Burkholderia (Basonym *Pseudomonas*) *cepacia* Binding to Lipid Receptors

FRANCISCO A. SYLVESTER,¹ UMADEVI S. SAJJAN,² AND JANET F. FORSTNER^{2*}

Divisions of Gastroenterology¹ and Research Biochemistry,² The Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada

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Piliated *Burkholderia* (formerly *Pseudomonas*) *cepacia* from sputa of cystic fibrosis patients in Toronto, Canada, were shown earlier to bind to purified mucins and to a protein receptor on epithelial cells via a 22-kDa adhesin located on unique cable pili. However, a second receptor, thought to be lipid in nature, was also identified on cells and appeared to serve as the major cell receptor for poorly piliated or nonpiliated isolates. In the present study in vitro approaches were used to identify putative lipid receptors for *B. cepacia* and to explore the nature of the binding interaction. As judged by thin-layer chromatography overlay assays, the best receptors were digalactosylceramide and globotriosylceramide (Gb₃). Both contain an unsubstituted terminal Gal α 1-4Gal sequence. *B. cepacia* also bound moderately to galactosylceramide, gangliosylceramide, and gangliotetraosylceramide. Binding to Gb₃ of a nonpiliated isolate was stronger than that of piliated isolates, suggesting that nonpiliated isolates may have greater access to lipid receptors or a greater concentration of an adhesin for Gb₃. Binding to glycolipids was not affected by tetramethylurea, a hydrophobic-bond-breaking agent. Binding was influenced by the structure of the ceramide, which probably affects the presentation of the carbohydrate epitope to the bacteria. Gb₃ was also the major receptor in lipid extracts of human erythrocytes, human buccal epithelial cells and HEp-2 laryngeal epithelial cells. In a receptor-based enzyme-linked immunosorbent assay, binding to Gb₃ within a phospholipid-cholesterol mixture (a membrane-like environment) increased and then approached saturation as a direct function of increasing bacterial concentration. The calculated value of K_a (3.06×10^{-8} ml/CFU), the affinity constant, was almost identical to the K_a calculated earlier for *B. cepacia* binding to a set of lipid receptors in buccal epithelial cells (1.5×10^{-8} to 2.0×10^{-8} ml/CFU). Our findings suggest that within cell membranes, galactose-containing glycolipids, particularly Gb₃, are good candidates for receptors for *B. cepacia*, particularly for isolates in which cable pili are poorly expressed.

Burkholderia (formerly *Pseudomonas*) *cepacia* (46) is an opportunistic pathogen that has been increasingly isolated from the sputa of patients with cystic fibrosis (CF) in North American and European clinics (2, 6). The prevalence of colonization is often as high as 20 to 30% (2). The clinical outcome for colonized patients is variable but usually follows one of three courses: chronic asymptomatic carriage, slow but steady pulmonary deterioration over several months, or rapidly fatal deterioration with pneumonia and septicemia, the so-called “*cepacia* syndrome” (12). Overall, colonized CF patients have a worse prognosis than those who do not become colonized (43, 44).

The properties of *B. cepacia* that contribute to pulmonary colonization and the nature of the virulence factors in CF are still largely unknown. Factors that have been suggested include previous infections with *Pseudomonas aeruginosa* (30), bacterial pili (19, 34), a bacterial exopolysaccharide that is distinct from the alginate of *P. aeruginosa* (29), several exoenzymes (including a protease [26] and a lipase [25]), and iron-chelating siderophores (37). In our own laboratory, isolates from 19 of 22 CF patients at the Hospital for Sick Children, Toronto, Canada, were shown to bind to purified mucins (32). The mucin-binding adhesin was identified as a 22-kDa protein present on pili having a unique cable morphology (Cbl pili) (5, 33, 34). The same adhesin was shown to recognize a 55-kDa buccal epithelial cell (BEC) receptor (34). We therefore spec-

ulated that Cbl pili facilitate *B. cepacia* colonization of the mucus-filled airways of patients with CF. Scatchard plots of the binding of *B. cepacia* to BECs, however, pointed to the existence of a second adhesin-receptor system, in which the receptors were judged to be membrane lipids (32). Although the isolates used in these experiments all carried the *cblA* gene for the Cbl pilin subunit (35) and were genetically of the same clonal strain (42), at least two isolates (BC61 and BC45) did not bind to mucin and were noted by electron microscopy to express few or no Cbl pili. These isolates bound to BECs by using predominantly the second receptor system (34).

