in a PSA deletion background, because PSA is the most abundantly expressed polysaccharide [produced by 79% of all bacterial cells in culture (11)]. We subsequently screened mutants by PCR using promoter-specific primers to isolate clones with all Mpi-regulated promoters in the ''locked-off'' orientation. Similar to previous findings (20), cultures of wild-type *B. fragilis* are heterogeneous with regard to polysaccharide expression and are in phase variation displaying both ''on'' and ''off'' promoters [Fig. 1*B* and supporting information (SI) Table 1]. Upon mutagenesis, we isolated one mutant [*B. fragilis* CPM1 (capsular polysaccharide mutant)] with seven of the eight characterized promoters in the ''off'' orientation at the time of *mpi* deletion (PSC is not controlled by Mpi). When *mpi* was introduced into CPM1 by transcomplementation, each of the Mpi-controlled polysaccharide promoters regained the ability to invert (Fig. 1*B*). We phenotypically verified the presence or absence of CPS synthesis with immunoblot analysis using antisera specific for each molecule. A complete loss of polysaccharide expression from all Mpi-controlled loci was observed in strain CPM1 compared with wild-type *B. fragilis* (Fig. 1*C*).

Expression of the remaining known capsular polysaccharide (PSC) in *B. fragilis* is predicted to not be controlled by promoter inversion, although it shares other critical transcriptional regulatory elements with the seven Mpi-regulated CPS (11). Indeed, immunoblot analysis showed an increase in PSC production for CPM1 over that for wild-type cultures (Fig. 1*C*). Consistent with this observation, PSC was expressed in 100% of cells for the Mpi mutant strain CPM1 but in only 4.2% of wild-type cultures when measured by flow cytometry with PSC-specific antiserum (Fig. 1*D*). Thus, expression of PSC appears to represent a default mechanism for capsule expression in the absence of Mpi-regulated polysaccharides.

Capsule Mutants Spontaneously Revert to Express Capsular Polysaccharides. To characterize the role of polysaccharide production in *B. fragilis*, we introduced a PSC deletion vector into CPM1 to generate mutants defective in the production of PSC. The strain bearing this deletion (named CPM2) unexpectedly showed a dramatic defect in culture growth compared with that of wild-type *B. fragilis* or the mutant strain CPM1, as assessed by optical density and plating for colony-forming units (Fig. 2*A* and data not shown). Furthermore, the mutant strain aggregated upon *in vitro* growth in culture (*SI Materials and Methods* and SI Fig. 5). As previously shown for *B. fragilis* and other organisms, the absence of a capsule layer may expose surface adhesive molecules leading to aggregation (21, 22). We next investigated the possible mechanism(s) that may explain the growth attenuation resulting from deletion of polysaccharide biosynthesis in *B. fragilis*. When CPM2 was grown in laboratory culture media and sequentially passaged for 5 days, we observed a progressive restoration of growth (Fig. 2*A*). Furthermore, passaged cultures displayed a recovery of CPS synthesis, as demonstrated by the presence of high-molecular-weight species after immunoblot analysis of whole-cell lysates using antisera raised against whole bacteria (Fig. 2*B*). Thus, the absence of polysaccharide production and the attenuation of growth are either genetically or phenotypically linked, demonstrating the importance of polysaccharide molecules to *B. fragilis*. The 5-day-passaged strain that exhibits wild-type growth characteristics may have acquired a genetic mutation to overcome the selective pressure of a growth defect; accordingly, we termed this revertant strain CPM3.

Promoter Inversion Occurs in the Absence of Mpi. What mediates polysaccharide expression in an *mpi* mutant strain? Initially, we tested the hypothesis that alternate promoters directed in the ''off'' orientation may be driving transcription of capsular polysaccharides in CPM3. Promoter fusions to transcriptional reporters determined that ''off''-oriented promoters were incapable of mediating polysaccharide expression (SI Fig. 6). To examine the possible existence of a pathway secondary to Mpi that is capable of catalyzing promoter inversion, we screened the

