Applied and Environmental Microbiology

Peptidoglycan from Bacillus cereus Mediates Commensalism with Rhizosphere Bacteria from the Cytophaga-Flavobacterium Group

Snow Brook Peterson, Anne K. Dunn, Amy K. Klimowicz, et al.

2006. Peptidoglycan from Bacillus cereus Mediates Commensalism with Rhizosphere Bacteria from the Cytophaga-Flavobacterium Group. Appl. Environ. Microbiol. 72(8):5421-5427. doi:10.1128/AEM.02928-05.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2006, p. 5421–5427 Vol. 72, No. 8
0099-2240/06/$08.00+0 doi:10.1128/AEM.02928-05

Peptidoglycan from *Bacillus cereus* Mediates Commensalism with Rhizosphere Bacteria from the *Cytophaga-Flavobacterium* Group

Snow Brook Peterson,[1,2] Anne K. Dunn,[1,3]† Amy K. Klimowicz,[1] and Jo Handelsman[1]*

Department of Plant Pathology,[1] Microbiology Doctoral Training Program,[2] and Department of Bacteriology,[3] University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 12 December 2005/Accepted 24 May 2006

Previous research in our laboratory revealed that the introduction of *Bacillus cereus* UW85 can increase the populations of bacteria from the *Cytophaga-Flavobacterium* (CF) group of the *Bacteroidetes* phylum in the soybean rhizosphere, suggesting that these rhizosphere microorganisms have a beneficial relationship (G. S. Gilbert, J. L. Parke, M. K. Clayton, and J. Handelsman, Ecology 74:840–854, 1993). In the present study, we determined the frequency at which CF bacteria coisolated with *B. cereus* strains from the soybean rhizosphere and the mechanism by which *B. cereus* stimulates the growth of CF rhizosphere strains in root exudate media. In three consecutive years of sampling, CF strains predominated among coisolates obtained with *B. cereus* isolates from field-grown soybean roots. In root exudate media, the presence of *B. cereus* was required for CF coisolate strains to reach high population density. However, rhizosphere isolates from the phylum *Proteobacteria* grew equally well in the presence and absence of *B. cereus*, and the presence of CF coisolates did not affect the growth of *B. cereus*. Peptidoglycan isolated from *B. cereus* cultures stimulated growth of the CF rhizosphere bacterium *Flavobacterium johnsoniae*, although culture supernatant from *B. cereus* grown in root exudate media did not. These results suggest *B. cereus* and CF rhizosphere bacteria have a commensal relationship in which peptidoglycan produced by *B. cereus* stimulates the growth of CF bacteria.

Studies of diverse habitats such as the human oral cavity, anoxic marine sediments, and soil have highlighted the importance of beneficial interactions between microorganisms. The mechanisms by which microorganisms interact are proving to be as diverse as the environments in which these interactions occur. To advance our understanding of beneficial microbial interactions, we chose to investigate interactions between microorganisms in the rhizosphere, which is the area surrounding and influenced by plant roots. A diverse microbial community, differing from the community present in bulk soil, inhabits the rhizosphere (17) and is influenced by carbon-rich root exudates, which can account for as much as 30% of a plant's fixed carbon (7, 23). A number of antagonistic interactions between microorganisms in the rhizosphere have been detected or postulated from studies in vitro. For example, many bacterial and fungal strains isolated from the rhizosphere protect their host plants against particular pathogens through direct or indirect antagonism (32). A subset of these organisms exhibiting disease-suppressive activity also produce antibiotics, which may contribute to their ability to outcompete other rhizosphere residents (27). Relatively little is known about beneficial microbial interactions in the rhizosphere, but the presence of multiple-species biofilms on plant roots suggests that metabolic interactions between species occurs (28).

Bacillus cereus interacts with numerous other microorganisms in the rhizosphere. *B. cereus* strains suppress plant diseases caused by oomycete (protist) pathogens and enhance crop productivity (3, 11, 26, 30, 31). *B. cereus* strain UW85 also enhances nodulation of soybean plants by *Bradyrhizobium japonicum* (10), and culture supernatant from the rhizosphere bacterium *Pseudomonas aureofaciens* 30-84 causes changes in gene expression in *B. cereus* UW85 in vitro (8). Additionally, the introduction of *B. cereus* UW85 into the soybean rhizosphere can affect the community structure (9).

Two observations suggest that bacteria from the *Cytophaga-Flavobacterium* group (CF) benefit from an association with *B. cereus* in the rhizosphere. A prominent aspect of the change in microbial community structure caused by the introduction of *B. cereus* UW85 in the rhizosphere was an increase in the number of CF bacteria present (9). We have also observed that *B. cereus* rhizosphere isolates that initially appear to be in pure culture will sometimes (1 to 5% of isolates) exhibit outgrowth of a CF bacterium "coisolate" after 2 to 4 weeks of incubation at 4°C. CF bacteria have been detected in the rhizosphere of many plants and are common in soil, and one study found that CF bacteria were seasonally dominant among organisms carrying enzymes for carbon turnover in the barley rhizosphere (15). However, little else is known about their role in rhizosphere ecology or the extent to which they interact with other rhizosphere microorganisms. In the present study, we measured the frequency of coisolation of CF bacteria with *B. cereus* from the soybean rhizosphere, characterized the interactions between these bacteria in culture media containing alfalfa or soybean root exudates, demonstrated that peptidoglycan fragments produced by *B. cereus* stimulate the growth of CF bacteria, and detected peptidoglycan-hydrolyzing activity secreted by CF bacteria.

MATERIALS AND METHODS

Culture conditions. Except as noted, all bacterial strains were propagated on 1/10th-strength tryptic soy agar (TSA) and grown in liquid culture in half-strength tryptic soy broth (TSB) at 28°C with vigorous shaking. Morpholinepro-

* Corresponding author. Mailing address: Department of Plant Pathology, University of Wisconsin—Madison, 1630 Linden Dr., Madison, WI 53706. Phone: (608) 263-8783. Fax: (608) 265-5289. E-mail: joh@plantpath.wisc.edu.

