A *Cronobacter turicensis* O1 Antigen-Specific Monoclonal Antibody Inhibits Bacterial Motility and Entry into Epithelial Cells

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Cronobacter turicensis is an opportunistic foodborne pathogen that can cause a rare but sometimes lethal infection in neonates. Little is known about the virulence mechanisms and intracellular lifestyle of this pathogen. In this study, we developed an IgG monoclonal antibody (MAB; MAB 2G4) that specifically recognizes the O1 antigen of *C. turicensis* cells. The antilipopolysaccharide antibody bound predominantly monovalently to the O antigen and reduced bacterial growth without causing cell agglutination. Furthermore, binding of the antibody to the O1 antigen of *C. turicensis* cells caused a significant reduction of the membrane potential which is required to energize flagellar rotation, accompanied by a decreased flagellum-based motility. These results indicate that binding of IgG to the O antigen of *C. turicensis* causes a direct antimicrobial effect. In addition, this feature of the antibody enabled new insight into the pathogenicity of *C. turicensis*. In a tissue culture infection model, pretreatment of *C. turicensis* with MAB 2G4 showed no difference in adhesion to human epithelial cells, whereas invasion of bacteria into Caco-2 cells was significantly inhibited.

Cronobacter spp. are opportunistic pathogens and are known to be rare but important causes of severe infections, including meningitis, necrotizing enterocolitis, and systemic sepsis, particularly in premature and low-birth-weight neonates (1). Recent reports have also highlighted an increased risk for immunocompromised adults (2, 3). The genus *Cronobacter* was proposed in 2008 and currently consists of seven species that are clearly distinguishable by biochemical and molecular means (4–8). Infection of humans may occur by ingestion of contaminated food or through environmental exposure (9–11). For neonates and infants, however, oral infection by powdered infant formula contaminated with *Cronobacter* seems to be the most likely route (10, 12, 13).

Only little is known about the major mechanisms of pathogenicity in *Cronobacter* spp. that are responsible for adhesion to and invasion of human intestinal cells. Most strains tested so far were able to attach to human epithelial cells (14, 15), and it has been reported that human isolates of *Cronobacter sakazakii* bind to human enterocytes more efficiently than environmental strains (16). Diffuse adhesion and cluster formation of the bacteria on the cell surface could be observed (14), and several studies demonstrated the ability of *Cronobacter* spp. to invade human intestinal cells (17, 18). The outer membrane proteins OmpA and Inv as well as the host cytoskeleton have especially been shown to play an important role during invasion (19–21). Inside the host, however, a pathogen has to cross the mucosal barrier before approaching the intestinal cells. Therefore, motility and chemotaxis are crucial for infection for many pathogenic bacteria (22). In *Cronobacter turicensis* 3032, seven filamental proteins of flagella were identified by proteomic profiling (23). The absence of flagella in mutants of *C. sakazakii* strain ES5 strongly reduced the ability to attach to host cells (24). In *Salmonella enterica* serovar Typhimurium, it has previously been shown that motility and the ability to invade epithelial cells could be inhibited by an IgA monoclonal antibody (MAB) that binds to the O antigen (25). The antibody compromised protein secretion as well as bacterial outer membrane integrity and energetics, resulting in an avirulent phenotype (26).

Within the genus *Cronobacter*, 17 serogroups have been defined on the basis of the O-antigen-encoding gene clusters (27–32). For *C. turicensis*, three O serotypes (serotypes O1 to O3) have been identified (27, 28, 30), and the chemical structure of the O antigen of the *Cronobacter turicensis* HPB3287 strain has been determined (33). However, little is known about the reactivity of antibodies with O-antigenic determinants. An O-antigen serotyping scheme based on rabbit antisera has recently been developed for *C. sakazakii*, allowing the identification of seven serotypes (31). In addition, monoclonal antibodies reactive to hypothetical outer membrane proteins have been described, but they did not bind to live untreated bacteria, and no neutralization properties were reported (34).

In this study, we were able to generate and characterize an O1-specific IgG antibody against *C. turicensis* 3032 (LMG23827^T), a strain that caused the death of two newborn children and for which the complete and annotated genome sequence is available (35). The antibody showed relevant and reproducible neutralizing activity *in vitro*. Binding of the antibody to the O antigen affected the bacterial membrane integrity, which led to greatly reduced motility and inhibited bacterial entry into epithelial cells. The antibody described here may be used as an additional tool for the

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TABLE 1 Characteristics of bacterial strains used in this study

Strain	Origin	Reference or source	Result ^b	
			ELISA	IF
<i>C. sakazakii</i> E767	Milk powder	IFSH ^a	—	—
<i>C. sakazakii</i> E601	Human	68	—	—
<i>Cronobacter malonaticus</i>	Human	69	—	—
<i>Cronobacter universalis</i>	Water	69	—	—
<i>Cronobacter dublinensis</i> subsp. <i>dublinensis</i>	Environment	69	—	—
<i>C. dublinensis</i> subsp. <i>lausannensis</i>	Water	69	—	—
<i>C. dublinensis</i> subsp. <i>lactaridi</i>	Environment	69	—	—
<i>Cronobacter muytjensii</i>		68	—	—
<i>Cronobacter condimenti</i>	Food	6	—	—
<i>C. turicensis</i> 3032	Human	35, 69	+	+
<i>C. turicensis</i> E609	Food	IFSH	—	—
<i>C. turicensis</i> E625	Baby food	IFSH	—	—
<i>C. turicensis</i> E688	Food	IFSH	+	+
<i>C. turicensis</i> 1053	Environment	IFSH	—	—
<i>Enterobacter helveticus</i>	Fruit powder	70	—	—
<i>Enterobacter turicensis</i>	Fruit powder	70	—	—
<i>Enterobacter pulveris</i>	Fruit powder	71	—	—
<i>E. coli</i> K-12		72	—	—
<i>S. Typhimurium</i>	Human	IFSH	—	—
<i>S. Infantis</i>	Human	IFSH	—	—

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^b The positive (+) and negative (−) results obtained by ELISA and immunofluorescence (IF) using MAb 2G4 are indicated.

detection of *C. turicensis* and will certainly be of value for the investigation of *Cronobacter* virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All strains were grown in Luria-Bertani (LB) medium at 37°C with constant shaking. For solid media, 15 g/liter agar was added. To grow *C. turicensis* 3032 under cell culture conditions (37°C, 7% CO₂, without shaking), a 2% inoculum from an overnight culture was prepared in fresh, prewarmed LB and incubated for 2 h at 37°C with shaking. After these 2 h, the cells reached an optical density at 600 nm (OD₆₀₀) of 0.45. After collecting cells by centrifugation, washing the cells, and resuspending the cells in RPMI 1640 without fetal calf serum (FCS; Biochrom, Berlin, Germany), bacteria were inoculated into RPMI 1640 to an OD₆₀₀ of 0.05 or 0.45. The measurement of the OD₆₀₀ was carried out in intervals of 20 min over 2 h using a spectrophotometer (Eppendorf, Hamburg, Germany). To measure the number of CFU, bacteria were quantified by plating 10-fold serial dilutions and colony counting on LB agar plates.