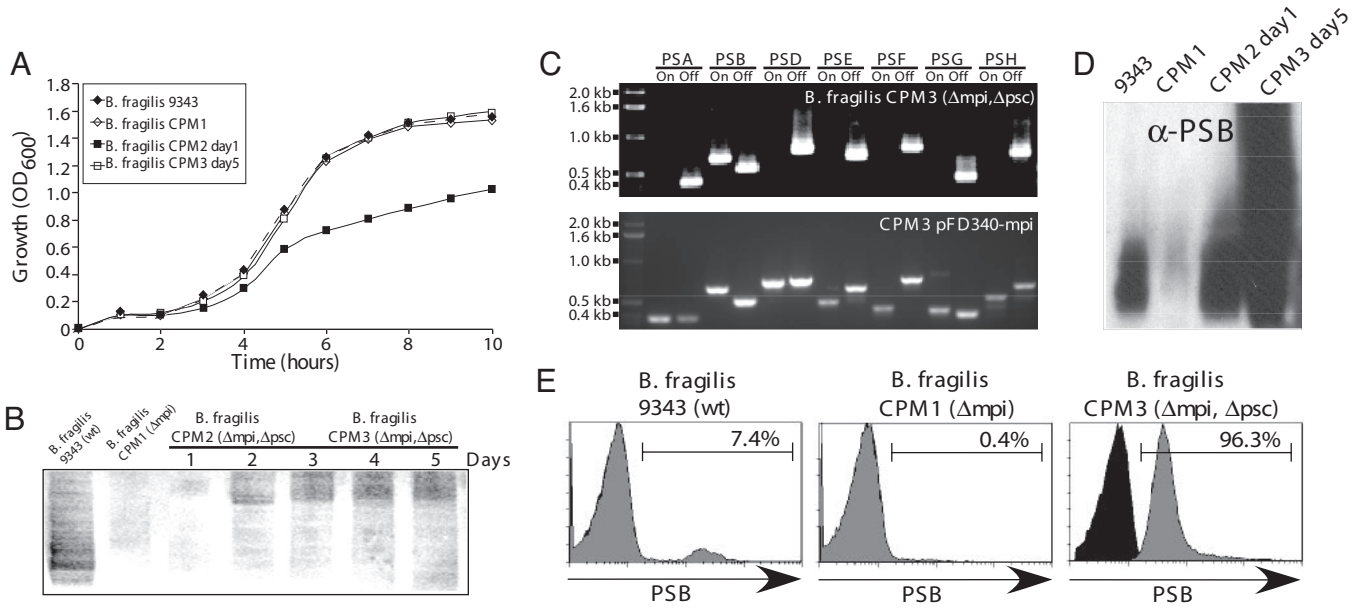

Fig. 2. Phenotypic reversion of *B. fragilis* mutants defective in CPS production. (*A*) Growth curve of bacterial cultures after specific deletion of PSC from the *mpi* mutant CPM1 shows severe growth attenuation of CPM2 (day1). A spontaneous reversion after serial passage of the bacterial cultures for 5 days restores *in vitro* growth (CPM3 day5). (*B*) Immunoblot analysis shows the overall reduction in CPS production in *mpi* mutant strains (CPM1 and CPM2) and the subsequent stepwise increase in polysaccharide production in CPM3 coincident with growth restoration. (*C*) Promoter analysis of CPM3 after 5 days of passage demonstrates that the PSB promoter undergoes reversion to the ''on'' orientation. CPM3 complemented with the *mpi* gene (CPM3 pFD340-*mpi*) displays restoration of phase variation at every promoter. (*D*) Immunoblot analysis with specific antisera shows the loss of PSB expression in CPM1 and a spontaneous recovery of PSB production under the selective pressure imposed by *psc* deletion (CPM2 day1). A phenotypic enrichment of PSB expression is found in CPM3. (*E*) Flow cytometry analysis of surface-displayed PSB reveals expression in nearly all bacterial cells after reversion in CPM3 but in only 7.4% of wild-type cultured cells. Black histograms represent control antibody staining; gray histograms (α-PSB) show PSB on the surface of *B. fragilis* CPM3.