† Present address: Department of Microbiology, University of Georgia, Athens, Georgia.

panesulfonic acid AA3 (MES AA3) medium with DL-malate or glucose as a carbon source was prepared as described previously, except the concentration of $MnSO_4 \cdot H_2O$ was 1.5 μM (22). Growth of *Flavobacterium johnsoniae* on peptidoglycan as a sole carbon source was tested in G-Tris medium (2) made without glucose.

Collection of coisolates of *Bacillus cereus* and random isolates. Soybean (cultivar Sturdy) plants were grown at the Arlington Agricultural Research station in 2001, 2002, and 2003. Plants were harvested on 31 July to 1 August, 21 to 23 August, and 10 September in 2001; 27 to 28 August and 14 September in 2002, and 22 to 23 July and 29 to 30 August in 2003. After harvest, plant roots were rinsed briefly with tap water to remove loosely adherent soil, and then each root was cut into three 3-cm sections, measured from the crown. Root sections were placed in 10 ml sterile, deionized water and sonicated in a bath sonicator (Model 2210; Branson, Danbury, CT) for 30 s, and then the sonicated sample was diluted and cultured on half-strength TSA containing cycloheximide (100 μg/ml, to reduce fungal growth) and polymyxin B (2 μg/ml, to enrich for *B. cereus*), except for the 10 September 2001 samples, which were cultured on 1/10th-strength TSA containing cycloheximide (100 μg/ml); half-strength TSA containing cycloheximide (100 μg/ml); and half-strength TSA containing cycloheximide (100 μg/ml), polymyxin B (2 μg/ml), and ampicillin (10 μg/ml, also to enrich for *B. cereus*). All plates were incubated for 14 to 18 h at 25°C.

At each sampling, ~500 *B. cereus* colonies were selected on the basis of broad, flat, opaque colony morphology and were purified by repeated streaking for single colonies on 1/10th-strength TSA (containing no antibiotics) until only one colony morphology was apparent after 14 to 18 h of incubation at 28°C. Single colonies from purified isolates were patched onto 1/10th-strength TSA and incubated for 14 to 16 h at 28°C. Plates were then sealed with parafilm and stored at 4°C for 60 days. Isolates exhibiting outgrowth of a different colony morphology after incubation at 28°C or up to 2 days of incubation at 4°C were noted and not included in subsequent analysis. Outgrowth of an additional colony morphology from patches of *B. cereus* after 3 or more days was considered indicative of the presence of a "coisolate" bacterium. Coisolates were separated from *B. cereus* isolates by repeated streaking for single colonies on 1/10th-strength TSA.

From the plants collected on 27 to 28 August 2002, samples of sonicate were also cultured on solid 1/10th-strength TSA containing cycloheximide (100 μg/ml) for 24 h at 28°C, from which 47 isolates were randomly collected. Randomness was established by assigning each plate a number and placing a grid of 50 squares on each plate and then randomly choosing 47 pairs of numbers corresponding to a plate and a square within the plate. The colony closest to the center of the square represented by the coordinates was then isolated and purified as described above for coisolates. Sensitivity of random isolates to polymyxin was determined by growing each strain for 24 to 48 h in half-strength TSB at 28°C, diluting cultures 1:100 in half-strength TSB, spotting 10 μl of each culture onto 1/10th-strength TSA containing 10, 2, and 0 μg/ml polymyxin, and monitoring growth after 24 and 48 h at 28°C.

Taxonomic identification of coisolates by 16S rRNA gene sequencing. Genomic DNA was extracted from 0.5 ml of cultures of each coisolate grown for 14 to 16 h and lysed by vortexing for 90 s in the presence of 100 μl of 0.1-mm-diameter silicon beads, followed by extraction with a Miniprep Express Matrix (ISC, Kaysville, UT), or genomic DNA was extracted using the Easy DNA kit (Invitrogen, Carlsbad, CA). 16S rRNA genes were amplified by PCR using bacterial 16S rRNA gene primers 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′TACGGYTACCTTGTTACGACTT3′). Partial (at least 500 bp) or full sequence of PCR products was obtained using primers 27F, 1492R, and 787R (5′CTACCRGGGTATCTAAT3′).

Preparation of root exudate media. Soybeans (cultivar Sturdy) were surface sterilized by soaking in 6% sodium hypochlorite for 10 min, rinsed 10 times with sterile deionized water, soaked for 2 h in sterile deionized water, transferred to water agar plates, and incubated for 3 days in the dark at 24°C to promote germination. Seedlings were transferred to Magenta boxes (three per box) in which holes had been drilled in the bottom to allow passage of roots, and roots were suspended in 250 ml sterile, modified Hoagland's plant growth solution (12). Spent growth solution was collected from around the plant roots after 10 to 15 days in a growth chamber (12-h photoperiod, 24°C); filter sterilized; amended with 1 mM of the amino acids glutamine, arginine, isoleucine, methionine, phenylalanine and tryptophan; and stored at −20°C until used as a bacterial growth medium.

Alfalfa root exudate medium was prepared from alfalfa seeds surface sterilized by soaking for 7 min in concentrated sulfuric acid, rinsed 10 times with sterile deionized water, placed in a 250-ml flask with sufficient sterile deionized water to cover the seeds, and incubated for 2 days at 28°C with vigorous shaking to promote germination. Seedlings were then either transferred to Magenta boxes (three per box) and suspended in plant growth solution as described above for soybean root exudate, or they were individually transferred to 20-mm-diameter test tubes containing 5 ml sterile plant growth solution. Plants were grown in a growth chamber (12-h photoperiod, 24°C), and spent growth solution was collected, filtered, and amended after 10 to 20 days as described above for soybean root exudate media.