Immunogen preparation. To prepare immunogenic extracts of *C. turicensis* 3032, 50 ml of an overnight culture was centrifuged at 10,000 × g for 10 min at 4°C. After washing with sterile phosphate-buffered saline (PBS), the bacterial pellet was resuspended in 1 ml digesting solution containing (per ml) 2 mg polymyxin B-sulfate, 40 µg DNase, 0.95 mg MgCl₂, 8 µg RNase (Sigma-Aldrich, Steinheim, Germany), and 150 µl protease inhibitor solution (Complete mini; Roche Diagnostics, Penzberg, Germany), and the suspension was incubated for 1.5 h at 37°C with slight shaking. The bacterial solution was centrifuged again at 14,000 × g for 30 min at 7°C. The supernatant (lysate) was filtered (pore size, 0.22 µm) and concentrated with Amicon ultracentrifugal filter units with a pore size cutoff of 30 kDa. For storage for longer than 1 week, the sample was frozen at −20°C. The protein concentration of the lysate was determined using the Bradford method with the Bradford reagent obtained from Sigma.

Immunization and hybridoma production. Immunizations of mice for generating monoclonal antibodies were conducted in compliance with the German Law for Protection of Animals. Study permission was obtained by the Government of Upper Bavaria (permit number 55.2-1-54-2531.6-1-08). Female mice [of the BALB/c strain and a hybrid strain of BALB/c × (NZW × NZB)] were immunized by intraperitoneal injection of 30 µg of immunogen dissolved in 100 µl PBS and mixed with Freund's adjuvant at a ratio of 1:3. The mice were boosted 14 weeks after the primary injection, using the same composition and amount of the immunogen. If necessary, the immunization was repeated. The final booster injection of 60 µg of antigen in 300 µl PBS without Freund's adjuvant was carried out 3 days before fusion. Cell fusion using X63-Ag8.653 myeloma cells was performed as previously described (36). Culture supernatants were tested for *C. turicensis* 3032-specific antibodies 12 days after fusion by enzyme-linked immunosorbent assay (ELISA), and positive clones were separated by limiting dilution. Production and purification of the MAbs from supernatants on a larger scale were performed as described earlier (37). In brief, selected clones were mass produced in a Mini-Perm bioreactor (Sarstedt, Nümbrecht, Germany), and the MAbs were isolated and purified by affinity chromatography on protein A-agarose (Bio-Rad, Munich, Germany). The immunoglobulin subtype of the MAbs was determined by using mouse monoclonal antibody isotyping reagents purchased from Sigma-Aldrich. Fab fragments were produced with a Pierce Fab micropreparation kit (Thermo Fisher, Munich, Germany).

ELISA. To demonstrate antibody binding to living bacterial cells, microtiter plates were coated with *C. turicensis* 3032, using the standard ELISA protocol (37) with minor modifications. In brief, cell pellets harvested from 1 ml overnight culture were washed, suspended in 1 ml PBS, and serially diluted in bicarbonate buffer. After incubation overnight at ambient temperature, the free binding sites of the wells were blocked with 150 µl 3% casein-PBS solution per well for 30 min. Prior to addition of 100 µl of MAb solution (1 µg/ml), the plate was washed three times and then incubated for 1 h. Further steps, including data analysis, were carried out in accordance with the standard protocol.

Serotype-specific PCR. PCR-based identification of the three serotypes described so far (serotypes O1 to O3) was performed using the primers and following the protocols described in the respective publications (27, 28, 32). The DNA templates were isolated using a Qiagen DNeasy blood and tissue kit (Hilden, Germany).

Construction of a *wzx* gene deletion mutant. The O-antigen flippase gene (*wzx*) was chosen as a target for gene deletion. The mutant was constructed as described by Philippe et al. in 2004 (38).

Bacterial agglutination and motility assay. Bacterial agglutination was performed using living *C. turicensis* 3032 cells grown to mid-logarithmic growth phase. The cells were washed once, resuspended in PBS at 5 × 10⁸ cells/ml, and incubated with different concentrations of MAb for 10 min or 1 h at 37°C. The agglutination of bacterial cells was assessed by light microscopy of 10-µl aliquots of the bacterium-MAb mixes spotted onto a glass microscope slide. For agar motility assays, LB agar plates containing 0.3% agar and different concentrations of MAbs were stab inoculated with 1 µl of an overnight culture of *C. turicensis* 3032 and incubated upright at 37°C. The motility was determined by measuring the diameter of the motility zone every hour.

Cell culture. Human colon epithelial (Caco-2; ACC 169) cells were received from the German Collection of Microorganisms and Cell Cultures (DMSZ) and cultured in a 7% CO₂ atmosphere at 37°C in RPMI 1640 medium with stable glutamine containing 10% FCS, unless indicated otherwise. All cell culture media, additives, and PBS were obtained from Biochrom (Berlin, Germany). Cells were passaged twice a week using a dilution rate of 1:3. Cell viability was determined by trypan blue staining. Trypsin-treated epithelial cells were seeded into 24-well cell culture plates at a concentration of 2.5 × 10⁵ cells/well for the 1-day-old Caco-2 cell monolayer or of 5 × 10⁴ cells/well to obtain polarized epithelial cells. The 24-well plates were incubated for 1 to 16 days until infection,

depending on the degree of differentiation and maintenance of tight junctions. The cell culture medium was replaced every 2 days.

Bacterial invasion and adhesion assays in Caco-2 cells. In order to examine the effect of MAb 2G4 on bacterial invasion and adhesion to Caco-2 cells, a gentamicin protection assay (18) with minor modifications was performed. In brief, the cell monolayers were washed twice with PBS and infected at a multiplicity of infection (MOI) of 100 by covering each well with 500 μ l RPMI 1640 without FCS mixed with the corresponding amount of bacteria from logarithmic growth phase and the MAb at various concentrations, as indicated below. Prior to infection, this solution was incubated for 10 min at room temperature. The bacteria were prepared by transferring 2% inoculum from an overnight culture into fresh prewarmed LB, followed by incubation for 2 h at 37°C with constant shaking, in which the bacteria reached an OD₆₀₀ of 0.45 (18). Bacteria were collected by centrifugation, washed, and resuspended in RPMI 1640 without FCS. A murine monoclonal antibody (MAb HT-2, an in-house MAb) directed against HT-2 toxin, a trichothecene mycotoxin, was used as an isotype control for MAb 2G4. After an infection period of 1.5 h at 37°C in a 7% CO₂ atmosphere, the infected cells were washed twice with PBS and incubated for another 1 h in 500 μ l fresh RPMI 1640 without FCS containing 100 μ g/ml gentamicin to kill any remaining extracellular bacteria. Following this invasion period, the monolayer was again washed three times with PBS and lysed in 1 ml cold Triton X-100 (0.1%). The released intracellular bacteria were quantified by plating 10-fold serial dilutions and counting the colonies on LB agar plates. To assess the number of cell-associated bacteria, adhesion assays were performed as described above, except that the infection time was reduced to 30 min and the gentamicin step was omitted. The number of cell-associated bacteria and the level of bacterial invasion were calculated by the formula (number of bacteria recovered/number of bacteria inoculated) $\times$ 100. The normalized percentage of CFU was determined by comparison with the count for the control (which was not treated with MAb 2G4), the number of CFU of which was set to 100%. The assays were run in triplicate and replicated three times.