promoters of polysaccharide operons in CPM3 for their ''on'' or ''off'' orientation. We were surprised to observe that the promoter upstream of the PSB biosynthesis locus displayed a reversion to the ''on'' orientation in CPM3 (the *mpi* mutant background), but only after PSC deletion (Fig. 2*C Upper*). Only the PSB promoter reverted in 8 of 10 trials (PSD and PSH once each); thus, there appears to be a requirement for production of at least one capsular polysaccharide, with a strong (but not absolute) bias for PSB production in the absence of Mpi. Immunoblot analysis of whole-cell lysates using antiserum specific to PSB demonstrated the phenotypic production of PSB (Fig. 2*D*). CPM3 exhibited recovery of all Mpi-regulated promoter inversions when *mpi* was provided *in trans* (Fig. 2*C Lower*). To quantify the frequency of reversion, we used flow cytometry to enumerate the proportion of bacteria expressing PSB on their surfaces. Unlike wild-type cultures, in which only 7.4% of cells in a mixed population express PSB, nearly all CPM3 revertants assembled PSB as part of their bacterial envelopes (Fig. 2*E*). A previously developed assay using cleavage of PCR products from invertible promoters demonstrated that the PSB promoter in strain CPM3 is found exclusively in the ''on'' orientation (SI Fig. 7) (11). Quantitative PCR digestion and flow cytometry showed that every bacterial cell expresses PSB, thus strongly suggesting that production of at least one capsular polysaccharide is essential for *B. fragilis* viability. This reversion resulting in the expression of PSB occurred only after deletion of a non-Mpi-controlled polysaccharide (PSC). A further link between the expression of PSB and PSC was shown through the significant increase in PSC-expressing bacteria upon deletion of only PSB (*B. fragilis* 9343ΔPSB), as measured by flow cytometry (SI Fig. 8). Taken together, these data demonstrate that *B. fragilis* has developed a profound and unusual requirement for the production of at least one capsular polysaccharide.

***B. fragilis* Employs Multiple Pathways for Capsular Polysaccharide Expression.** The finding that the PSB promoter reverted to the ''on'' orientation in CPM3 suggests that this strain acquires a ''gain-of-function'' phenotype to invert promoters under the physiologic stress of multiple polysaccharide deletion. To test whether CPM3 has the ability to catalyze promoter recombination in the absence of *mpi*, we introduced a reporter plasmid with the PSB promoter in the ''off'' orientation into several *B. fragilis* strains. We measured inversion of the PSB promoter within the plasmid from ''off'' to ''on'' orientation by PCR. The increased activity of promoter inversion in CPM3 was evidenced by recombination of the PSB promoter reporter, similar to the function found in wild-type bacteria (Fig. 3*A*). CPM1, also deleted in *mpi* but not displaying a selective pressure to revert to polysaccharide production, is incapable of flipping the ''off''-positioned PSB promoter. Furthermore, analysis of reporter plasmids for the remaining polysaccharide promoters illustrates that they are incapable of flipping to the ''on'' orientation in CPM3 (SI Fig. 9), corroborating the specificity of CPM3's activity for the PSB promoter.

In addition to *mpi*, the genome sequence of the prototype *B. fragilis* strain NCTC9343 has been shown to contain two other serine site-specific recombinase genes: *ssr1* and *ssr3* (also known as *finB*) (Fig. 3*B*) (19, 20). We hypothesized that one of these homologues may serve the nonredundant function of catalyzing recombination at invertible promoters in the absence of Mpi. To determine whether either gene product could mediate promoter inversion, we used reporter plasmids with the PSB promoter in the ''off'' orientation that also expressed either Ssr1 or Ssr3. These reporter constructs were subsequently introduced into *B. vulgatus*, a related species previously shown to be incapable of inverting the polysaccharide promoters of *B. fragilis* (20). As demonstrated in Fig. 3*C*, Ssr1 was unable to mediate PSB promoter inversion in *B. vulgatus*, as assessed by PCR. In

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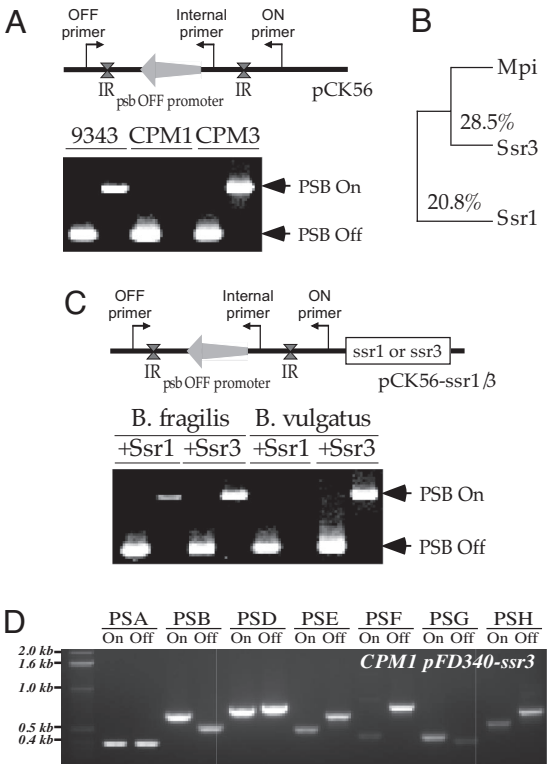