Growth promotion experiments. The ability of one bacterial strain to promote the growth of another was measured by comparing growth of the two strains in coculture in MES AA3 or soybean or alfalfa root exudate medium with growth in pure culture in the same medium. In a typical growth promotion experiment, both strains were first grown individually for 14 to 16 h in half-strength TSB, 1-ml samples of each culture were removed, and the cells in each sample were washed twice with sterile deionized water. Cells were then resuspended in sterile deionized water, and three cultures were inoculated per experiment with ~10^7 cells of the strain to be tested for its growth promotion ability, as well as 10^4 to 10^6 cells from the strain to be measured for its ability to grow, and three parallel cultures were inoculated with the second strain in the absence of the first. The ability of filter-sterilized supernatant from 5-day-old *B. cereus* or *B. cereus*/*Flavobacterium johnsoniae* CI04 cocultures grown in alfalfa root exudate medium as described above to promote growth of *F. johnsoniae* CI04 was measured by inoculating parallel cultures of root exudate media and either 100% culture supernatant or 50% supernatant/50% root exudate media with *F. johnsoniae* cells grown and washed as described above. After 5 days at 28°C with vigorous shaking, all cultures were dilution plated on 1/10th-strength TSA containing antibiotics to select for the strain for which growth was being measured (100 μg/ml polymyxin or 10 μg/ml chloramphenicol, chosen on the basis of the strains' intrinsic resistance levels).

Promotion of *F. johnsoniae* growth by *B. cereus* through a membrane. Five milliliters of root exudate medium from 15-day-old soybean plants was placed in each of three 60-mm by 15-mm sterile petri plates. Approximately 10^7 *B. cereus* cells that were grown and washed as described above were added to two of the three plates. Three 10-mm-diameter tissue culture inserts with 0.2-μm-pore-size anopore membrane bottoms (Nunc, Rochester, NY) were then placed in each plate and filled with 0.5 ml soybean root exudate medium. Approximately 10^5 *F. johnsoniae* cells grown and washed as described above were added to each of the inserts. An additional ~10^7 *B. cereus* cells were also added to the interior of one of the inserts along with the *F. johnsoniae* cells. All plates were then incubated at 28°C for 5 days. Inserts were then removed, placed individually in test tubes containing 5 ml sterile deionized water, sonicated for 30 s in a bath sonicator, and diluted and cultured on solid medium as described above for growth promotion experiments.

Purification of peptidoglycan from *B. cereus*. Peptidoglycan was purified from cultures of *B. cereus* grown for 14 to 16 h in 500 ml half-strength TSB according to the method for gram-positive bacteria described by Rosenthal and Dziarski (28a), with the following modifications. DNase and RNase digestions were performed simultaneously using RNase at 2 mg/ml (Sigma, St. Louis, MO) and 10 U RQ1 DNase (Promega, Madison, WI) in a 10-ml total volume of RQ1 DNase buffer. Proteins were removed by 18 h of digestion with pronase at 2 mg/ml (Sigma). Activity was tested both before and after extraction with 5% trichloroacetic acid (TCA), which removes teichoic acids, and no differences were noted between the effects of the different preparations. For peptidoglycan hydrolysis assays, crude cell wall preparations of 1-liter *B. cereus* cultures (grown in half-strength TSB) were extracted with 10 M LiCl according to a method described previously (14) and were concentrated to a final volume of 10 ml in sterile deionized water.

Peptidoglycan hydrolysis assays. To measure peptidoglycan hydrolysis on plates, 1 ml of *B. cereus* crude cell extract was mixed with 25 ml molten half-strength TSA and solidified. Isolates tested were grown first for 16 to 18 h in half-strength TSB, and then diluted 1:100 into TSB containing molten, 1.5% agar, which was used to fill wells cut into the cell-wall-containing plate. The test plate was then incubated for 48 h at 28°C. Cultures were prepared for measuring peptidoglycan hydrolytic activity of culture supernatants by growing isolates for 16 to 18 h in half-strength TSB, diluting the cultures 1:100 into alfalfa root exudate medium containing 2 mg/ml purified *B. cereus* peptidoglycan, and incubating the resulting cultures for 48 h at 28°C. We then filter sterilized culture supernatants and mixed 200-μl samples of filtrate with 2 μl crude *B. cereus* cell wall preparation. The mixtures were incubated for 48 h at 28°C, and the optical density at 600 nm was monitored over time using a Wallac-VICTOR2 plate reader (Perkin Elmer, Wellesley, MA).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in the course of this study can be found in GenBank under the accession numbers DQ530064 to DQ530169.

