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Defects in Gliding Motility in Mutants of *Cytophaga johnsonae* Lacking a High-Molecular-Weight Cell Surface Polysaccharide

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We previously observed (W. Godchaux, L. Gorski, and E. R. Leadbetter, J. Bacteriol. 172:1250–1255, 1990) that two mutants (strains 21 and NS-1) of the gliding bacterium *Cytophaga johnsonae* that were totally deficient in motility-dependent colony spreading, movement of rafts (groups) of cells as observed with a microscope, and movement of polystyrene-latex spheres that attached to the cell surface (observed in wet mounts) were also deficient in a high-molecular-weight cell surface polysaccharide (HMPS) and suggested a role for that substance in gliding motility. Antisera have been prepared against the purified HMPS, and these were used to select mutants specifically and highly deficient in the polysaccharide. All five such mutants had rates of colony spreading and raft movement that were much lower than those of the parent strain, but the rate of increase in colony diameter was higher than that found for strains NS-1 and 21 (which do not undergo raft movement at all). Unlike these latter two strains, the HMPS mutants retained the ability to move polystyrene-latex spheres over their surfaces. Hence, HMPS deficiency results in defective motility but not nonmotility, and the HMPS deficiency cannot fully explain the phenotype of mutants 21 and NS-1; in these strains, gliding must be affected by additional biochemical lesions. The HMPS may, nonetheless, be advantageous in that it supports greater gliding speeds.

Bacterial gliding motility, the ability to move over solid surfaces but not to swim through liquids, is commonly assessed by several means. Colonies of motile cells on solid media spread (increase in diameter) much faster than can be accounted for by growth alone; among the simple gliding bacteria, colony morphology is distinct, as the colonies are thin and veil-like and not compact and convex as are those of nonmotile strains. When a drop of a suspension of motile cells is deposited on nonnutrient agar (nutrients inhibit motility), rafts, groups of cells gliding together, can be observed (by phase-contrast microscopy) to form and move away from the originally smooth edge of the zone of deposited cells; their speed can be measured easily. A possibly related phenomenon is that many cytophagas (13, 17) bind polystyrene-latex spheres to their surfaces, after which the spheres can be observed (in wet mounts) to move over the cell surface.

In our continuing investigation of the mechanism of gliding motility in *Cytophaga johnsonae*, we reported earlier (9) that a sulfonolipid-deficient mutant (strain 21) that exhibited no colony spreading, no raft formation or movement, and no sphere movement was deficient in a high-molecular-weight cell surface polysaccharide (HMPS). Restoration of the sulfonolipid content of this strain by provision of a specific sulfonolipid precursor led also to restoration of the HMPS and also of colony spreading, raft movement, and sphere movement. Clearly, the lesion in HMPS is secondary to that in sulfonolipid, and we considered the hypothesis that the HMPS is the component required for motility and that the lipids are required for its synthesis or emplacement in the outer membrane. This view was reinforced by two additional observations. A second mutant totally deficient in spreading and raft and sphere movement, strain NS-1, was also deficient in HMPS (though normal in sulfonolipid content). Fink and Zissler (7) screened a large number of transposon-induced mutants of *Myxococcus xanthus* and found five that were unable to bind a monoclonal antibody to the O antigen of the lipopolysaccharide of that species. All five were deficient in adventurous gliding (movement by isolated cells) but exhibited normal social gliding (rafts of cells moving together).

The mutants of *C. johnsonae* mentioned above were selected as nongliders; one of them (strain 21) exhibits obvious pleiotropy, and the other might have lesions other than its HMPS deficiency as well. Clearly, in order to define the effect of HMPS deficiency on gliding, it was necessary to select mutants solely on the basis of that deficiency and then to examine their motility. This has been done by using antisera to the purified HMPS, and we report here that the resulting severely HMPS-deficient strains are defective in colony spreading and raft movement, both being about threefold slower than those in the parent strain, but are not totally devoid of these activities. The mutant cells moved polystyrene-latex spheres attached to their surfaces with approximately the same speed and frequency as the parent strain.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were derived from *C. johnsonae* ATCC 43786. Strain SR-1 is a spontaneous streptomycin-resistant mutant derived from the original strain by selection on medium containing the antibiotic. Strain NS-1, selected as a nonmotile mutant but also polysaccharide deficient, has been described previously (9).