Immunofluorescence. To stain untreated live *Cronobacter* cells and cells of other *Enterobacteriaceae*, an overnight culture was diluted (1:20) in PBS and pelleted by centrifugation at 8,500 $\times$ g and room temperature for 15 min. The cells were resuspended in fresh PBS and incubated with the primary antibody (MAb 2G4) at a concentration of 1 μ g/ml in 1% Tween 20-PBS for 30 min at room temperature with constant shaking. After washing once with PBS, bacterial cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen GmbH, Darmstadt, Germany) secondary antibody at a concentration of 2.5 μ g/ml in PBS for 30 min at room temperature with constant shaking. Bacteria were collected by centrifugation, washed, and resuspended in PBS. Five to 10 μ l of this cell suspension was pipetted onto a glass slide, a cover slip was placed on the slide, and the cells were analyzed with a Keyence immunofluorescence microscope.

To localize intracellular bacteria, confluent monolayers of Caco-2 cells (6×10^4) in chamber slides (Nunc, Wiesbaden, Germany) were infected with *C. turicensis* 3032 as described above. After gentamicin protection, the cells were fixed with ice-cold methanol at -20°C for 10 min, permeabilized by treatment with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS. The preparations were blocked with 5% inactive goat serum for 40 min and then sequentially incubated with the primary antibody (MAb 2G4) and Alexa Fluor 488-conjugated antimouse secondary antibody at concentrations of 1 μ g/ml and 10 μ g/ml, respectively, in 1% bovine serum albumin (BSA)-PBS for 1 h. The nuclei of Caco-2 cells were stained with DAPI (4',6-diamidino-2-phenylindole). The coverslips and slides were analyzed using a Keyence immunofluorescence microscope.

Measurement of membrane potential by flow cytometry. Changes in the electrical membrane potential ($\Delta\Psi$) of the *C. turicensis* 3032 and *S. Typhimurium* ST4/74 (39) strains was measured with JC-1, a membrane potential sensor cationic dye (Invitrogen GmbH, Darmstadt, Germany),

as described by Becker et al. (40) and Forbes et al. (26). Bacterial cells loaded with JC-1 were resuspended in 1 ml M9 medium (pH 7.0) including 0.5% glucose and incubated for 15 min at room temperature. *C. turicensis* 3032 cells were then diluted 1:10 in PBS and incubated with 5 μ g/ml of MAb 2G4 for 20 min. The sample without the addition of the antibody served as a negative control. Fluorescence intensity was analyzed by a FACSCalibur flow cytometer using CellQuest software (BD Bioscience, USA).

Extraction and SDS-PAGE analysis of LPS. Lipopolysaccharide (LPS) was prepared from a 1-ml overnight culture by the rapid phenol-chloroform method described previously (41, 42), with an additional step being performed at the end of procedure. Before dissolving the LPS in 50 μ l distilled water, the precipitated LPS was dried at 45°C for 10 min in order to evaporate the rest of the ethanol. LPS extracts were mixed with an equal amount of the loading buffer (Laemmli buffer) and subjected to SDS-PAGE or stored at 4°C. Standard LPSs from *Escherichia coli* and *S. Typhimurium* were provided by Sigma (catalog numbers L6143 and L4391, respectively). Twenty microliters of each LPS extract and 10 μ l of LPS standards were separated by 12.5% SDS-PAGE and subsequently silver stained, as described by Tsai and Frasch (43), or used for immunoblotting analysis.

Western blot analysis. The SDS-gel was first incubated for 30 min in cold (4°C) distilled water, and then the separated LPS was transferred to a polyvinylidene difluoride membrane in a Trans-Blot cell (Bio-Rad, Germany) using Towbin buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol). The transfer conditions were 10 V and 30 to 40 mA for 16 h at 4°C with permanent stirring of the buffer solution. The membrane was saturated with Tris-buffered saline (TBS) containing 5% nonfat skim milk and 0.1% Tween 20 for 1 h at room temperature and probed with the primary antibody (MAb 2G4) at a concentration of 2 μ g/ml in TBS (containing 3% nonfat skim milk, 0.1% Tween 20) for 48 h at 4°C. After three washing steps with TBS, the membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated horse antimouse secondary antibody (1:2,000), obtained from Cell Signaling Technology (Danvers, MA, USA). LPS bands were visualized using SuperSignal West Femto maximum-sensitivity substrate (Pierce, Rockford, IL, USA).

TEM and immunogold labeling. The presence of flagella was analyzed by negative staining and transmission electron microscopy (TEM). Bacteria were prepared as described above for the bacterial agglutination assay with PBS, mixed with control antibody HT-2 (10 μ g/ml) or MAb 2G4 (20 μ g/ml), and incubated at 37°C without shaking for 2.5 h. Then, 10 μ l of the bacterial culture was placed on a carbon/Pioloform-coated copper 200-mesh grid, and the cells were allowed to adhere for 10 min. The grids were subsequently washed twice in distilled water for 2 min each time, negative stained with 2% uranyl acetate for 1 min, and examined using a FEI 120 kV Tecnai G2 BioTwin transmission electron microscope.

For immunogold labeling, 10 μ l of the bacterial culture was placed on carbon/Formvar copper 200-mesh grids, and cells were allowed to adhere for 10 min. The grids were then washed twice in distilled water and blocked in PBS containing 3% BSA and 0.01% Tween 20 for 30 min at room temperature. After five washes in PBS with 0.01% Tween 20 for 5 min at room temperature, the grids were incubated for 2 h with MAb 2G4 (diluted 1:200 in dilution buffer) at 37°C. Next, the grids were washed again five times in PBS for 5 min each time and incubated with secondary colloidal gold (18-nm)-conjugated goat anti-mouse IgG (diluted 1:25 in dilution buffer) for 1 h at room temperature. Before the bacteria on the grids were examined by TEM, five washes in PBS with 0.01% Tween 20, two washes in distilled water, and negative staining with 2% uranyl acetate for 1 min were performed.