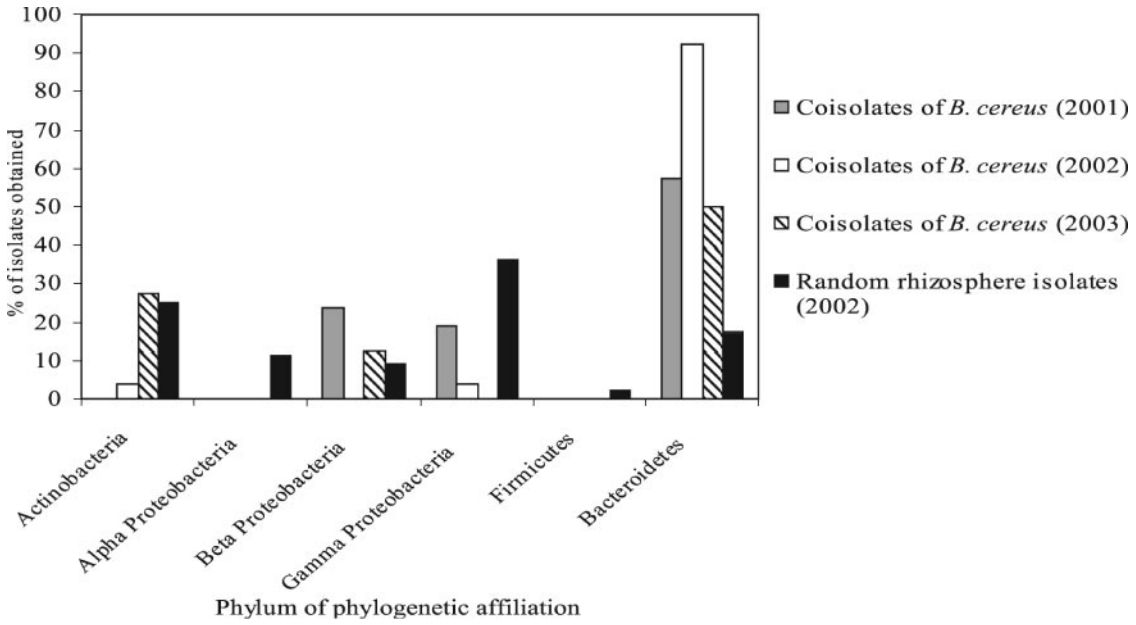

FIG. 1. *Bacteroidetes* bacteria are more prevalent among *B. cereus* coisolates than among randomly sampled rhizosphere isolates. Approximately 1,000 *B. cereus* soybean root isolates were tested for coisolates in each year sampled. Phylogenetic classification of isolates was determined on the basis of 16S rRNA gene sequences, which can be found in GenBank under the accession numbers DQ530064 to DQ530169.

RESULTS

Characterization of coisolates of *B. cereus*. To determine whether bacteria from the *Cytophaga-Flavobacterium* group are consistently coisolated from *B. cereus* strains, we collected and purified ~1,000 *B. cereus* isolates from field-grown soybeans in 2001, 2002, and 2003, from which we obtained 21, 29, and 16 coisolates, respectively. Most of the coisolates collected were affiliated with the CF group of the *Bacteroidetes* phylum (12/21 in 2001, 24/29 in 2002, and 10/16 in 2003) (Fig. 1). To determine whether the predominance of CF bacteria among coisolates simply reflects their relative abundance among readily culturable rhizosphere bacteria, we compared the diversity of coisolates from 2002 with that of 47 isolates randomly selected from colonies obtained from the same soybean roots. CF bacteria represented a much higher proportion of the coisolates (83%; Fig. 1) than of the random isolates (17%; Fig. 1). Because polymyxin (2 μg/ml) was included in media to select for *B. cereus* in most of the rhizosphere platings but not in the media used for collecting random isolates, this could have influenced the proportions of each population represented by CF bacteria. However, if random isolates sensitive to 2 μg/ml polymyxin in half-strength TSA were excluded from the analysis, CF bacteria still represented a higher proportion of 2002 coisolates (83%; Fig. 1) than random isolates (21%; data not shown).

***B. cereus* UW85 promotes growth of *F. johnsoniae* and other CF strains.** To begin to study the interactions between *B. cereus* and CF isolates in the rhizosphere, we compared the growth of four CF coisolates and four additional, phylogenetically representative coisolates in alfalfa root exudate medium in the presence and absence of *B. cereus*. After 5 days, the CF coisolates had achieved populations 4- to 30-fold higher in the presence than in the absence of *B. cereus*, and coisolates from other divisions grew equally well in the presence and absence of *B. cereus*, with the exception of *Stenotrophomonas maltophilia*, which reached a 4-fold higher population in the presence than the absence of *B. cereus* (Fig. 2). *Flavobacterium johnsoniae* was selected for further experiments, because it is the best-characterized CF species among the coisolates and was obtained in all three years investigated. *B. cereus* promoted the growth of *F. johnsoniae* in both soybean and alfalfa root exudate media, but in a defined medium containing no plant exudates, *F. johnsoniae* growth was inhibited by the presence of *B. cereus* (Table 1). *B. cereus* growth was not affected by the presence of *F. johnsoniae* in any of the three media types (data not shown).

We also tested whether sporulation affected the ability of *B. cereus* to promote *F. johnsoniae* growth. Cultures of *B. cereus* that were sporulated at the time of addition to cocultures promoted *F. johnsoniae* growth as well as vegetative cultures (data not shown). However, it is possible that *B. cereus* spores germinated in the root exudate medium and the resulting vegetative cells promoted growth of *F. johnsoniae*. Additionally, an uncharacterized mutant derived from *B. cereus* UW85 that is unable to sporulate promoted *F. johnsoniae* growth as well as the wild type, demonstrating that spore release is not required for growth promotion to occur (data not shown).

These results demonstrate that *B. cereus* enhances growth of CF isolates in the presence of compounds found in the rhizosphere. To determine whether this effect is specific to *B. cereus*, we tested other bacterial strains for promotion of *F. johnsoniae* growth. *Erwinia herbicola* LS005 promoted *F. johnsoniae* growth, *B. subtilis* 168 had no effect, and *Pseudomonas* sp. strain CI12 reduced the *F. johnsoniae* growth (Table 1).

***B. cereus* promotes *F. johnsoniae* growth through a membrane.** To test the hypothesis that an extracellular metabolite in *B. cereus* cultures promotes *F. johnsoniae* growth, we tested the growth-stimulatory potential of cell-free filtrate from root