Cultivation of bacteria. The liquid medium used has been described previously (11); LTY medium contained 0.1% yeast extract, 0.1% Bacto-tryptone and 1.8% Bacto-agar; the glucose minimal medium contained 40 mM glucose, 10 mM KNO_3, 6 mM K_2HPO_4, 0.5 mM Na_2SO_4, a mineral base (6), and 1.8% Bacto-agar. All media for the cultivation of SR-1

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and its derivatives contained streptomycin sulfate (50 μg/ml, filter sterilized). Incubation was done at 25°C; liquid cultures were grown in a rotary shaker.

Purification of polysaccharide. Previously (9), we had purified the HMPS and the low-molecular-weight lipopolysaccharidelike polysaccharide (LMPS) from a membrane preparation; for preparation of bulk quantities, we have used a Westphal-like whole-cell extraction (12), modified in that wet, packed cells of *C. johnsonae* ATCC 43786 (25 g, grown in the liquid medium) were extracted with 65 ml of water and 80 ml of 90% phenol. The phenol phase from the first extraction was reextracted with 60 ml of phenol-saturated water (at room temperature), and the two aqueous phases were combined. To these were added 1/10 their combined volume of 4 M sodium acetate and three times their combined volume of 95% ethanol; the mixture was stored at −20°C overnight, and the precipitate was collected by centrifugation. The precipitate was redissolved in 30 ml of water and reprecipitated as described above. The precipitate was then dissolved in 30 ml of water, mixed with 3.3 ml of 1 M Tris HCl buffer (pH 7.8), 34 μmol of $MgCl_2$, and 2 mg of DNase I, and incubated at 25°C for 15 min; 60 μmol of NaEDTA (pH 7) and 2 mg of RNase A were added, and the incubation was repeated. The preparation (35 ml) was then reextracted (at room temperature) with an equal volume of water-saturated phenol; the phenol phase was reextracted with 35 ml of phenol-saturated water, and the polysaccharide from the combined aqueous phases was precipitated twice as described above. The final precipitate (crude polysaccharide) was dissolved in 10 ml of water.

The HMPS and LMPS were separated and further purified by gel filtration on Sephadex G-200 as described previously (9) except that the procedure was scaled up to employ a 2.5 cm (diameter)-by-90 cm column loaded with the crude polysaccharide from 25 g of cells (wet weight) in 15 ml, and the elution buffer was changed to that used by Rivera et al. (containing deoxycholate) (19). Elution profiles were similar to those previously reported (9) except that the use of deoxycholate (instead of the sodium dodecyl sulfate [SDS] previously employed) resulted in baseline separations between the HMPS and the LMPS. They were precipitated from their respective pooled fractions, redissolved, and reprecipitated as described above; the final precipitates were washed with ethanol, ethanol-diethyl ether (1:1), and ether (twice) and then air dried. A typical yield of HMPS was 250 mg (dry weight) from 25 g of cells (wet weight).

Preparation of antibodies to HMPS. Three New Zealand White outbred female rabbits, 6 to 8 lb (ca. 2.7 to 3.6 kg), were injected subcutaneously at four sites (over each shoulder blade and haunch) with 0.2 ml (per site) of an emulsion of equal volumes of HMPS (2 mg/ml in 0.9% NaCl) and Freund's complete adjuvant. Emulsification was accomplished by repeated passage of the mixture between two syringes connected by 18-gauge (inside diameter) tubing. After 2 weeks and again at 5 weeks following the initial immunization, each rabbit was reimmunized with 0.2 ml of aqueous antigen without adjuvant; at 8 weeks, each was reimmunized intraperitoneally with 100 μg of HMPS in 1 ml of the saline. Blood was collected from the central artery of the ear; sera obtained during the 5 to 10 weeks after the initial immunization were all equally active. The ability of the sera to agglutinate cells of strain SR-1 that had been suspended in 10 mM Tris HCl–150 mM NaCl (TBS) to an A_{650} of 0.8 to 1.2 was determined by direct examination with a phase-contrast microscope.