Very little is known about the binding of *B. cepacia* to specific lipid receptors, although Krivan et al. (16) reported that a *B. cepacia* isolate from a CF patient in Texas bound to gangliosylceramide (Gg₃ or asialo-GM₂) and gangliotetraosylceramide (Gg₄ or asialo-GM₁). The present study was carried out to identify more specifically the major class of lipid receptors for Toronto CF isolates of *B. cepacia* and to compare the lipid binding properties of a nonpiliated isolate with those of representative piliated isolates.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Clinical isolates of *B. cepacia* (designated BC5, BC7, BC61, NBC1, NBC2, and NBC3) were obtained from patients seen in the CF outpatient clinic or hospitalized at The Hospital for Sick Children in Toronto between 1986 and 1989 (BC5, BC7, and BC61) and during 1993 (NBC1, NBC2, and NBC3). The organisms were identified in the Clinical Mi-

* Corresponding author. Mailing address: The Hospital for Sick Children, Division of Research Biochemistry, 555 University Ave., Toronto, Ontario M5G 1X8, Canada.

crobiology Laboratory at the Hospital for Sick Children as previously described (32). All isolates were shown earlier to have identical ribotype and pulsed-field gel electrophoresis patterns and to carry the *cbiA* gene for the major Cbl pilin subunit (35, 42). The piliation status of the isolates was also determined earlier by electron microscopy (5, 32, 35). Unlike the other isolates, BC61 expressed almost no surface Cbl pili (32, 34). Each culture was suspended in sterile water, transferred onto brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.), grown for 36 to 48 h at 37°C, and maintained at -70°C in vials containing 5% trisodium citrate in 40% glycerol.

In preparation for a thin-layer chromatography (TLC) overlay binding assay, bacteria were subcultured on brain heart infusion agar plates and grown for 36 to 48 h at 37°C, and single colonies were inoculated into 10 ml of Trypticase soy broth (Difco Laboratories) supplemented with 0.1 mCi of L-[³⁵S]methionine (specific activity, 800 Ci/mmol; New England Nuclear Research Products, Boston, Mass.). The bacteria were grown under aerobic conditions in an Orbit shaker (CanLab, Mississauga, Ontario, Canada) at 150 rpm for 16 to 18 h at 37°C. The broth was centrifuged at 10,000 × g for 10 min at 4°C, and the bacterial pellet was washed three times by resuspension and centrifugation with 20 ml of 10 mM phosphate-buffered saline (pH 7.2) (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.). The pellet was resuspended in PBS-1% BSA to give a concentration suitable for binding assays (2.5×10^8 to 2.5×10^9 CFU/ml). An optical density of 0.1 at 650 nm was equal to 2.52×10^8 CFU/ml as determined by plating serially diluted bacteria. The specific activity of each bacterial suspension was measured in a Beckman beta counter (Beckman Instruments, Fullerton, Calif.); values were typically 1.5×10^2 to 2.5×10^2 CFU/cpm. For a receptor-based enzyme-linked immunosorbent assay (R-ELISA) (see below), the bacteria were grown and prepared as described above except that the culture broth contained no radioactive label.

TLC overlay binding assay. The method of Hansson et al. (9), with modifications (32, 49), was used for the TLC overlay binding assay. Digalactosylceramide (Gb₂) was a gift from C. A. Lingwood (Hospital for Sick Children). All other glycolipids were purchased from Supelco Canada Ltd. Phospholipids and cholesterol were from Sigma Chemical Co. Individual lipids (5 mg in chloroform-methanol [2:1]) or 20 mg of a ganglioside mixture was separated by TLC on plastic-based silica gel plates (80 by 80 mm) (Polygram SilG; Brinkmann Instruments, Rexdale [Toronto], Ontario, Canada) with chloroform-methanol-water (60:40:9, vol/vol/vol) as a solvent system. The glycolipids were stained with Iodine reagent (Sigma Chemical Co.), and the phospholipids were stained with iodine vapor, to visualize their positions on the silica gel. Nonstained TLC plates were blocked with 1% gelatin (Bio-Rad, Richmond, Calif.) in PBS overnight at 37°C with shaking at 90 rpm, washed three times with PBS-1% BSA, and incubated with 10 ml of ³⁵S-labeled bacteria for 90 min at 37°C with shaking at 90 rpm. In a separate experiment the gelatin blocking step was preceded by treatment of the plates with 0.1% polyisobutylmethacrylate in acetone for 1 min and air drying for 1 h. To remove nonbound bacteria, the plates were washed five times (5 min each) with PBS-1% BSA, air dried, and processed for autoradiography with X-ray film (Kodak X-OMAT-AR; Eastman Kodak Co., Rochester, N.Y.) and an automated X-ray film developer (Kodak X-OMAT M35 processor; Eastman Kodak Co.).