RESULTS

Specificity of monoclonal antibody 2G4. In order to develop a monoclonal antibody specific to *Cronobacter turicensis* 3032, mice were immunized with a filtered cell lysate. After fusion of mouse splenocytes with myeloma cells, screening for positively reacting

FIG 1 Detection of *C. turicensis* 3032 cells by immunofluorescence using MAb 2G4. (A) Fluorescence microscopic image of *C. turicensis* from an overnight culture. (B) Caco-2 cells infected with *C. turicensis* and stained with MAb 2G4 (green fluorescent bacteria). DNA was stained with DAPI.

hybridoma cell clones was performed using an indirect ELISA. One clone (2G4) which secreted a monoclonal antibody of the IgG2a subtype showing a high affinity to the immunogen as well as to polymyxin B-treated cells was identified in the indirect ELISA. The specificity of MAb 2G4 was further tested by noncompetitive ELISA using different *Cronobacter* strains and other strains of the *Enterobacteriaceae* (Table 1) as coating antigens. MAb 2G4 selectively recognized *C. turicensis* 3032 and *C. turicensis* E688. No cross-reactivity with other *Cronobacter* spp. or more distantly related bacteria was detected. To demonstrate the reactivity of the MAb with untreated bacterial cells, MAb 2G4 was also employed in immunofluorescence staining experiments using the same bacterial strains. In agreement with the ELISA results, fluorescence signals were obtained only for *C. turicensis* 3032 and *C. turicensis* E688 (Table 1). For strong and reproducible immunofluorescence staining of the bacterial cells, 1 µg/ml of the MAb 2G4 was sufficient. To assess the suitability of MAb 2G4 for detection of single *C. turicensis* 3032 cells in an infection model, e.g., the promising zebrafish model, the antibody was first tested in a cell culture model. Therefore, Caco-2 cells were infected with *C. turicensis* 3032, following by several washing steps and gentamicin incubation to kill and remove extracellular bacteria. By applying MAb 2G4 and an Alexa Fluor 488-conjugated antimouse secondary antibody, *C. turicensis* 3032 cells were detected both in liquid culture (Fig. 1A) and in the infected and gentamicin-treated Caco-2 cells (Fig. 1B).

Identification of the MAb 2G4 antigen. MAb 2G4 showed a strong reactivity with live bacteria as well as with heat-killed *C.*

FIG 3 Detection of *C. turicensis* serotypes by specific PCR-based O-antigen serotyping. Three different serotype-specific primers sets, z3032-wzwF5/z3032-wzwR4 (323 bp), wl-44241/wl-44242 (438 bp), and E609-wzxR1/E609-wzxR1 (236 bp), were used for the detection of serotype O1, O2, and O3 strains, respectively. Lane 1, *C. turicensis* 3032; lane 2, *C. turicensis* E688; lane 3, *C. turicensis* E609; lane 4, *C. turicensis* E625; lane 5, *C. turicensis* E1053; lane 6, negative control.

turicensis 3032 cells, suggesting that the antibody recognized a heat-stable component of the cell, possibly polysaccharides. In order to determine the surface antigen to which MAb 2G4 binds, LPS was extracted from *C. turicensis* strains and analyzed by SDS-PAGE, followed by silver staining or immunoblotting. The SDS-PAGE profiles of the LPS extracts revealed a characteristic ladder pattern (Fig. 2A). LPSs from *E. coli* and *S. Typhimurium* showed various bands of high molecular weight, indicating a smooth LPS structure containing full-length O-chain polysaccharides (29). The LPSs of all *C. turicensis* strains, however, seemed to be less smooth, showing an O-antigen chain length distribution with fewer and less intensely stained bands of lower molecular weight. To determine the antigenic specificity of purified MAb 2G4, immunoblotting of LPSs extracted from four *C. turicensis* strains and *E. coli* and *S. Typhimurium* strains was performed (Fig. 2B). MAb 2G4 displayed a strong and specific reaction with LPSs from *C. turicensis* 3032 and E688, whereas no signals were detected for other *C. turicensis* or non-*Cronobacter* strains. This led to the suggestion that the O-specific polysaccharide chain could potentially be the epitope of MAb 2G4. Therefore, a serotype-specific PCR was performed (Fig. 3) and confirmed that the *C. turicensis* 3032 and E688 strains, which reacted positively with MAb 2G4, were members of the O1-serotype group, while *C. turicensis* E609 and

FIG 2 LPS analysis by silver staining, Western blotting, and immunogold labeling. (A) Silver-stained polyacrylamide gel showing the O-antigen LPS profiles of various *C. turicensis* strains, *E. coli*, and *S. Typhimurium*. Lane 1, *C. turicensis* E609; lane 2, *C. turicensis* E688; lane 3, *C. turicensis* E625; lane 4, *C. turicensis* 3032; lane 5, *E. coli*; lane 6, *S. Typhimurium*. Standard LPSs (10 µg) from *E. coli* O111:B4 (lane 7) and *S. Typhimurium* (lane 8) were obtained from Sigma. (B) Immunoblotting of purified LPS of *C. turicensis* using the anti-LPS O-antigen MAb 2G4 (2 µg/ml) and secondary horse anti-mouse IgG (1:2,000). Lane 1, *C. turicensis* 3032; lane 2, *C. turicensis* E609; lane 3, *C. turicensis* E625; lane 4, *C. turicensis* 3032; lane 5, *E. coli*; lane 6, *S. Typhimurium*; lane 7, standard LPS from *S. Typhimurium* (10 µg); lane 8, *C. turicensis* E688. (C) Transmission electron micrograph of *C. turicensis* 3032 labeling with MAb 2G4 followed by goat anti-mouse IgG conjugated to 18-nm gold spheres. Magnification, $\times 26,500$.

FIG 4 Growth inhibition of *C. turicensis* 3032 in the presence of different concentrations of MAb 2G4 at 37°C. Bacterial cells were grown in LB to an OD₆₀₀ of 0.45 at 37°C, harvested, and transferred to RPMI 1640 (without FCS) containing 0, 1, 10, and 20 µg/ml MAb 2G4 (time zero). Two cultures with different cell densities, one with an OD₆₀₀ of 0.05 and one with an OD₆₀₀ of 0.45 (B), were prepared before addition of MAb 2G4 and grown at 37°C in 7% CO₂ without shaking. An HT-2 toxin-specific antibody, MAb HT-2, served as a negative control. Ampicillin (Amp) and chloramphenicol (Cm) were used as controls for bactericidal and bacteriostatic reagents, respectively. The x axis shows the time (t) after addition of MAb 2G4. (C) Growth curves of the samples described for panel B, expressed as numbers of CFU/ml. The results are representative of those from three independent experiments performed in triplicate.

E625 belonged to the O3-serotype group. The O serotype of the *C. turicensis* E1053 strain could not be determined with the available primer sets. The ability of MAb 2G4 to bind to the surface of *C. turicensis* 3032 was further confirmed by immunoelectron transmission microscopy (Fig. 2C). The localization and density of the gold spheres on the bacterial surface also suggest that MAb 2G4 binds to the O antigen because LPS can account for up to 70% of the surface of the outer monolayer (44). To further characterize the O-specific polysaccharide side chain specificity of MAb 2G4, a *wzx* gene (O-antigen flippase/translocase) deletion mutant (45) available from a *C. turicensis* 3032 mutant collection (Institute for Food Safety and Hygiene) was tested by immunostaining. The deletion of the *wzx* gene completely abolished the binding of MAb 2G4 to *C. turicensis* 3032 (data not shown). Altogether, these results prove that the lipopolysaccharide of *C. turicensis* acts as the antigen and that the O-specific polysaccharide chain of the O1 serotype represents the epitope specifically recognized by MAb 2G4.