Selection of HMPS-deficient mutants. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was added (0.3 mg/ml) to exponential-phase liquid cultures (A_{650} of 0.3 to 0.6) of strain SR-1, incubation was continued for 60 min, and then a portion of the culture was diluted 100-fold with fresh medium and allowed to grow to an A_{650} of about 0.6. (The MNNG treatment was omitted for the spontaneous mutant which we isolated.) Cells were collected by centrifugation and suspended to an A_{650} of 0.8 to 1.2 in TBS, and 1 ml of this suspension was mixed with 70 μl of undiluted serum. After incubation for 10 min at 25°C, the preparation was centrifuged for 2 min at half-maximum speed in a bench top clinical centrifuge (this procedure sedimented agglutinated cells but did not sediment cells that had not been mixed with antiserum), and the supernatant was transferred to 50 ml of fresh medium. This cycle of growth and enrichment for nonagglutinable cells was repeated four times, and the final supernatant was diluted and spread on LTY medium containing 20 mM glucose to inhibit gliding (20). Individual clones were picked, transferred to liquid medium, and grown to an A_{650} of about 0.6. Cells collected by centrifugation were suspended in TBS as described above; 0.1 ml of the suspension was mixed with 7 μl of the antiserum. After 10 min at 25°C, the preparation was mixed by being gently drawn in and out of a Pasteur pipet 10 times and then immediately examined under the microscope. Agglutinability was scored as (i) none at all, (ii) complete (more than 90% of the cells in aggregates), or (iii) partial (less than 90% agglutinated).

Chemical methods. Hydrolysis of crude extracts (described below) or of purified polysaccharides (2 mg) was carried out in 4 N HCl (0.5 ml) at 90°C for 12 h under N_2; the preparations were then dried under a stream of N_2. In one experiment, the sugars in the hydrolysates were reduced with $NaBH_4$ (14), and the hydrolysates were again dried. O trimethylsilylation was carried out as described by Chaplin (5); trimethylsilylation was always preceded by storage of the sample overnight in a vacuum desiccator over P_2O_5 and KOH. Combined gas chromatography-mass spectrometry (GC-MS; electron-impact ionization) was performed as previously described (1) except that the temperature was programmed to hold at 140°C for 2 min and then to rise to 250°C at 8°C/min. With the exception of one sugar (a deoxyhexosamine for which standards were not available), sugars were identified by the correspondence of their retention times and mass spectra to those of standards.

Determination of HMPS content. A rapid, microscale method was used to prepare crude extracts containing HMPS for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or by GC-MS of hydrolysates. A culture (40 ml at an A_{650} of about 0.6) was centrifuged, and the cells were suspended in 0.6 ml of water and placed in a microcentrifuge tube. After the addition of 70 μl of 20% (wt/vol) SDS, the mixture was sonicated until no longer viscous, mixed with 0.7 ml of water-saturated phenol, and allowed to stand at room temperature for 30 min with occasional vigorous mixing. The preparations were centrifuged, and the aqueous phases were removed and extracted four times with 2-ml portions of diethyl ether to remove phenol, after which the polysaccharide and nucleic acid were twice precipitated with sodium acetate and ethanol as described above. Each precipitate was dissolved in 0.15 ml of water, and the A_{260} of an appropriate dilution was determined. Readings at 280 and 310 nm indicated that there was no interference from residual phenol or protein or from light scattering; hence, the A_{260} represented cellular nucleic acid. The samples were then incubated with DNase I and RNase A (40 μg each; 10 min at

25°C) and prepared for SDS-PAGE, electrophoresed, and stained with the periodic acid-Schiff reagents, as previously described (9). The samples applied to the gels contained 3 A_{260} units of nucleic acid; since the nucleic acid content should not vary much among the strains, the samples represented equivalent amounts of cells.

For sugar analysis by GC-MS, the aforementioned procedure was scaled up fourfold, the nuclease treatment was omitted, a *m*-inositol internal standard was added in the amount of 1 $\mu g/A_{260}$ unit of nucleic acid, and the material was hydrolyzed. The dried hydrolysates were taken up in 1 ml of water, mixed with 0.4 ml of a 30% (vol/vol) suspension of Dowex-1 (×4, 200/400 mesh, chloride form) to remove organophosphate nucleic acid degradation products, and centrifuged. The supernatant was again taken to dryness, O trimethylsilylated, and analyzed by GC-MS.

Raft movement. Nonnutrient agar contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; sodium salt, pH 7.0), 1 mM $CaCl_2$, 1 mM $Mg(SO_4)_2$, 30 mM NaCl, and 1.8% Bacto-agar. Approximately 0.3 ml of the molten mixture was placed on a large coverslip and covered with a second coverslip supported by two pieces cut from a microscope slide, to generate a flat agar slab of optically correct thickness. After the agar solidified, one of the coverslips was removed. Cultures (A_{650} of about 0.6) were centrifuged, and the cells were suspended to an A_{650} of 1.5 in a solution containing the buffer and salts of nonnutrient agar; 2 μl of this mixture was deposited on the agar surface, and the preparation was observed under a phase-contrast microscope. Rafts could be observed to form and move soon after the liquid deposited had been absorbed by the agar gel; the rate of movement of their leading edges, over a 10-μm course, was measured with a calibrated eyepiece micrometer and a stopwatch.