Fig. 3. An Mpi-independent pathway for promoter inversion. (*A*) Upon introduction of reporter plasmid pCK56 (containing the PSB promoter in the ''off'' orientation), wild-type *B. fragilis* and CPM3 are competent to mediate conversion of the ''off'' promoter to the ''on'' position. CPM1 (Δ*mpi*) lacks the ability to invert the locked-off *psb* promoter. (*B*) Phylogenetic analysis demonstrating the relatedness of the three serine site-specific recombinases (Ssr) in *B. fragilis* NCTC9343 generated by CLUSTAL W analysis. The percent identity values indicate direct sequence comparisons of Ssr3 and Ssr1 to Mpi, respectively. (*C*) PCR products from a reporter plasmid containing the *psb* promoter in the ''off'' orientation and each of the Mpi homologues, Ssr1 and Ssr3. The *psb* promoter inverts in *B. vulgatus* only in the presence of Ssr3. Ssr3 overexpression in wild-type *B. fragilis* shows an increase in inversion activity. (*D*) PCR promoter orientation assay of CPM1 complemented with *ssr3* (CPM1 pFD340-*ssr3*). All Mpi-regulated promoters in the ''off'' orientation undergo phase variation when Ssr3 is constitutively expressed. Compare complementation to the expression of Mpi (Fig. 1*B*).

contrast, the expression of Ssr3 was sufficient to catalyze this reaction. The ability of Ssr3 to facilitate promoter inversion is consistent with the previous observation that this molecule binds to invertible promoters upstream of polysaccharide biosynthesis operons in *B. fragilis* (23).

We next investigated whether Ssr3 was capable of mediating inversion of the other capsular polysaccharide promoters in *mpi* deletion mutants. *ssr3* was cloned into the expression vector pFD340 and was ectopically overexpressed in CPM1 (with all Mpi-regulated invertible promoters in the ''locked-off'' orientation). As shown by PCR analysis using promoter-specific primers, Ssr3 is capable of catalyzing recombination at all Mpi-regulated invertible promoters in the absence of Mpi (Fig. 3*D*). The recovery of phase variation is also recapitulated in the mutant strain CPM3 (SI Fig. 10). In addition to capsular polysaccharides, it has been shown that Mpi regulates promoter inversion at six additional genomic loci, the products of which all encode for unknown proteins. Mpi is capable of catalyzing promoter inversion at all of these sites; Ssr3 is able to flip five of the six promoters, again demonstrating its ability to substitute for the lack of Mpi (SI Fig. 11). None of these ORFs encode for predicted surface proteins, and, unlike capsular polysaccharides, none are affected by the deletion of PSC (data not shown). Taken together, in addition to Mpi, we have revealed that a second enzyme is capable of mediating promoter inversion in *B. fragilis* and appears to provide a ''fail-safe'' function to ensure production of capsular polysaccharides.

Mutants Expressing a Single Capsular Polysaccharide Are Defective for Intestinal Colonization of Animals. It has been proposed that control of multiple polysaccharide loci through promoter inversion allows for the generation of extensive surface diversity during bacterial colonization of the mammalian gastrointestinal tract (11, 18, 19). We reasoned that if the purpose of this system is to generate a multiplicity of surface structures (256 possible combinations of the 8 known polysaccharides), the homogeneous polysaccharide-expressing strains of *B. fragilis* we have created may display defects during colonization. Initially, germ-free mice (animals born and raised in the absence of microbial contamination) were readily colonized to similar levels by wild-type and mutant strains upon monoassociation with bacteria (Fig. 4*A*). Thus, all strains are competent for growth in animals. However, germ-free animals provide a model for the direct comparison of two bacterial strains for initial colonization (mimicking the events after a sterile birth) and thus accurately reflect each strain's capacity for competitive colonization without the confounding effects of a complete microbiota. We coassociated germ-free animals with wild-type and mutant *B. fragilis* strains and compared their ability to establish intestinal colonization. Competition experiments between wild-type and CPM1 strains demonstrated that wild-type *B. fragilis* quickly outcompeted the single polysaccharide (PSC)-producing CPM1 strain in terms of intestinal colonization (Fig. 4*B*). After 7 days, 95% of the bacteria recovered from the competition experiment in germ-free animals were wild-type organisms. Coculture of wild-type bacteria with CPM1 during serial passage in laboratory media consistently resulted in no growth defect at any time point (Fig. 4*C*); this finding illustrates that the colonization phenotype observed is due to factors found in the intestinal environment of animals. The same phenotype for cocolonization is also observed for CPM3, which expresses only PSB (SI Fig. 12), as well as for a previously characterized PSA-only-expressing strain Δ*mpi* mut44 (data not shown) (20). Thus, expression of PSA, PSB, or PSC alone is insufficient to allow bacterial competition against wild-type *B. fragilis*.