Preparation of a polyclonal antibody to *B. cepacia*. An antibody was raised in a 2.5-kg New Zealand male rabbit by using a Polytron-disrupted bacterial suspension of isolate BC7 as the antigen, according to the protocol of Harlow and Lane (10). Four subcutaneous injections of antigen (10 mg of protein in Freund's adjuvant) were performed at biweekly intervals. The reactivity of the antibody with *B. cepacia* isolates was tested by using a slot blot assay with 250 ng to 2 mg of bacterial homogenate protein on each slot and antibody dilutions ranging from 1:500 to 1:10,000 (vol/vol) in 1% BSA in 10 mM Tris-buffered saline (pH 7.4), with 0.01% Tween 20 (TTBS). Incubations were carried out for 2 h at 25°C, following which the membranes were washed six times with TTBS. A second antibody (goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate [Bio-Rad]) at 1:3,000 (vol/vol) in 1% BSA-TTBS was incubated for 2 h at 25°C. Membranes were washed once again, and bound antibody was detected with the substrate nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) according to the Bio-Rad protocol. Colored bands corresponding to binding of antibody to bacterial proteins were visible with a 1:10,000 dilution of the primary antibody. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) were also performed on homogenates of isolates BC5, BC7, and BC61 (2 to 10 mg of protein per lane), with sequential exposure to the same antibodies. Multiple bacterial proteins ranging in size from 96 to 14 kDa were detected. BSA used as an antigen served as a negative control, as did experiments in which the primary antibody was omitted.

R-ELISA. For quantitation of the binding of *B. cepacia* to commercially purified globotriosylceramide (Gb₃), an R-ELISA based on a technique developed for verocytotoxin binding to Gb₃ (24) was developed. The assay was carried out in 96-well polystyrene microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.). Glycolipid exposure to ligands has been shown to be enhanced by mixing the lipid with phosphatidylcholine (PC) and cholesterol, thereby mimicking a more physiologic membrane lipid environment (7). Initially experiments were performed to establish the optimum mixture of PC, cholesterol, and glycolipid needed to ensure that there was a linear relation between the concentration of Gb₃ applied to the wells and bacteria bound in the presence of excess added bacteria. In all cases the ratio of PC to cholesterol was maintained at 2:1

(wt/wt), but the total amount of each lipid was varied. This latter parameter and the method of mixing of the total lipid mixture (i.e., PC, cholesterol, and glycolipid) were both found to be critical for obtaining reproducible results and are thus provided in some detail. The procedure consisted of preparing a solution of glycolipid in 0.2 ml of methanol and making successive 1:2 (vol/vol) dilutions in methanol. Before each dilution, thorough dispersion of the glycolipid was achieved by vortexing the tube for 30 s, sonicating for 30 s, and vortexing again for 30 s. A 2:1 mixture of PC and cholesterol (0.5 mg of PC and 0.25 mg of cholesterol to 0.06 mg of PC and 0.03 mg of cholesterol) in methanol was then added to give a total volume of 0.2 ml. The final mixture was thoroughly dispersed as before, and 0.05-ml aliquots were transferred to each of three microtiter plate wells such that the final content of glycolipid ranged from 0.125 to 4 mg per well. Control wells contained PC and cholesterol without added glycolipid. Samples were air dried overnight, and each well was blocked for 2 h at 37°C with 2% BSA in PBS and then washed three times with PBS. *B. cepacia* (a suspension of 2.5×10^8 CFU/ml) was added in a volume of 0.05 ml of PBS containing 1% BSA and incubated at 37°C for 90 min in an Orbit shaker at 90 rpm. The wells were then washed five times with 0.1 ml of PBS. The anti-*B. cepacia* antibody (1:1,000 [vol/vol] in 2% BSA-100 mM Tris-buffered saline) (0.05 ml per well) was then added, and the incubation mixtures were left for 2 h at 25°C. After the wells were washed, the second antibody (0.05 ml of a 1:2,000 [vol/vol] dilution of anti-rabbit immunoglobulin G-horse radish peroxidase conjugate) (Bio-Rad) was added, and the incubation mixtures were left for 1 h at 25°C. The wells were washed again, and the bound antibody was detected colorimetrically according to the Bio-Rad protocol. Nonspecific color development in control wells containing the PC-cholesterol mixture but no Gb₃ was <10% of the total absorbance of the test samples and was subtracted from the values obtained from comparable Gb₃-containing wells. Three assays were performed for each variable tested, and means $\pm$ standard errors were determined. For all concentrations of Gb₃ used (0.125 to 4 mg per well), the optimum concentrations of PC and cholesterol were found to be 0.125 and 0.06 mg per well, respectively, and these concentrations were used in subsequent experiments.