Impact of MAb 2G4 on *C. turicensis* 3032 growth. The effect of MAb 2G4 on the growth of bacteria was determined in RPMI 1640 without FCS. Before addition of MAb 2G4, cells were grown in LB until the OD₆₀₀ was 0.45 (logarithmic growth phase), harvested, and set to different cell densities (OD₆₀₀s, 0.05 and 0.45) in RPMI 1640 (time zero). Different antibody concentrations were added to the bacteria, and the OD₆₀₀ and the numbers of CFU were monitored for 2 h at 20-min and 30-min intervals, respectively. As shown in Fig. 4, bacterial growth was reduced in an antibody concentration-dependent manner, independently of the density of the bacterial culture. However, the inhibitory effect of MAb 2G4 at 10 and 20 µg/ml after it was added at the low cell count (OD₆₀₀, 0.05; Fig. 4A) was more pronounced than that after it was added at the higher cell count (OD₆₀₀, 0.45; Fig. 4B). Addition of the control antibody, HT-2, did not influence bacterial growth. Moreover, comparison of the results with those obtained after the addition of ampicillin and chloramphenicol during growth demonstrated that MAb 2G4 shows no bactericidal effect but seems to have an inhibitory activity causing the reduced growth of *C. turicensis* 3032. To exclude the possibility that an

artifact of antibody-mediated agglutination can indirectly impact the light transmission and antibody-induced cell death, the numbers of CFU were determined at the time points indicated in Fig. 4C. At all MAb 2G4 concentrations used during the course of the experiment (2 h), an increase in the numbers of CFU in comparison to the numbers observed for both antibiotic-treated controls was observed. With respect to untreated *C. turicensis* 3032 cells at the end time point of 120 min, the number of CFU of cells treated with 1 µg MAb 2G4 was reduced by 1.5-fold, the number of CFU of cells treated with 10 µg MAb 2G4 was reduced by 3.1-fold, and the number of CFU of cells treated with 20 µg MAb 2G4 was reduced by 3.5-fold. In contrast, the difference in the numbers of CFU between chloramphenicol-treated and untreated *C. turicensis* 3032 cells was much higher (19.4-fold); i.e., even at the highest MAb 2G4 concentration of 20 µg/ml, full bacteriostasis was not reached. These results show that MAb 2G4 reduces the cell growth of bacteria by binding to the cell surface and exhibits antibacterial activity but is not bactericidal or bacteriostatic at the concentrations tested.

MAb 2G4 inhibits the motility of *C. turicensis* 3032 cells independently of agglutination. The results described above suggested that MAb 2G4 is able to inhibit *C. turicensis* 3032 growth, and the decrease of the optical density is not based on clumping of bacterial cells. Typically, antibodies binding to the O antigen are highly agglutinating. Agglutination is caused by the binding of bivalent molecules, such as IgG, to the surface of two bacterial cells, leading to the formation of a three-dimensional complex consisting of the antibodies and bacterial cells. To examine the agglutinating potential of MAb 2G4, the bacterial cells were incubated with different concentrations of MAb 2G4 or with control antibody HT-2 for 10 min and for 1 h and assessed by light microscopy. Untreated *C. turicensis* 3032 cells and cells incubated with the control antibody demonstrated normal swimming behavior. In contrast, the addition of MAb 2G4 (1 to 20 µg/ml) inhibited the swimming of *C. turicensis* 3032 cells in a concentration-dependent manner (Table 2; only data for MAb 2G4 concentrations which were used in further experiments are shown). Con-

TABLE 2 Motility inhibition and agglutination by MAb 2G4^d

MAb concn (μg/ml)	Motility inhibition			Agglutination ^b		
	IgG	IgG + secondary Ab ^a	Fab	IgG	IgG + LPS	Fab + LPS
1	+	++	+	—	—	—
2	++	++	+	—	+	—
5	++	+++	++	—	++	—
10	+++	+++	++	—	+++	—
20	+++	+++	++	(+)	+++ ^c	—

^a The secondary antibody (Ab) was goat anti-mouse IgG (H+L).

^b Positive results were observed within 20 to 30 s only when 10 μl LPS prepared from *C. turicensis* 3032 was added after the incubation of MAb 2G4 with bacterial cells.

^c Visible to the naked eye as granular clumping.

^d Agglutination of whole IgG molecule and Fab fragments. The incubation time was 1 h. The incubation of *C. turicensis* 3032 with MAb 2G4 for 10 min showed the same results for the inhibition of motility; motility was only slightly decreased. —, no motility or agglutination; (+), <10% of total bacterial cells were agglutinated; +, <25% of total bacterial cells were immotile or agglutinated; ++, ~50% of total bacterial cells were immotile or agglutinated; +++, >90% of total bacterial cells were immotile or agglutinated.

concentrations of MAb 2G4 of >5 μg/ml were sufficient to completely arrest the motility of *C. turicensis* 3032. Moreover, when the bacterial cells from an overnight culture (stationary growth phase) were directly used for the agglutination tests, the inhibitory effect was observed even at lower concentrations. Here, the minimum concentration of MAb 2G4 required to inhibit motility was as low as 3 to 4 μg/ml. This enhanced inhibition of motility can be explained by the different surface compositions of the bacterial cells during these two growth phases (logarithmic and stationary phases): in the stationary growth phase the cell surface contains more LPS, which is increasingly formed during the transition from logarithmic into stationary growth phase, and therefore, the cells present more O antigen for MAb 2G4. Agglutination could not be observed in any of the experiments, except for those with the 20-μg/ml concentration, where the sporadic aggregation of a few bacteria occurred.

To exclude the possibility that motility inhibition was due to cross-linking of LPS molecules, the experiments were repeated using monovalent Fab fragments of MAb 2G4 (Table 2). Up to a concentration of 5 μg/ml of Fab fragments, the level of motility inhibition was comparable to that achieved with the whole IgG molecule; i.e., up to 50% of total bacterial cells were immotile. At concentrations of 10 μg/ml and 20 μg/ml, however, motility could not be reduced further by the Fab fragments.

Lastly, we investigated the binding of bivalent or monovalent MAb 2G4 to the O antigen by indirect agglutination using a mixed-antigen agglutination assay. In this assay, the free antigen, i.e., isolated LPS of *C. turicensis* 3032, was added to MAb 2G4-treated bacterial cells after 1 h of incubation to saturate the second valence of the IgG molecule. A positive reaction (agglutination) was observed within 20 to 30 s (Table 2) and was visible under the microscope as a three-dimensional network. At 20 μg/ml of MAb 2G4, the addition of free LPS even led to a visible white precipitate. These data strongly suggest that the bivalent MAb 2G4 mainly binds monovalently to the O antigen on the bacterial cell surface and that the remaining free binding sites of MAb 2G4 were able to react with the antigenic determinants of soluble LPS but were probably sterically hindered from binding to the O antigen on a second bacterial cell.