We next sought to determine whether the colonization defect for CPM1, a single-polysaccharide-producing mutant of *B. fragilis*, could be reversed by restoring expression of the variable surface polysaccharides with ectopic expression of either Mpi or Ssr3. In competitive co-colonization experiments, CPM1 strains complemented with Mpi or Ssr3 rapidly outcompeted the PSC-only-producing mutant CPM1 (Fig. 4 *D* and *E*). This result provides additional evidence that single-polysaccharide-expressing *B. fragilis* mutants exhibit severe defects in host colonization when challenged by the presence of a *B. fragilis* strain that can vary its surface polysaccharide expression. In summary, examinations within this article demonstrate that *B. fragilis* has a strong propensity to elaborate at least one surface polysaccharide for *in vitro* growth, and expression of a single polysaccharide appears insufficient to allow bacterial colonization of the mammalian host comparable with that by wild-type bacteria.

Discussion

The intestinal microbiota of humans contains 10 times more cells than the human body and 100 times the number of genes than the human genome (24). Although we are beginning to understand the identities of microorganisms that inhabit the gastrointestinal tract, the mechanisms they use to establish colonization are almost entirely unknown. The *Bacteroides* represent one of the

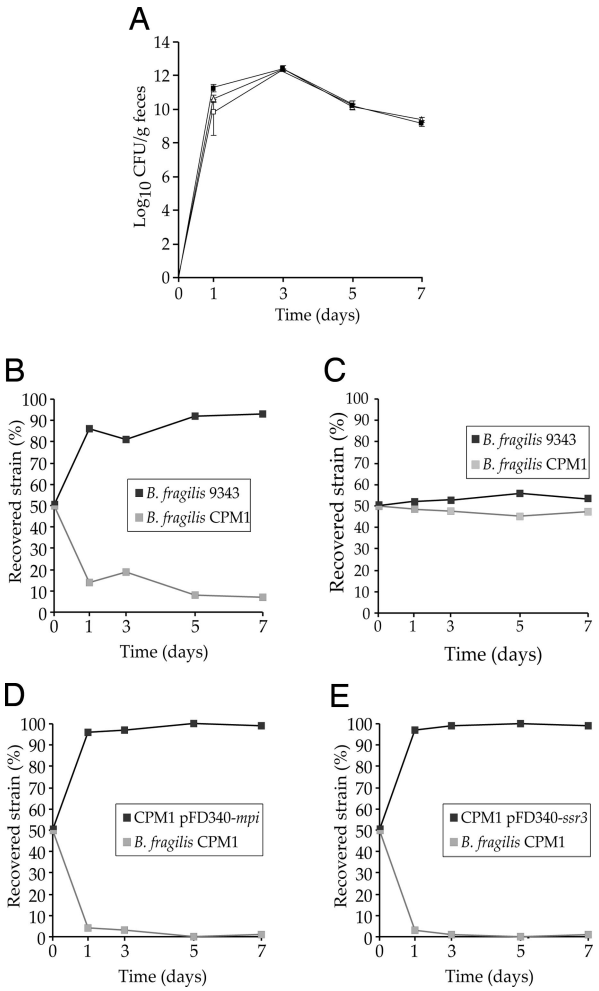

Fig. 4. Comparison of germ-free animal colonization by *B. fragilis* mutants with limited diversity of surface polysaccharides. (*A*) Counts of colony-forming units (CFU) recovered from feces of germ-free animals after oral inoculation of bacteria show the ability of both wild-type and mutant strains to colonize laboratory animals during monoassociation. Filled squares, wild-type *B. fragilis* 9343; open triangles, *B. fragilis* CPM1; open squares, *B. fragilis* CPM3. (*B*) Percentages of each strain recovered from feces of animals after dual colonization of wild-type *B. fragilis* and *B. fragilis* CPM1 strain, premixed at a 1:1 ratio before gavage. Upon plating on selective media, wild-type bacteria quickly outcompete CPM1. (*C*) Percentages of each strain recovered from laboratory media after daily serial passages during coculture demonstrate that CPM1 displays no growth defect in culture competition with wild-type *B. fragilis*. Overnight cultures were subcultured daily into fresh media at a 1:100 dilution. (*D*) Percentages of each strain recovered from feces of animals after dual colonization of CPM1 and CPM1 complemented with *mpi*. (*E*) Percentages of each strain recovered from feces of animals after dual colonization of CPM1 and CPM1 complemented with *ssr3 in trans*.

most numerically prominent constituents of the human microbiota, and all members of this genus encode multiple capsule loci and contain Mpi homologues (9). Are capsular polysaccharides key molecular components that mediate various interactions between symbiotic *Bacteroides* and their mammalian hosts? The importance of these molecules is suggested by our finding that *B. fragilis* requires expression of at least one capsular polysaccharide for *in vitro* growth. When we selected for a mutant that was genetically unable to express all seven Mpi-regulated polysaccharides, we observed that every viable cell phenotypically expressed the default polysaccharide PSC. When we deleted PSC in this strain, we were able to recover only a poorly growing strain that overcame its growth defect and reverted to express another polysaccharide. To our knowledge, this is the first demonstration that the lack of capsule expression adversely affects bacterial growth. We