Binding assays were then performed with a fixed amount of Gb₃ (2.5 mg per well) and increasing bacterial concentrations (2.5×10^8 to 2.5×10^9 CFU/ml). Assays with six pairs of wells were done for each bacterial concentration, with each pair consisting of a test well with Gb₃ and a control well without Gb₃. Net binding was obtained by subtracting background binding to the PC-cholesterol mixture alone from binding to the mixture containing Gb₃. A mean ($\pm$ standard error) of net binding was then obtained for each concentration of bacteria added to the wells.

Optical density readings were converted into CFU bound by using a standard curve relating optical density to bacterial radioactivity (not shown). To construct the curve, ³⁵S-labeled BC61 cells were applied in six different concentrations (range, 2×10^5 to 1.5×10^7 CFU/ml, each in triplicate) to microtiter wells, dried at 42°C, and fixed at 65°C for 20 min. With the anti-*B. cepacia* antibody, an ELISA was performed as outlined above and optical density values were measured. The radioactive bound bacteria were then removed by washing the wells three times with 2% SDS. No radioactivity was detected with a fourth wash. On the basis of the known specific radioactivity of the bacterial suspension, CFU were calculated and plotted against optical density. There was a linear relation between CFU and optical density over the range of bacterial concentrations used in R-ELISAs.

Cell culture and lipid extraction and fractionation. HEP-2 laryngeal carcinoma cells (American Type Culture Collection) were used as a model for human respiratory epithelial cells because they are known to contain membrane Gb₃, grow readily in culture, and provide a sufficiently abundant source for lipid extractions (45). They were grown at 37°C in 5% CO₂ in modified Eagle's medium with glutamine, 15% decompartmented fetal calf serum, 0.1% NaHCO₃, 1% amphotericin B, and 2% penicillin-streptomycin. All reagents were purchased from Canadian Life Technologies (Burlington, Ontario, Canada), except for fetal calf serum (Cansera, Rexdale, Ontario, Canada).

The method of Vance and Sweeley (47) was used to extract lipids from suspensions of 50 ml of packed human erythrocytes (blood group O Rh⁻) devoid of the buffy coat and supernatant. After lysis by freeze-thaw steps, cells were extracted twice with chloroform-methanol (2:1, vol/vol). For HEP-2 cells and buccal epithelial cells from healthy volunteers (2 g [wet weight]), the extraction procedure of Smith and Prieto (36) was followed. The lower phases were fractionated on silica gel columns (silica gel 60 [70-230 mesh] ASTM; EM Science, Gibbstown, N.J.) to yield neutral lipids (fraction 1), crude glycosphingolipids (fraction 2), and phospholipids (fraction 3). The samples were flash evaporated, dissolved in 1 ml of chloroform-methanol, and stored in amber glass vials at -20°C. Routinely 0.01 ml of each fraction was used for TLC overlay binding assays.