MAb 2G4 inhibits swimming of *C. turicensis* 3032 in agar motility assay. In addition, the swimming behavior of *C. turicensis* 3032 in the presence of different amounts of MAb 2G4 was subsequently validated and evaluated using a soft agar motility assay which shows the combination of the effects of flagellar motility, chemotaxis, and growth on the formation of a circular colony on agar plates. The motility was determined by measuring the diameter of the diffuse growth area (motility halo) at the site of inoculation every hour for up to 18 h. Two different motility phenotypes were observed (Fig. 5): on plates containing the control antibody (HT-2), *C. turicensis* 3032 showed a low-density motility zone substantially larger than that on plates containing different MAb 2G4 concentrations, on which the *C. turicensis* 3032 motility zones were smaller and of higher density. As shown in Fig. 5, exposure of *C. turicensis* 3032 to the control antibody had no measurable effect on bacterial motility, i.e., the bacterial cells moved fast (0.54 cm/h in logarithmic growth phase) and formed a motility zone of ~3.8 cm. In contrast, addition of MAb 2G4 (1 to 20 μg/ml) to the medium resulted in a significant (48 to 95%) reduction of bacterial motility, depending on the MAb 2G4 concentration (Fig. 5A). At concentrations of 10 and 20 μg/ml of MAb 2G4, *C. turicensis* 3032 was nonmotile and was able to grow only at the site of inoculation (Fig. 5B). The minimum concentration of MAb 2G4 required to reduce significantly the motility of *C. turicensis* 3032 in soft agar was 0.7 μg/ml.

In order to exclude the possibility of steric interference with flagellum rotation after binding of MAb 2G4 as the cause of motility inhibition, MAb 2G4 was pretreated with anti-mouse IgG secondary antibodies at a molar ratio of 1:1 in PBS for 15 min at

FIG 5 MAb 2G4 inhibits the motility of *C. turicensis* 3032 cells. (A) Bacterial motility was assayed using 0.3% (wt/vol) soft agar plates to which different concentrations of MAb 2G4 were added (white bars) or 10 μg/ml of control HT-2 antibody was added (black bar). Bars represent the means and standard deviations of quadruplicate measurements of the motility zone diameter after 9 h. The results are representative of those from three independent experiments. *, significant reduction in motility in comparison to that achieved with HT-2 ($P < 0.0001$, determined by Student's t test). (B) Increase of motility zone (in cm/h) during logarithmic growth phase according to the concentration of MAb 2G4.

room temperature and then used in the motility assay. The increase in antibody size by binding of a secondary antibody did not influence the inhibitory effect (data not shown). The motility assay was also carried out with Fab fragments of MAb 2G4. At a concentration of Fab fragments of up to 2 $\mu\text{g/ml}$ during the treatment of *C. turicensis* 3032 cells, there was no significant inhibition of bacterial motility. At concentrations of Fab fragments above 5 $\mu\text{g/ml}$, the motility in soft agar was significantly reduced by more than 50%. Hence, these and former studies (25) suggest that it is highly unlikely that MAb 2G4, when bound to LPS on the cell surface of *C. turicensis* 3032, sterically interferes with flagellum rotation but shows an indirect inhibitory effect on flagellum-based motility.

MAb 2G4 reduces the membrane potential of *C. turicensis* 3032. Flagellum-based motility is an energy-dependent process that needs the bacterial proton motive force (PMF), which is a membrane-localized electrochemical gradient in Gram-negative bacteria consisting of the electrical membrane potential ($\Delta\Psi$) and the proton gradient (ΔpH), to function (46). To analyze the influence of MAb 2G4 on $\Delta\Psi$, bacterial cells were loaded with JC-1, a lipophilic fluorescent cationic dye that is sensitive to the membrane potential (40). The fluorescent emission of JC-1 shifts reversibly from red (590 nm) to green (530 nm) with decreasing $\Delta\Psi$ when it is excited at 488 nm, and the green fluorescence/red fluorescence ratio provides an estimate of $\Delta\Psi$. The $\Delta\Psi$ measurement was performed using flow cytometry. The *S. Typhimurium* ST4/74 ΔrpoE mutant strain served as a positive control. In *Salmonella*, the alternative sigma factor σ^E , encoded by the *rpoE* gene, is required for PMF maintenance (40, 47). The deletion of the *rpoE* gene resulted in membrane depolarization, represented by a substantial increase in the green fluorescence/red fluorescence ratio in comparison to that for the *Salmonella* wild-type strain, as described by Becker et al. (40). To assess the effect of MAb 2G4 on the membrane potential of *C. turicensis* 3032, cells were exposed to MAb 2G4 (5 $\mu\text{g/ml}$) or PBS for 20 min. The treatment of *C. turicensis* 3032 with MAb 2G4 resulted in a shift of the fluorescence comparable to that for the positive control, indicating membrane depolarization upon addition of the antibody (Fig. 6A). A 3.84-fold increase in the green fluorescence/red fluorescence intensity ratio was observed in MAb 2G4-treated *C. turicensis* 3032 cells (Fig. 6B). These findings indicate that the binding of MAb 2G4 to the surface of *C. turicensis* 3032 cells leads to a reduction of the PMF, and this possibly leads to the loss of flagellum-based motility.

MAb 2G4 inhibits entry of *C. turicensis* 3032 into epithelial cells. It has been shown for *Enterobacteriaceae* that motility is required for bacteria to invade epithelial cells *in vitro* (48–50). Therefore, MAb 2G4's effect on *C. turicensis* 3032's flagellum-based motility might also influence adhesion and invasion capacities. Our adhesion experiments showed that with an MOI of 100, $7.10 \times 10^5 \pm 3.47 \times 10^4$ CFU/ml (for unpolarized Caco-2 cells) and $5.97 \times 10^5 \pm 3.02 \times 10^4$ CFU/ml (for polarized Caco-2 cells) of *C. turicensis* 3032 were recovered after 30 min of infection. Hence, the level of adhered bacteria at this time point corresponded to 1.16% of the initial inoculum of *C. turicensis* 3032 for both differentiation stages of epithelial cells. At the 2.5-h time point after infection, which corresponds to bacterial invasion, $2.98 \times 10^4 \pm 4.57 \times 10^3$ CFU/ml (for unpolarized Caco-2 cells) and $2.95 \times 10^4 \pm 9.41 \times 10^3$ CFU/ml (for polarized Caco-2 cells) of *C. turicensis* 3032 were recovered. Consequently, the levels of

FIG 6 Impact of MAb 2G4 on the membrane potential of *C. turicensis* 3032. (A) Fluorescence profile of PBS-treated (negative control, white area) and MAb 2G4-treated (gray area) *C. turicensis* 3032 cells. (B) Measurement of the membrane potential as the ratio of JC-1 green fluorescence (depleted potential) to red fluorescence (intact potential). *C. turicensis* 3032, an *S. Typhimurium* wild-type strain, and an *S. Typhimurium* ΔrpoE mutant strain in PBS served as controls. The data are representative of those from three independent experiments. Significant differences were determined by comparison to the results for the *Salmonella* wild-type or PBS-treated *C. turicensis* 3032 strain and were determined by Student's *t* test. Significant differences are indicated: *, $P = 0.0032$; **, $P < 0.0001$. Ctu, *C. turicensis* 3032; Stm, *Salmonella enterica* serovar Typhimurium ST4/74.