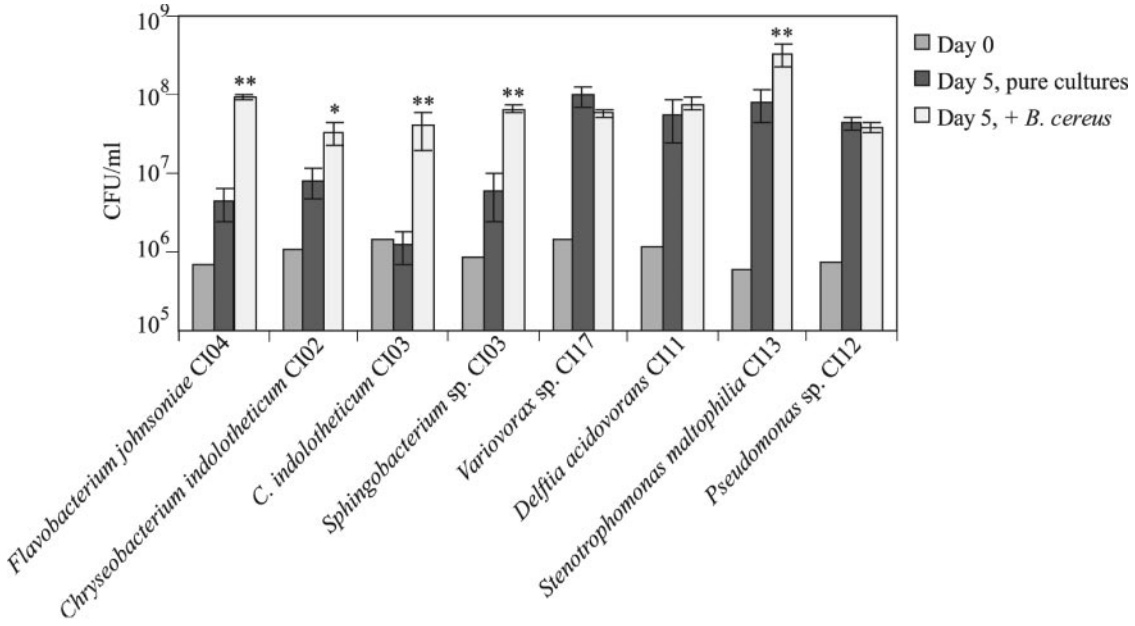

FIG. 2. *B. cereus* stimulates the growth of coisolates from the CF group of the *Bacteroidetes* phylum in alfalfa root exudate medium. Means and standard errors are shown from one experiment (representative of three independent experiments) in which the growth of *Bacteroidetes* and *Proteobacteria* coisolates was measured in the presence and absence of *B. cereus*. The log-transformed growth of the coisolate was significantly higher in the presence of *B. cereus* at $P < 0.05$ (indicated by an asterisk) or at $P < 0.001$ (indicated by a double asterisk) in analysis of variance, in which experiments were treated as blocks.

exudate cultures of *B. cereus*. Neither the cell-free filtrate of *B. cereus* cultures nor that of *B. cereus*/*F. johnsoniae* cocultures stimulated *F. johnsoniae* growth (Table 1). To determine whether direct contact between *B. cereus* and *F. johnsoniae* cells was required for *F. johnsoniae* growth promotion, we measured *F. johnsoniae* growth when the strains were separated by a 0.2-μm-pore anopore membrane. *B. cereus* promoted growth of *F. johnsoniae* even when the two species were separated by the membrane (Table 1).

Peptidoglycan purified from *B. cereus* stimulates *F. johnsoniae* growth. Since members of the CF group grow on diverse complex carbohydrates (13), we reasoned that peptidoglycan from

TABLE 1. Growth of *F. johnsoniae* in various media amended with other bacterial strains or culture supernatant

Expt no.	Medium	Amendment	Amt of *F. johnsoniae* (CFU/ml) at 3 or 5 days[a]
1	MES AA3	None	8.43×10^9 (8.50×10^6)
	MES AA3	*B. cereus* UW85	2.45×10^9 (1.10×10^6)
2	Soybean root exudate	None	2.3×10^6 (0.25×10^6)
	Soybean root exudate	*B. cereus* UW85	5.3×10^7 (0.85×10^6)*
3	Alfalfa root exudate	None	1.1×10^6 (0.43×10^6)
	Alfalfa root exudate	*B. cereus* UW85	1.0×10^7 (0.10×10^7)*
	Alfalfa root exudate	*B. subtilis* 168	1.1×10^6 (0.65×10^6)
	Alfalfa root exudate	*E. herbicola* LS005	2.8×10^7 (1.7×10^7)*
	Alfalfa root exudate	*Pseudomonas* sp. strain CI12	8.5×10^4 (1.7×10^4)*
4	Alfalfa root exudate	None	4.9×10^6 (1.7×10^6)
	Alfalfa root exudate	*B. cereus* UW85	1.9×10^8 (0.49×10^7)*
	Alfalfa root exudate	*B. cereus* UW85 culture supernatant	6.7×10^6 (4.4×10^6)
	Alfalfa root exudate	*B. cereus* UW85/*F. johnsoniae* coculture supernatant	6.1×10^6 (4.4×10^6)
5	Alfalfa root exudate	None	2.2×10^6 (0.72×10^6)
	Alfalfa root exudate	*B. cereus* UW85	5.8×10^7 (1.0×10^7)*
	Alfalfa root exudate	*B. cereus* UW85, membrane[b]	6.2×10^7 (0.58×10^7)*

[a] Growth was measured after 3 days in experiments 1 and 2 and after 5 days in subsequent experiments. Means and one standard error (in parentheses; $n = 3$) are shown from single experiments representative of three independent experiments conducted for each combination of conditions. Growth of *F. johnsoniae* was significantly affected by the amendment across all three experiments conducted at $P < 0.001$ (indicated by an asterisk) in analysis of variance, in which experiments were treated as blocks, and Tukey pairwise comparisons were tested.

[b] *F. johnsoniae* was grown separated from *B. cereus* by a 0.2-μm-pore membrane.