RESULTS

Binding of *B. cepacia* to lipids. Binding assays were performed with ³⁵S-labeled *B. cepacia* isolates BC5, BC7, BC61, NBC1, NBC2, and NBC3 overlaid onto blots of TLC-separated commercial lipid standards. All of the isolates exhibited

FIG. 1. Overlay of ^{35}S -labeled piliated (BC5) and nonpiliated (BC61) clinical isolates of *B. cepacia* onto purified lipids separated by TLC. Binding was detected by autoradiography. Arrows indicate the best receptors (Gb_2 and Gb_3) as judged by band density. Lanes: 1, Gb_2 ; 2, glucosylceramide; 3, galactosylceramide; 4, lactosylceramide; 5, Gb_3 ; 6, Gb_4 ; 7, GM_1 and GM_2 ; 8, Gg_4 (asialo- GM_1); 9, Gg_3 (asialo- GM_2); 10, phosphatidylethanolamine; 11, phosphatidylserine; 12, PC; 13, cholesterol.

binding specificities similar to those presented in Fig. 1 for isolate BC5 (Cbl^+) and isolate BC61 (Cbl^-). As judged by the band density on autoradiographs, the strongest binding was to Gb_2 and Gb_3 , both of which carry the $\text{Gal}\alpha 1\text{-}4\text{Gal}$ sequence at the nonreducing terminus of the carbohydrate (Fig. 2). Galactosylceramide was intermediate, and Gg_3 and Gg_4 were next in order of density (Fig. 1). Very weak binding was noted for lactosylceramide, glucosylceramide, and PE. These experiments were repeated six more times with bacterial suspensions (BC5, B7, and BC61) with equivalent specific activities, and it was noted without exception that BC61 bound more strongly to (i.e., produced darker bands with) the galactose-containing glycolipids than BC5 or BC7. This suggests that the adhesin for galactolipids is not a component of *Cbl* pili and may be present in a higher concentration or have greater access to lipid receptors in poorly piliated or nonpiliated *B. cepacia* than in piliated *B. cepacia*. The results also suggest that the terminal galactose in the sugar moiety of glycosphingolipids is important for receptor recognition but that the optimal receptor epitope is an unsubstituted $\text{Gal}\alpha 1\text{-}4\text{Gal}$ sequence. Blocking with TBS-BSA instead of PBS-gelatin and the use of polyisobutylmethacrylate (49) before the blocking step did not affect the relative binding strengths or specificities (data not shown).

Effect of ceramide composition on binding. Commercially available galactosylceramide, lactosylceramide, and Gb_3 standards migrated as doublets in silica gel. This phenomenon has

Common name

Terminal sugars

Glc Cer	Glc $\beta 1\text{-}1\text{Cer}$
Gal Cer	Gal $\beta 1\text{-}1\text{Cer}$
Lac Cer	Gal $\beta 1,4$ Glc $\beta 1\text{-}1\text{Cer}$
Gb_2	Gal $\alpha 1,4$ Gal $\beta 1\text{-}1\text{Cer}$
Gb_3	Gal $\alpha 1,4$ Gal $\beta 1\text{-}1\text{Cer}$
Gb_4	Gal $\alpha 1,4$ Gal $\beta 1\text{-}1\text{Cer}$
Gg_3 (asialo GM_2)	GalNAc $\beta 1,3$ Gal $\alpha 1,4$ Gal $\beta 1,4$ Glc $\beta 1\text{-}1\text{Cer}$
Gg_4 (asialo GM_1)	GalNAc $\beta 1,4$ Gal $\beta 1,4$ Glc $\beta 1\text{-}1\text{Cer}$
	Gal $\beta 1,3$ GalNAc $\beta 1,4$ Gal $\beta 1,4$ Glc $\beta 1\text{-}1\text{Cer}$

FIG. 2. Terminal sugar epitopes of glycolipids used in *B. cepacia* binding experiments. Glc Cer, glucosylceramide; Gal Cer, galactosylceramide; Lac Cer, lactosylceramide.

FIG. 3. TLC overlay of ^{35}S -labeled *B. cepacia* (isolate BC61) on galactosylceramide. Arrows indicate the upper (less polar) and lower (more polar) bands of galactosylceramide. (A) Orcinol stain; (B) autoradiography.

been noted by others and is a reflection of molecular species having differences in the composition of the ceramide moiety, including fatty acid length, saturation of double bonds, and sphingosine hydroxylation (14). Alterations (hydroxylation or a shorter fatty acid) of the ceramide portion of the molecule that result in a more polar glycolipid result in relatively less migration in nonpolar organic solvents on TLC plates. It has also been reported that the ceramide moiety of glycolipids may influence the presentation of the polar carbohydrate to various ligands (13, 27). We observed *B. cepacia* to bind to both bands, but in most cases there was preferential binding to the upper (less polar) band of galactosylceramide (Fig. 3B). For Gb_3 , there was little preference exhibited between the upper and lower bands. Ceramide by itself was not a receptor for *B. cepacia* (not presented).