recovery of *C. turicensis* 3032 cells invading epithelial cells were 0.06% for unpolarized epithelial cells and 0.08% for polarized epithelial cells. We compared the ability of *C. turicensis* 3032 to adhere to Caco-2 cells without MAb 2G4 and with different MAb 2G4 concentrations (Fig. 7A). In comparison with the results for untreated *C. turicensis* 3032 cells (for which the level of adherence was set to 100%), approximately 84% of *C. turicensis* 3032 cells treated with MAb 2G4 at concentrations of 1 $\mu\text{g/ml}$ ($3.42 \times 10^5 \pm 4.08 \times 10^4$ CFU/ml) and 10 $\mu\text{g/ml}$ ($3.75 \times 10^5 \pm 5.18 \times 10^3$ CFU/ml), 67% of *C. turicensis* 3032 cells treated with MAb 2G4 at a concentration of 20 $\mu\text{g/ml}$ MAb 2G4 ($3.44 \times 10^5 \pm 2.71 \times 10^4$ CFU/ml), and 81% of *C. turicensis* 3032 cells treated with MAb HT-2 at a concentration of 10 $\mu\text{g/ml}$ ($5.22 \times 10^5 \pm 2.47 \times 10^4$ CFU/ml) were able to adhere to polarized Caco-2 cells. There were no significant differences between the adhesion efficiency obtained with 1 to 10 μg MAb 2G4 and that obtained with the control antibody, whereas the adhesion efficiency of MAb 2G4 at 20 $\mu\text{g/ml}$ showed a minor decrease compared with that seen with control MAb HT-2. In adhesion to unpolarized Caco-2 cells, no significant differences between MAb 2G4-treated *C. turicensis* 3032 cells ($4.46 \times 10^5 \pm 3.21 \times 10^4$ to $5.52 \times 10^5 \pm 1.86 \times 10^4$ CFU/ml) and the HT-2 control ($6.38 \times 10^5 \pm 1.26 \times 10^4$ CFU/ml) were observed; however, we recovered slightly higher numbers of bacteria from nonpolarized Caco-2 cells. Thus, MAb 2G4-treated *C. turicensis* 3032 cells, which showed no flagellar motility, were able to attach to epithelial cells, and this ability was not significantly different from that of control bacteria, nor was it dependent on the differentiation stage of the epithelial cells (Fig. 7A).

The capacity of MAb 2G4-treated *C. turicensis* 3032 to invade Caco-2 cells was tested next, and in contrast to the findings of the adhesion experiments, bacterial invasion was significantly reduced in an antibody concentration-dependent manner (Fig. 7B). Compared with the results for untreated *C. turicensis* 3032 cells, with treatment with MAb 2G4 at a concentration of 10 $\mu\text{g/ml}$, bacterial invasion was reduced by approximately 70% in both differentiation stages of Caco-2 cells (for 1-day-old epithelial cells,

FIG 7 MAb 2G4 inhibits invasion of Caco-2 cells by *C. turicensis* 3032. Cell monolayers that were either undifferentiated (1 day old) or polarized (16 days old) were infected with *C. turicensis* 3032 at an MOI of 100 mixed with different concentrations of MAb 2G4. MAb HT-2 served as a negative control. (A) At 30 min after infection, Caco-2 cells were washed three times with PBS and lysed to obtain the number of cell-associated bacteria. (B) To test the invasion capacity of *C. turicensis* 3032, a gentamicin protection assay was performed, and the normalized percentages of CFU were determined by comparison with the counts for the control (to which MAb 2G4 was not added). The results are representative of those from three independent experiments performed in triplicate. Significant differences were determined by comparison to the results for negative control MAb HT-2 and were determined by Student's *t* test. *, $P = 0.0018$ to 0.0164 ; **, $P = 0.0002$.

$1.23 \times 10^4 \pm 2.34 \times 10^3$ CFU/ml; for polarized epithelial cells, $1.18 \times 10^4 \pm 5.99 \times 10^3$ CFU/ml). At a concentration of $20 \mu\text{g/ml}$ MAb 2G4, bacterial invasion was reduced by 70% ($8.83 \times 10^3 \pm 2.98 \times 10^3$ CFU/ml) in 1-day-old Caco-2 cells and by up to 95% ($1.37 \times 10^3 \pm 8.06 \times 10^2$ CFU/ml) in polarized Caco-2 cells. These observations indicate that the developed antibody blocks bacterial entry into epithelial cells but not cell adherence.

To examine the bacterial cells of *C. turicensis* 3032 in more detail, we determined by electron transmission microscopy whether or not the bacteria were flagellated after treatment with MAb 2G4 (Fig. 8). The TEM images revealed that the morphology and flagella of *C. turicensis* 3032 cells were unaltered after 2.5 h of incubation with PBS, control antibody HT-2, or MAb 2G4 (Fig. 8A to C), and structurally intact flagellar filaments were observed on the surface of the bacterial cells. The new finding is that the monovalent binding of IgG to the bacterial cell wall is sufficient to inhibit invasion into epithelial cells.

DISCUSSION

The pathogenesis of *Cronobacter* spp. is still poorly understood, and the identification and characterization of the major virulence factors of clinical isolates remain challenging tasks. *C. turicensis* 3032, a strain responsible for lethal infections in neonates, has been well characterized genetically (35) as well as by proteomics (23). Therefore, *C. turicensis* 3032 served as a model strain for the generation of antibodies as tools for studies on the virulence factors necessary for adhesion to and invasion of Caco-2 cells. MAb 2G4 selectively recognized the *C. turicensis* 3032 and E688 strains (Fig. 2B and Table 1) and showed no cross-reactivity with other *Cronobacter* spp. or several other members of the *Enterobacteriaceae*. Both the *C. turicensis* 3032 and E688 strains belong to the O1 serotype, and we identified the O-specific polysaccharide chain of LPS to be the epitope that is specifically recognized by this antibody. To our knowledge, this is the first MAb able to react with untreated live cells of *Cronobacter*. MAb 2G4 was able to bind to bacterial cells in liquid culture as well as inside eukaryotic cells and inhibited bacterial invasion into Caco-2 cells. In addition, the high affinity to live and heat-treated bacteria will enable the development of immunochemical methods for the simple and rapid de-

tection of the pathogenic *C. turicensis* 3032 strain and other *C. turicensis* O1-serotype strains in food, clinical, and environmental samples.

During the characterization of MAb 2G4, we found that the antibody inhibited the growth of *C. turicensis* 3032 in a nonbactericidal manner without inducing bacterial agglutination. In particular, the mixed-antigen agglutination assay demonstrated that the antibodies bind only with one valence and that the second valence is free but cannot react with the O1 antigen of another bacterial cell. These findings indicate that the antigen is not lo-

FIG 8 Transmission electron micrographs of *C. turicensis* 3032. Bacterial cells and surface flagella were negatively stained with uranyl acetate after 2.5 h of pretreatment with PBS (A), $10 \mu\text{g/ml}$ control antibody HT-2 (B), or $20 \mu\text{g/ml}$ MAb 2G4 (C). (D) A higher-magnification view of the flagellar origin (arrows).

cated on the immediate surface but might be located in a less accessible, deeper structure or between prominent structures of the bacterial envelope.