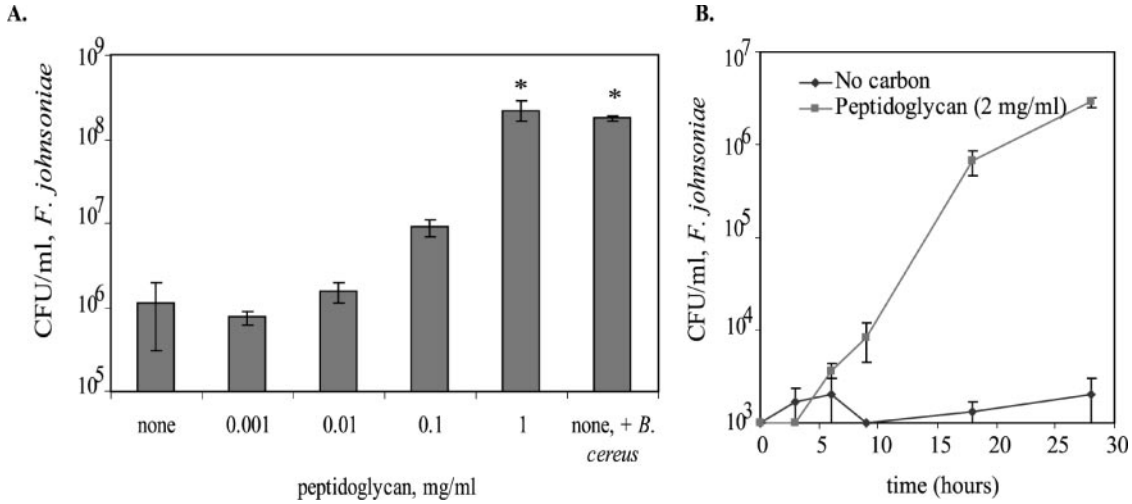

FIG. 3. Peptidoglycan purified from *B. cereus* stimulates *F. johnsoniae* growth in root exudate medium, and *F. johnsoniae* grows in minimal medium with purified *B. cereus* peptidoglycan as the sole carbon source. Means ($n = 3$) and standard errors are shown from one experiment that is representative of three independent experiments conducted for each treatment. A) *F. johnsoniae* growth after 5 days in alfalfa root exudate medium with increasing amounts of purified peptidoglycan (also containing teichoic acids). *F. johnsoniae* growth was significantly enhanced across all experiments at $P < 0.001$ (indicated by an asterisk) in analysis of variance, in which experiments were treated as blocks. B) Growth over time of *F. johnsoniae* diluted 1:1,000 into minimal G-Tris medium (3) containing purified *B. cereus* peptidoglycan (2 mg/ml) or no carbon.

B. cereus might be responsible for promotion of *F. johnsoniae* growth. Peptidoglycan is present on the surface of *B. cereus* cells and is released by turnover during growth, so it could be accessed by diffusion of *F. johnsoniae* enzymes across the 0.2-μm-pore membrane or could be at a low concentration in *B. cereus* supernatant due to rapid turnover and recycling. Peptidoglycan purified from *B. cereus* cultures stimulated *F. johnsoniae* growth in alfalfa root exudate medium approximately 100-fold when added at a concentration of 1 mg/ml, which is comparable to stimulation by live *B. cereus* cells (Fig. 3A). *F. johnsoniae* growth stimulation was directly proportional to the concentration of peptidoglycan added to the medium; no stimulation was detected at concentrations of 0.01 mg/ml and below (Fig. 3A). Additionally, *F. johnsoniae* grew when purified *B. cereus* peptidoglycan (1 mg/ml) served as the sole carbon source in a defined medium (Fig. 3B).

CF isolates produce extracellular peptidoglycan-hydrolyzing activity. The ability of *B. cereus* to stimulate *F. johnsoniae* growth across a 0.2-μm-pore membrane despite the lack of stimulation by *B. cereus* culture supernatant suggests that *F. johnsoniae* secretes a diffusible enzyme that passes through the membrane and liberates diffusible peptidoglycan fragments from *B. cereus*. We found that on TSA plates containing a crude *B. cereus* cell wall extract, all four CF strains tested generated a visible zone of clearing of the opaque cell wall preparation (Fig. 4A), compared to only one of four non-CF rhizosphere isolates tested, *Stenotrophomonas maltophilia* CI13. Interestingly, the growth of *S. maltophilia* CI13 was also

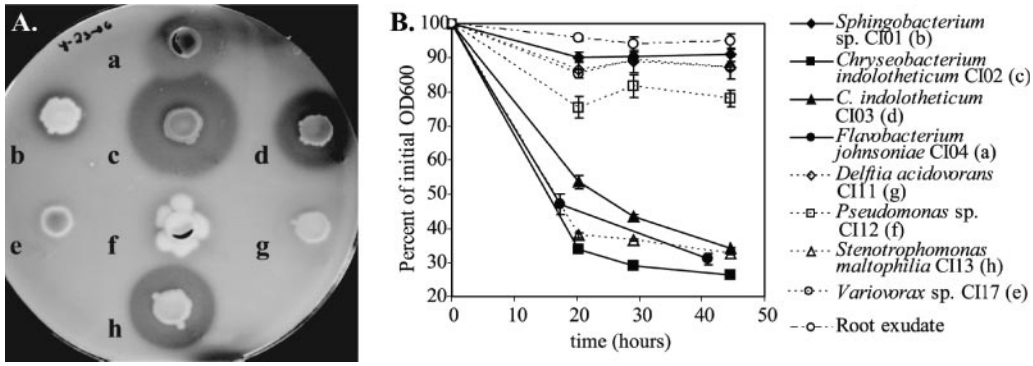

FIG. 4. CF bacteria secrete a *B. cereus* cell-wall-degrading activity. A) Zones of clearing on media containing a crude *B. cereus* cell wall preparation, produced by coisolate bacteria (a) *F. johnsoniae* CI04, (b) *Sphingobacterium* sp. strain CI01, (c) *Chryseobacterium indolotheticum* CI02, (d) *C. indolotheticum* CI03, (e) *Variovorax* sp. strain CI17, (f) *Pseudomonas* sp. strain CI12, (g) *Delftia acidovorans* CI11, and (h) *Stenotrophomonas maltophilia* CI13. B) *B. cereus* cell wall degradation by coisolate bacteria culture supernatants. Optical density at 600 nm (OD600) of supernatants mixed with crude *B. cereus* cell wall extract was measured over time and is presented as a percentage of the initial optical density. Solid lines indicate results from CF bacteria, while dashed lines represent non-CF strains and the root exudate-alone control. Means and standard errors of degradation by three samples of each culture supernatant are shown, and results are representative of three independent experiments.

significantly stimulated by *B. cereus* in root exudate medium cocultures (Fig. 2).