The commercial Gb_3 standard used in these experiments was obtained from porcine erythrocytes. To confirm that *B. cepacia* was also capable of recognizing Gb_3 in human cells, we extracted and separated the glycolipids from human erythrocytes (O Rh^-) and performed TLC overlay binding assays. As judged by Orcinol staining, the human cell lipid extract, unlike the porcine extract, produced a band for Gb_3 only in the upper (less polar) position. This band was well recognized by *B. cepacia* (Fig. 4). *B. cepacia* isolates also bound to Gb_3 in lipids extracted from freshly obtained human BECs and cultured human respiratory HEp-2 cells. For these cell lines Gb_3 appeared as a single broad band rather than a true doublet, and bacterial binding was consequently more diffuse.

Effect of tetramethylurea on binding. Because *B. cepacia* showed a preference for the relatively less polar upper band of galactosylceramide, we investigated the possibility that hydrophobic interactions were important in the adherence phenomenon. Tetramethylurea, a disrupter of hydrophobic associations (32), was used for this purpose. A TLC overlay binding

FIG. 4. TLC overlay of ^{35}S -labeled *B. cepacia* (isolate BC61) on globosides extracted from human O Rh^- erythrocytes, BECs, and respiratory HEp-2 cells. (A) Orcinol stain of porcine commercial standards (lane 1) and human erythrocytes (lane 2); (B) autoradiography of binding to human erythrocytes (lane 3), BECs (lane 4), and HEp-2 cells (lane 5). Arrows indicate a band corresponding to the position of Gb_3 . Gal-Cer, galactosylceramide; Lac-Cer, lactosylceramide.

FIG. 5. Effect of increasing concentrations of ^{35}S -labeled *B. cepacia* (isolate BC61) on binding to Gb_3 in TLC overlay binding assays. Autoradiograms were used to detect the position of bacteria bound to Gb_3 (5 mg) on silica plates. The bands were cut, and radioactivity was determined.

assay was performed as described in Materials and Methods, except that bacteria were suspended in tetramethylurea (10 to 100 mM in PBS-1% BSA) prior to incubation with TLC-separated lipids. There was no difference in binding to galactosylceramide between untreated and tetramethylurea-treated bacteria (data not presented). The same was true for other lipids, suggesting that hydrophobic associations are not a major determinant of *B. cepacia* binding to receptor glycolipids.

Effect of bacterial concentration on binding to Gb_3 . The nature of the binding of *B. cepacia* to Gb_3 was explored in more depth by two approaches. In the first set of experiments, a TLC overlay binding assay was performed in the presence of increasing concentrations (2.5×10^8 to 2.5×10^9 CFU/ml) of radiolabeled bacteria (BC61). Each bacterial suspension was overlaid onto separate silica gel plates containing 5 mg of Gb_3 . After autoradiography, the position of the Gb_3 band was determined by matching the Gb_3 band on the film with the silica plate. The band was cut out from the plate, placed into 10 ml of Ready Protein⁺ scintillation cocktail (Beckman Instruments), vigorously shaken, and allowed to stand overnight. The radioactivity in the vials was then counted in a Beckman beta counter. As shown in Fig. 5, Gb_3 saturation was approached at a concentration of bacteria of 2×10^9 CFU/ml. Half-maximal saturation required approximately 1×10^9 CFU/ml, giving a K_a (affinity constant) of approximately 1.25×10^{-8} ml/CFU.

To confirm these results in a more physiologic (i.e., membrane-like) environment, a second set of *in vitro* experiments was performed by using a quantitative R-ELISA in 96-microtiter well plates with a mixture of immobilized Gb_3 , PC, and cholesterol (2.5, 0.125, and 0.06 mg, respectively) and increasing concentrations of added bacteria ranging from 2.5×10^8 to 2.5×10^9 CFU/ml. Bacterial binding increased as the concentration of *B. cepacia* increased, with a plateau of binding approached at a bacterial concentration of 1.2×10^9 CFU/ml (Fig. 6). A reciprocal plot (1/added CFU per milliliter versus 1/bound CFU) gave a straight line, suggesting that there is a single class of binding sites in Gb_3 having a K_a of 3.06×10^{-8} ml/CFU and a maximum binding of 5.2×10^4 CFU/mg of Gb_3 . The value of K_a is similar to that calculated earlier (34) from Scatchard plots of the binding of isolate BC61 to whole BECs (i.e., 2.04×10^{-8} ml/CFU). Assuming a molecular weight of 1,000 for Gb_3 and an average cell membrane content of Gb_3 in