demonstrate that *B. fragilis* contains an alternative recombinase (Ssr3) that is capable of inverting polysaccharide promoters. We were, however, unable to generate mutants of Ssr3 (because it is present on a multicopy plasmid) to directly assess whether it is responsible for the CPM3 phenotype. Also, genomic sequencing of the PSB promoter or the *ssr3* gene and upstream regulatory elements yielded no mutations between any of the CPM mutants and wild-type bacteria (data not shown). Importantly, Ssr3 can compensate for the absence of Mpi in both catalyzing promoter inversion and complementing defects in animal colonization. An inhibition of growth upon capsule deletion, a redundancy of mechanisms to ensure capsule biosynthesis, and defects in colonization upon limiting the capsular polysaccharide repertoire attest to the evolutionary importance for regulation of surface architecture by *B. fragilis* during colonization.

The bacterial cell envelope can be viewed as the structural interface between microorganisms and their countless environments, mediating essential functions required for microbial attachment and colonization (25). The many examples of adherence mechanisms used by bacterial pathogens during infection include molecules such as pili, fimbriae, and surface proteins (26). However, mechanisms involved in the establishment of colonization by multitudinous and important symbiotic organisms remain largely uncharacterized. Pioneering work by Comstock and colleagues (11) first demonstrated that *B. fragilis* contains at least eight distinct capsular polysaccharides; the recent findings from genome sequences that three other *Bacteroides* species contain multiple capsule structures indicate that this is a unique and distinguishing feature of these important human commensals (9). We demonstrate that *B. fragilis*, a numerically prominent symbiotic organism of the human microbiota, is attenuated for intestinal colonization when it can no longer express a diverse repertoire of surface structures. Specifically, our data show that *B. fragilis* strains able to synthesize only a single polysaccharide cannot compete with wild-type bacteria for survival in the gastrointestinal tract of germ-free animals. Restoration of surface diversity in these mutants through complementation with either Mpi or its alternative homolog, Ssr3, rescues defects in both capsular polysaccharide production and intestinal colonization. All sequenced *Bacteroides* contain orthologs of Mpi, presumably to create extensive surface diversity in these human commensals. Thus, our results with the well studied model organism *B. fragilis* may extend to other numerically significant commensal bacteria that inhabit the gastrointestinal tract of humans. Because the gut presents a dynamic and changing environment to colonizing organisms (i.e., nutrient changes, immune responses, bacteriophage attacks), perhaps sustained association of commensal bacteria with mammals requires intricate and dynamic processes unlike expression of a single toxin or adhesin used by many pathogens to establish acute infections. The regulation of surface architecture during intestinal colonization appears to be critically involved for establishing the commensal association of *B. fragilis* with its mammalian host. Recent efforts to define the "normal" sequence of colonization by bacterial species after birth have resulted in a deeper understanding of the evolutionary partnership between humans and their microbiota (27). Our findings provide the framework to compel future studies to reveal the mechanisms by which the bacterial envelope contributes to the vital process of host–bacterial mutualism.