We confirmed that the activity responsible for degrading *B. cereus* cell walls was extracellular by measuring the optical density over time of cell-free supernatants mixed with crude *B. cereus* cell wall extract. The extent of *B. cereus* cell wall degradation by culture supernatants generally correlated with the size of clearing zones produced by the isolates on media containing *B. cereus* cell walls (Fig. 4). Culture supernatants from three of four CF rhizosphere isolates tested consistently degraded *B. cereus* cell walls, resulting in 70 to 80% reduction in optical density of the mixture over 48 h (Fig. 4B). In some experiments, culture supernatant from CF isolate *Sphingobacterium* sp. strain CI01 caused a decrease of up to 60% in optical density of *B. cereus* cell walls (data not shown). The non-CF rhizosphere isolate that produced zones of clearing of *B. cereus* cell walls in TSA plates, *S. maltophilia* CI13, also produced cell-wall-degrading culture supernatant, but the supernatants of other non-CF rhizosphere isolates tested exhibited little to no activity (Fig. 4B).

DISCUSSION

To advance our understanding of the mechanisms by which microbes interact in the rhizosphere, we sought to characterize the interactions between *B. cereus* and CF bacteria. Previous work suggested that *B. cereus* stimulates the colonization of plant roots by CF species (9). In the present study, we demonstrated that *B. cereus* directly stimulates the growth of CF rhizosphere isolates in root exudate medium. Several lines of evidence indicate that peptidoglycan produced by *B. cereus* stimulates growth of CF species by serving as a source of carbon and energy. Peptidoglycan purified from *B. cereus* stimulated *F. johnsoniae* growth in root exudate medium in the absence of live *B. cereus*, and *F. johnsoniae* grew in a minimal medium in which peptidoglycan from *B. cereus* was provided as a sole carbon source. Additionally, the populations of *F. johnsoniae* achieved in root exudate medium were proportional to the concentration of purified *B. cereus* peptidoglycan. Finally, we demonstrated that CF rhizosphere isolates secrete a moiety that degrades *B. cereus* cell walls.

The ability of *B. cereus* to promote *F. johnsoniae* growth through a membrane and the production of extracellular peptidoglycan-hydrolyzing activity by CF bacteria suggest that CF bacteria acquire *B. cereus* peptidoglycan fragments by hydrolyzing the outer layers of *B. cereus* cell walls. However, this activity did not affect the growth of *B. cereus* in cocultures. Peptidoglycan hydrolases secreted in membrane vesicles by *Pseudomonas* spp. have been shown to solubilize the outer layers of cells walls of a variety of other bacteria without affecting their growth in exponential phase (16). It remains to be determined whether hydrolysis of *B. cereus* peptidoglycan by CF bacteria has detrimental effects under different growth conditions.

The ability to use peptidoglycan or its derivatives as a source of carbon and energy may mediate interactions between CF bacteria and many of their bacterial neighbors in diverse environments. We found that *E. herbicola* also stimulates *F. johnsoniae* growth in root exudate medium, although we do not yet know whether this interaction is mediated by peptidoglycan. In autotrophic, nitrifying biofilms, CF and other heterotrophic bacteria grow in the absence of externally supplied carbon, indicating that carbon and energy to support their growth must be derived from their nitrifying neighbors (25). Evidence suggests that the CF bacteria in these biofilms receive carbon and energy from components of the peptidoglycan of the nitrifying bacteria (18, 25). Additionally, CF bacteria from a marine environment preferentially consumed *N*-acetyl-D-glucosamine, a major substituent of peptidoglycan (5). Although recent evidence conflicts with earlier reports (1), peptidoglycan is thought to be a major source of dissolved organic nitrogen in the ocean (21), so marine CF species consuming *N*-acetyl-D-glucosamine may be growing on peptidoglycan derivatives.

Peptidoglycan was once thought to play merely a structural role, but its diverse biological activities are becoming evident. In addition to its role as a growth substrate, fragments of peptidoglycan also play roles in pathogenic and mutualistic interactions with eukaryotes (19, 29). Peptidoglycan needs to be considered for its structural, nutritional, and signaling properties in microbial communities.

The relationship between CF bacteria and *B. cereus* appears to be commensal, because growth of *B. cereus* is unaffected by the presence of *F. johnsoniae*. Relatively little is known about the prevalence of commensalisms among microorganisms in nature. Studies in vitro have demonstrated that bacterial commensalism can arise when one organism serves a protective role by degrading or physically blocking access to a toxin (6, 20), or when a metabolic intermediate generated by one strain serves as a growth substrate for the other (4, 24). Interestingly, in these examples, the formation of biofilms was a critical component in establishing the commensalisms. The nature of biofilms formed by *B. cereus* and CF bacteria has not been investigated, but the consistent coisolation of CF bacteria with *B. cereus* from the rhizosphere suggests that the bacteria colocalize on plant roots. However, it is also possible that *B. cereus* inhibits growth of CF strains under some conditions, which would explain why coisolates appear with *B. cereus* only after prolonged incubation.

As the field of microbial ecology matures, evidence is accumulating about the contribution of microbial interactions to the establishment and function of microbial communities. The present study emphasizes the importance of context in determining the outcome of microbial interactions. Stimulation of CF bacterial growth by *B. cereus* was detected in root exudate medium and not in a rich medium, and peptidoglycan, a molecule that normally plays a structural role, mediated the interaction between two microorganisms. These results serve as a caution about making ecological inferences from experiments conducted under typical laboratory conditions and present a reminder of the additional roles that well-characterized microbial products may play.

ACKNOWLEDGMENTS

We thank Brian Manske and Sarah Ridderbusch for technical assistance and Bradley Borlee, Christian Riesenfeld, and Karen Cloud-Hansen for helpful discussions of this work.

This work was supported by a National Science Foundation predoctoral fellowship and the Ira Baldwin Distinguished Fellowship in Bacteriology to S.B.P. and by University of Wisconsin—Madison College of Agricultural and Life Sciences Hatch Project #4534.