a respiratory epithelial cell of 0.388×10^{-6} nmol per cell (45), the number of bacteria capable of binding to 10^6 cells would be approximately 2×10^4 if all Gb_3 binding sites were available for binding.

DISCUSSION

Binding specificity for $\text{Gal}\alpha 1\text{-}4\text{Gal}$. The Toronto CF isolates of *B. cepacia* used in the present study bound to galactose-containing glycolipids, particularly Gb_2 and Gb_3 . Gb_2 is not a physiologic component of cell membranes but was used because it contains the same terminal disaccharide as Gb_3 . Gb_3 was the major receptor in commercial (porcine) lipids and in lipid extracts of human erythrocytes and two different human respiratory epithelial cell lines. Our results differ somewhat from those of Krivan et al. (16), who reported that a single *B. cepacia* isolate from Galveston, Tex., bound best to Gg_3 and Gg_4 and did not recognize Gb_3 . The common structural feature in Gg_3 and Gg_4 is a $\text{GalNAc } \beta 1\text{-}4 \text{ Gal}$ sequence, and although all six Toronto isolates recognized both of these asialoglycolipids, Gb_3 was preferred. Our binding data were not influenced by polyisobutylmethacrylate (49) in TLC overlays, and thus the use of this agent in the study by Krivan et al. (16) does not explain our observed difference in specificities. Addition of GalNAc linked $\beta 1\text{-}3$ to the terminal Gal of Gb_3 (as is found in Gb_4) was not tolerated, suggesting that the digalactosyl receptor for our *B. cepacia* isolates must be properly oriented or stereochemically unhindered for a good fit between the (as yet unidentified) lectin-like bacterial adhesin and its lipid receptor (41). Since genotypic differences among geographically widespread *B. cepacia* isolates are common (40), it is possible that related phenotypic differences in the lectin-like adhesin structure may explain the observed differences in receptor specificity between the Toronto and Galveston strains.

The binding of Cbl^+ , and the even stronger binding of Cbl^- , isolates of *B. cepacia* to Gb_3 indicates that the involved adhesin is not part of the Cbl pilus structure. The only other type of pili present in both of these strains has been designated Msh (mesh) pili (5), and it is theoretically possible that these could mediate lipid adherence. Alternatively, the adhesin could be another component common to both isolates, such as an outer membrane protein. Since long Cbl pili project outward for 2 to 4 μm and are arranged in a peritrichious distribution over the

FIG. 6. Effect of increasing concentrations of ^{35}S -labeled *B. cepacia* (isolate BC61) on binding to Gb_3 (2.5 mg) immobilized in polystyrene wells. Binding was detected with an R-ELISA. The inset shows a double-reciprocal plot of bacteria added ($1/A$) against bacteria bound ($1/B$). The equation for the line is $y = 7.4560 \times 10^{-6} + 243.44x$ ($r^2 = 1.00$). K_a is 3.06×10^{-8} ml/CFU, and B_{max} is 5.2×10^4 CFU/mg of Gb_3 . OD₄₉₀, optical density at 490 nm.

surface of the bacteria (35), these pili may cause partial hindrance of the access of an outer membrane adhesin for cell lipid receptors. This might explain the reduced adherence of Cbl⁺ BC5 and BC7 to lipid compared with the Cbl⁻ isolate BC61. Future testing of isogenic mutants lacking one or both types of pili will be important to address these issues.

All isolates of *B. cepacia* bound better to the upper (less polar) band of galactosylceramide. The presence of two bands in silica gel was assumed to reflect differences (fatty acid length, saturation, and/or hydroxylation) in the polarity of the ceramide of glycosphingolipid molecules that have identical carbohydrate moieties (14). Support for this interpretation derives from other studies in which the ceramide fatty acid has been shown to influence glycolipid affinity, as in the cases of the class II adhesins of *Escherichia coli*, *Bordetella pertussis*, *Neisseria gonorrhoeae*, and others (14) and *E. coli* verocytotoxin, which recognizes the same Gal α 1-4Gal receptor sequence (24) recognized by our *B. cepacia* isolates.