Materials and Methods

Bacterial Strains, Plasmids, and Media. Bacterial strains and plasmids are described in SI Table 2.

Generation of Strains CPM1 and CPM2. To create the *B. fragilis* Δ*mpi* mutant CPM1, the plasmid pLEC80 (SI Table 2) was mobilized from DH5α cells by helper plasmid RK231 and conjugally transferred into a *B. fragilis* 9343ΔPSA mutant strain to generate a deletion of *mpi* and *tsr19*, a tyrosine site-specific recombinase that is not involved in Mpi-mediated capsular polysaccharide promoter inversion (28). Colonies selected for erythromycin resistance (Em^r), indicating integration of the suicide vector into the host chromosome, were passaged for 5 days and then plated on nonselective medium (BHI-S). The resulting colonies were replica-plated to BHI-S containing Em, and Em^s (sensitive) colonies were screened by PCR to distinguish wild-type revertants from strains with the desired mutation. To create mutant CPM2, a portion of the PSC locus was deleted from CPM1 by using plasmid pMJC2Δ.1 (SI Table 2). Complementation studies with *mpi* and *ssr3* were performed by cloning each gene into the *B. fragilis–Escherichia coli* shuttle plasmid pFD340 (SI Table 2).

Flow Cytometry. For surface staining, cell preparations were washed twice in ice-cold FACS buffer (PBS with 2% FBS) and resuspended in 100 μl of the same buffer. A total of 1×10^7 cells were incubated with rabbit polyclonal antisera to PSB and PSC at a 1:100 dilution for 30 min at 4°C. Cells were then washed, incubated with goat anti-rabbit fluorochrome-conjugated secondary antibodies, and analyzed by flow cytometry with an FC500 cytometer.

Germ-Free Animal Colonization Experiments. Male Swiss–Webster germ-free mice were purchased from Taconic Farms. Animals were screened for bacterial, viral, and fungal contamination by Gram staining, degenerate PCR, RapID ANA II System analysis, and plating of fecal samples under aerobic and anaerobic conditions. Animals were housed in gnotobiotic chambers or microisolator cages, and all food and bedding were sterilized by autoclave. Erythromycin (12.5 μg/ml) and gentamicin (125 μg/ml) were added to sterile drinking water. All *Bacteroides* strains (naturally resistant to gentamicin) contained the plasmid pFD340 conferring erythromycin resistance. Eight- to 13-week-old mice were orally inoculated with $\approx 1 \times 10^8$ colony-forming units of bacteria harvested from a log-phase culture and resuspended in PBS with 1.5% $NaHCO_3$. At each time point after bacterial introduction to animals, fresh fecal samples were collected, serially diluted, and plated for colony-forming units. To discern between strains, we introduced a plasmid pFD340-*cat* (SI Table 2), which confers chloramphenicol resistance. One hundred individual colonies were patched onto BHI-S agar with erythromycin and chloramphenicol to determine the ratio between strains during colonization. All animals were cared for under established protocols using Institutional Animal Care and Use Committee guidelines of Harvard Medical School and the California Institute of Technology.

Note added in proof. A recent report (29) shows that eight intestinal *Bacteroidales* contain multiple capsular polysaccharides, a feature not found in four oral *Bacteroidales* species, suggesting that regulation of surface architecture has evolved as a niche specific feature for intestinal organisms.

ACKNOWLEDGMENTS. We are very grateful to Dr. Laurie Comstock (Brigham and Women's Hospital, Harvard Medical School, Boston) for helpful discussions and reagents throughout the entire course of this work, and we thank the members of the S.K.M. laboratory for comments on the manuscript. D.L.K. acknowledges support from the National Institutes of Health/National Institute of Allergy and Infectious Diseases. S.K.M. acknowledges support from the Searle Scholars Program and the Crohn's and Colitis Foundation of America.

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