REFERENCES

1. **Aluwihare, L. I., D. J. Repeta, S. Pantoja, and C. G. Johnson.** 2005. Two chemically distinct pools of organic nitrogen accumulate in the ocean. Science **308:**1007–1010.
2. **Aronson, A. I., N. Angelo, and S. C. Holt.** 1971. Regulation of extracellular protease production in *Bacillus cereus* T: characterization of mutants producing altered amounts of protease. J. Bacteriol. **106:**1016–1025.
3. **Bullied, W. J., T. J. Buss, and J. K. Vessey.** 2002. *Bacillus cereus* UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: field studies. Can. J. Plant Sci. **82:**291–298.
4. **Christensen, B. B., J. A. J. Haagensen, A. Heydorn, and S. Molin.** 2002. Metabolic commensalism and competition in a two-species microbial consortium. Appl. Environ. Microbiol. **68:**2495–2502.
5. **Cottrell, M. T., and D. L. Kirchman.** 2000. Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. **66:**1692–1697.
6. **Cowan, S. E., E. Gilbert, D. Liepmann, and J. D. Keasling.** 2000. Commensal interactions in a dual-species biofilm exposed to mixed organic compounds. Appl. Environ. Microbiol. **66:**4481–4485.
7. **Dakora, F. D., and D. A. Phillips.** 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil **245:**35–47.
8. **Dunn, A. K., A. K. Klimowicz, and J. Handelsman.** 2003. Use of a promoter trap to identify *Bacillus cereus* genes regulated by tomato seed exudate and a rhizosphere resident, *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. **69:**1197–1205.
9. **Gilbert, G. S., J. L. Parke, M. K. Clayton, and J. Handelsman.** 1993. Effects of an introduced bacterium on bacterial communities on roots. Ecology **74:**840–854.
10. **Halverson, L. J., and J. Handelsman.** 1991. Enhancement of soybean nodulation by *Bacillus cereus* UW85 in the field and in a growth chamber. Appl. Environ. Microbiol. **57:**2767–2770.
11. **Handelsman, J., S. Raffel, E. H. Mester, L. Wunderlich, and C. R. Grau.** 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Appl. Environ. Microbiol. **56:**713–718.
12. **Hoagland, D. R., and D. I. Arnon.** 1950. The water-culture method for growing plants without soil, p. 1–32. California Agricultural Experiment Station Circular 347. California Agriculture Experiment Station, Berkeley, Calif.
13. **Holms, B.** 1992. The genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella*, p. 3620–3630. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. Schleifer (ed.), The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, vol. 4. Springer-Verlag, New York, N.Y.
14. **Iyer, J. L., P. Shetty, and J. S. Pai.** 2005. Evaluation of whole cells of *Bacillus subtilis* as substrate for measurement of autolysin activity. Proc. Biochem. **40:**1593–1597.
15. **Johansen, J. E., and S. J. Binnerup.** 2002. Contribution of *Cytophaga*-like bacteria to the potential of turnover of carbon, nitrogen, and phosphorus by bacteria in the rhizosphere of barley (*Hordeum vulgare* L.). Microb. Ecol. **43:**298–306.
16. **Kadurugamuwa, J. L., and T. J. Beveridge.** 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. J. Bacteriol. **178:**2767–2774.
17. **Kent, A. D., and E. W. Triplett.** 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. **56:**211–236.
18. **Kindaichi, T., T. Ito, and S. Okabe.** 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. Appl. Environ. Microbiol. **70:**1641–1650.
19. **Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman, and M. J. McFall-Ngai.** 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science **306:**1186–1188.
20. **Leriche, V., R. Briandet, and B. Carpentier.** 2003. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. Environ. Microbiol. **5:**64–71.
21. **McCarthy, M. D., J. I. Hedges, and R. Benner.** 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science **281:**231–234.
22. **Milner, J. L., S. J. Raffel, B. J. Lethbridge, and J. Handelsman.** 1995. Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Appl. Microbiol. Biotechnol. **43:**685–691.
23. **Nguyen, C.** 2003. Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie **23:**375–396.
24. **Nielsen, A. T., T. Tolker-Nielsen, K. B. Barken, and S. Molin.** 2000. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. Environ. Microbiol. **2:**59–68.
25. **Okabe, S., T. Kindaichi, and T. Ito.** 2005. Fate of ^{14}C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl. Environ. Microbiol. **71:**3987–3994.
26. **Osburn, R. M., J. L. Milner, E. S. Oplinger, R. S. Smith, and J. Handelsman.** 1995. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites. Plant Dis. **79:**551–556.
27. **Pierson, L. S., and E. A. Pierson.** 1996. Phenazine antibiotic production in *Pseudomonas aureofaciens*: role in rhizosphere ecology and pathogen suppression. FEMS Microbiol. Lett. **136:**101–108.
28. **Ramey, B. E., M. Koutsoudis, S. B. von Bodman, and C. Fuqua.** 2004. Biofilm formation in plant-microbe associations. Curr. Opin. Microbiol. **7:**602–609.

28a. **Rosenthal, R. S., and R. Dziarski.** 1994. Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol. **235:**253–285.

29. **Rosenthal, R. S., W. Nogami, B. T. Cookson, W. E. Goldman, and W. J. Folkening.** 1987. Major fragment of soluble peptidoglycan released from growing *Bordetella pertussis* is tracheal cytotoxin. Infect. Immun. **55:**2117–2120.
30. **Ryder, M. H., Z. N. Yan, T. E. Terrace, A. D. Rovira, W. H. Tang, and R. L. Correll.** 1999. Use of strains of *Bacillus* isolated in China to suppress take-all and rhizoctonia root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. Soil Biol. Biochem. **31:**19–29.
31. **Sadfi, N., M. Cherif, M. R. Hajlaoui, and A. Boudabbous.** 2002. Biological control of the potato tubers dry rot caused by *Fusarium roseum var. sambucinum* under greenhouse, field and storage conditions using *Bacillus* spp. isolates. J. Phytopathol. **150:**640–648.
32. **Whipps, J. M.** 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. **52:**487–511.