Functional implications of *B. cepacia* binding to glycolipids.

A general inference from the present study is that in addition to the Cbl pilin adhesin system, which, as shown earlier, is active in the adherence of *B. cepacia* to mucin and epithelial cells, the same bacteria have also evolved a second adhesin system, which is specific for galactolipids, especially Gb₃. The relative clinical significance of each adhesin-receptor system is unknown at present, although it is reasonable to speculate that both could be used to anchor bacteria to host surfaces during the initial stages of CF lung colonization. Because of its higher affinity (34), the second receptor system may serve as the principal means of epithelial cell attachment for isolates once they migrate through the mucus layer and reach the cell surface. Upregulation of Gb₃ expression for any reason might then enhance the infectious process. Gb₃ in human vascular endothelial cells has been shown in vitro to be upregulated by tumor necrosis factor and interleukin-1 (15, 48). In CF, the same cytokines are implicated in pulmonary inflammatory responses (8, 28). We might speculate, therefore, that if Gb₃ becomes upregulated in CF respiratory cells because of previous lung injury from viral or bacterial pathogens, the increased Gb₃ would be influential in mediating widespread adherence and colonization by opportunistic *B. cepacia*. In this context, it is interesting that formalin-fixed heat-killed *P. aeruginosa* has been reported to induce production of tumor necrosis factor alpha and interleukin-1 in peripheral blood mononuclear leukocytes in culture (38). Whether the same phenomenon occurs in epithelial cells has not been tested, to our knowledge.

Our studies indicated that *B. cepacia* isolates obtained from independent CF patients in Toronto between 1986 and 1989 (BC5, BC7, and BC61) and in 1993 (NBC1, NBC2, and NBC3) had identical lipid binding specificities. The maintenance of this specificity, particularly for Gb₃, over a period of several years suggests that the expression of an adhesin for Gb₃ may be important for the continued survival of this hypertransmissible strain (42) in the CF lung, where it has to compete continuously with other opportunistic pathogens.

With respect to other potential membrane lipid receptors, Saiman and Prince have shown an increase in Gg₄ in respiratory cells of CF patients (31). Since Gg₄ was also a receptor for *B. cepacia* in our experiments (although not as effective as Gb₃), a coordinated upregulation of both Gb₃ and Gg₄ might create a very attractive climate for *B. cepacia* adherence and colonization.

In addition to *B. cepacia*, many bacterial pathogens (e.g., *P. aeruginosa*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *B. pertussis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, certain *E. coli* strains, and *N. gonorrhoeae*) bind to the GalNAc

β 1-4 Gal sequence characteristic of Gg₃ and Gg₄ (3, 18). For some bacteria, binding to Gg₃ and Gg₄ is coupled with the capacity to bind to PE (as, for example, in the cases of *P. aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Helicobacter pylori*, and *Helicobacter mustelae*) (4, 17, 21, 23). It has been suggested that bacteria that exhibit both PE and Gg₃-Gg₄ binding possess an exoenzyme S-like adhesin, which was described first for *P. aeruginosa* (11) and then for *H. pylori* (22). Since we observed binding of *B. cepacia* to Gg₃, Gg₄, and PE (the latter very weakly, however), it is possible that *B. cepacia* can also express an exoenzyme S-like adhesin. If so, this would represent a third potential receptor-adhesin system. Further experiments will be needed to confirm this possibility.

Verocytotoxin (24), Shiga toxin (20), and the G adhesin in the Pap pilus of uropathogenic *E. coli* (1) also recognize the Gal α 1-4Gal moiety of the globo-series of glycolipids. Unlike the G adhesins, however, Shiga toxin, verocytotoxins 1 and 2, and the putative adhesin for *B. cepacia* do not tolerate sugar additions distal to the disaccharide Gal α 1-4Gal (41), as found in Gb₄, for example. Therefore, once the adhesin for Gb₃ can be identified in *B. cepacia*, it will be of interest to determine whether the protein has a binding epitope that is homologous with that of Shiga toxin or verocytotoxin, particularly verocytotoxins 1 and 2, which, like *B. cepacia*, bind to Gb₃ but not to Gb₄ (39).

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