Studies on the adhesion of *C. turicensis* to monolayers of Caco-2 cells revealed that attachment was not reduced in the presence of MAb 2G4. This result indicated that the LPS of *C. turicensis* 3032 is not substantially involved in binding to epithelial cells. Interestingly, after antibody treatment, *C. turicensis* 3032 cells were unable to invade epithelial cells *in vitro*, and this protective effect was clearly dependent on the antibody concentration and on the differentiation stage of Caco-2 cells. At a concentration of 20 $\mu\text{g/ml}$ MAb 2G4, *C. turicensis* 3032 was almost unable to invade polarized Caco-2 cells. The polarized epithelial cells resemble the epithelial cell layer of the intestinal tract of the host, and it has already been reported that dimeric monoclonal IgA antibody specific for LPS protects mice against oral challenge with a lethal dose of a virulent *S. Typhimurium* strain and *Shigella flexneri* (51–53). We tested if this could also apply for a monoclonal IgG antibody, such as MAb 2G4.

Since the IgG antibody did not influence adhesion and could not induce agglutination, we assumed a more indirect effect of the antibody on *Cronobacter* as the cause for the invasion defect. Interestingly, we found that exposure of *C. turicensis* 3032 to MAb 2G4 resulted in the loss of flagellum-based motility. Flagellar motility enables pathogens to colonize the epithelium of specific host organs and is therefore considered a major virulence factor (54). It has been previously reported for *S. Typhimurium* that flagella are necessary for full virulence (55). Also, aflagellate mutants from the *C. sakazakii* ES5 strain were not able to invade Caco-2 cells (K. Schauer, unpublished data). Together, these findings indicate that flagellum-based motility may play an important role during *Cronobacter* entry, especially into polarized epithelial cells. Inhibition of flagellar motility by LPS-specific antibodies has been described for only a few bacteria, such as *Salmonella* Typhimurium (25). In this study, IgA dimers and Fab fragments of the antibodies showed similar effects on motility, leading the authors to conclude that the antibody did not sterically hinder flagellum rotation. In our study, we could also show that it is unlikely that the binding of MAb 2G4 to the O1-specific chain of the LPS sterically interferes with flagellum rotation, because increasing the size of MAb 2G4 by a secondary antibody did not further decrease motility and reducing the size of MAb 2G4 through the creation of Fab fragments did not restore the motility of the negative control. As another possibility, weak cross-linking of the O1 antigen could induce envelope stress of the bacterial cells, which in turn could trigger signal transduction and physiochemical pathways which could reduce the proton motive force (PMF) (26, 53). We propose that the PMF, which plays a key role in bacterial motility, affects invasion into host cells. The PMF consists of two components, the electrical membrane potential ($\Delta\Psi$) and the proton gradient (ΔpH), both of which energize the rotation of the flagellum (46). The impact of MAb 2G4 on ΔpH results in the arrest of flagellum-based motility, because flagellar motor speed varies linearly with the PMF gradient (56). Also, the MAb 2G4-treated bacteria were affected in $\Delta\Psi$, as shown by flow cytometry with JC-1-loaded *C. turicensis* 3032 cells. However, the effect of MAb 2G4 on ΔpH and $\Delta\Psi$ did not lead to the loss of or obvious structural changes to the flagella. These results resemble the effects of a monoclonal IgA antibody on *S. Typhimurium* and *Shigella flexneri* observed by Forbes et al. (26, 53).

Interestingly, as a consequence of perturbation of the membrane integrity after antibody treatment, Forbes et al. (26) and other investigators (57, 58) also found a decrease of the intracellular ATP levels and a loss of functionality of the *Salmonella* pathogenicity island 1 (SPI-1)-encoded type 3 secretion system (T3SS) that translocates virulence-associated proteins into host cells. The suppression of T3SS by a murine monoclonal IgA antibody and loss of the ability to enter intestinal epithelial cells were also demonstrated for *Shigella flexneri* (53). It is tempting to presume that MAb 2G4 could similarly interfere with ATP-dependent secretion systems, like the general secretion system (Sec), T4SS, T6SS, and the flagellar secretion system, which were found in *C. turicensis* 3032 (23). The flagellar secretion system is structurally related to T3SS, and virulence factors may be secreted into the extracellular medium or exposed on the bacterial cell surface, where they might influence the invasion of human intestinal cells (59, 60). The protein CiaI of *Campylobacter jejuni*, for example, is involved in mediating cellular trafficking and plays a role in bacterial survival within cells (61). It was also reported that secretion of virulence proteins from *C. jejuni* is dependent on a functional flagellar export apparatus (62). Thus, one might speculate that a loss of functionality of the flagellar secretion system in *Cronobacter* may concomitantly lead to the inability to secrete virulence factors potentially influencing invasion and/or survival in host cells. However, the presence and/or function of such potential effector proteins remain to be elucidated.

The reduction in total ATP levels was demonstrated indirectly by growth experiments in the presence of MAb 2G4. Bacterial growth was retarded in a concentration-dependent manner, but it did not arrest completely, as would have been the case for bactericidal or static substances. In general, bacteria have different mechanisms of assessing their energy status (63), as shown, for example, for the F_1F_0 ATPase of *Streptococcus bovis* (64). The ATPase activity was regulated either directly or indirectly by the PMF (65). When bacteria are limited for energy, the free energy change of catabolic reactions is generally tightly coupled to the anabolic steps of cellular biosynthesis, and total energy flux can be divided into growth and maintenance functions (63). This can be observed in a resting state, when cells utilize energy sources in the absence of growth, or, as in our case, during retarded growth. Due to MAb 2G4-induced envelope stress, the cells presumably use most of the intracellular ATP to maintain cellular integrity, as shown for *E. coli*, which uses more than half of its energy to sustain the $\Delta\Psi$ (66). Furthermore, it was shown for *S. Typhimurium* that in IgA-treated bacteria, ATP levels rapidly decreased as a result of the reduction of the PMF. However, the PMF was restored within 30 to 40 min after using 5 $\mu\text{g/ml}$ of IgA (26). The distribution of total ATP into growth and maintenance functions as well as the prolonged regeneration time of the cellular ATP level after treatment with MAb 2G4 could be the reason for the reduced bacterial growth. However, much higher antibody concentrations (above 20 $\mu\text{g/ml}$) are probably necessary to achieve an antimicrobial activity comparable to the static or bactericidal effect of antibiotics. This was recently shown for an MAb against the LPS of *Burkholderia pseudomallei*, for which the minimum concentrations of antibody required to reach bacteriostatic and bactericidal effects were 62.5 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$, respectively (67). The concentration differences appear to depend on the MAb target and immunoglobulin isotype.

In summary, we were able to develop a monoclonal IgG anti-

body that specifically recognizes the O1 antigen from *C. turicensis* 3032 and is therefore able to detect live bacterial cells and to differentiate *Cronobacter* serotypes. Additionally, we have demonstrated that MAb 2G4 is a potent inhibitor of *C. turicensis* 3032 flagellum-based motility leading to a significantly reduced invasion of Caco-2 cells. Furthermore, this study underscores the model of monoclonal IgA antibodies acting on the bacterial cell envelope recently proposed by Forbes et al. (26, 53) and also demonstrates that monovalent binding of IgG to the O antigen of this Gram-negative pathogen causes an antibacterial effect.

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