Other methods. Sulfonolipid content (10) and polystyrene-latex sphere movement (9) were determined as previously described.

Materials. Freund's complete adjuvant, Dowex-1, sodium deoxycholate, MNNG, nucleases, and sugar standards were obtained from Sigma Chemical Co. (St. Louis, Mo.); Bacto-agar was obtained from Difco Laboratories (Detroit, Mich.). All other materials were obtained and/or prepared as previously described (1, 9).

RESULTS

Activity of the antisera. The antisera obtained from three different animals were all capable of agglutinating the parent strain (at an A_{650} of 1) almost quantitatively at a dilution of 1:20, with about 50% agglutination occurring at a dilution of 1:40 and little agglutination at 1:80; agglutination resulted in clumps containing hundreds of cells. The agglutination was blocked when the serum was mixed with an excess of purified HMPS prior to its addition to the cell suspension; normal rabbit serum did not cause agglutination. Two of the antisera did not agglutinate strain NS-1, known to be deficient in HMPS (9); the third did partially agglutinate that strain and was not used further.

Selection of HMPS-deficient mutants. After mutagenesis and outgrowth, viable cell counts of the initial cell suspension in TBS and the supernatant remaining after the first antiserum treatment and centrifugation indicated that over 90% of the cells were agglutinated; in contrast, after the fourth cycle of selection, these determinations indicated that fewer than 10% of the cells were agglutinated. Hence, selection for nonagglutinable cells clearly took place.

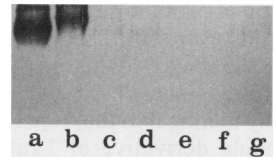

FIG. 1. SDS-PAGE of polysaccharide. Lanes: a, purified HMPS (30 μg); b through g, the crude extracts (samples representing equal amounts of cellular nucleic acid) from strains SR-1, NG-A, NG-B, NG-C, NG-D, and SP-A, respectively. Only the upper portion of the gel is shown; the stained band shown is the only one formed by the HMPS.

The nonagglutinated cells were then dilution plated on LTY medium containing 20 mM glucose (which inhibits gliding) (20); this motility-inhibiting medium was used to permit, as much as possible, the selection of clones without prejudice as to their motility and also to prevent any gliders present from overrunning and contaminating adjacent colonies. Despite these conditions, there was some size heterogeneity among the colonies. About 10% of the colonies fell into a class of uniform, larger size, which was about twice the diameter of the smallest colonies present (but still much smaller than those formed by parent strain cells in the absence of glucose); the remainder were of a distinctly smaller size. If the inhibition by glucose was less than complete, this variation in colony size could have reflected some differences in residual colony spreading. In order to avoid prejudice of the results and ensure selection solely on the basis of HMPS deficiency, we examined the agglutinability of cells grown from colonies of both types. Among 22 clones forming colonies of the larger size class (4 to 6 from each mutagenesis and selection procedure), 21 showed essentially complete agglutination by the anti-HMPS (parental phenotype), only one showed partial agglutination, and none were nonagglutinable. In contrast, among 48 clones forming distinctly smaller colonies (10 to 14 from each independent procedure), 9 showed complete agglutination, 18 showed partial agglutination, and 21 showed no agglutination at all (the behavior expected of HMPS-deficient mutants). Given that the larger colonies constituted only 10% of the total, then nearly 40% of the clones exhibited the mutant phenotype. One nonagglutinable clone from each of four independent mutagenesis procedures was selected for further study (designated NG-A, -B, -C, and -D).

In contrast to the results with MNNG, when the selection procedures were applied to nonmutagenized populations, by the fourth cycle about 90% of the cells were still being agglutinated. By examining many colonies obtained after this cycle, we were able to find one nonagglutinable clone in one such preparation, but found none in another. The one spontaneous mutant obtained is designated SP-A.

HMPS content of the mutants. SDS-PAGE of crude extracts from the parent strain, SR-1, (Fig. 1, lane b) revealed a heavily stained band corresponding to HMPS (lane a). In contrast, the mutants (Fig. 1, lanes c through g) did not contain detectable amounts of this material.

This result was confirmed by sugar analysis. The HMPS contains both galactosamine and a sugar that has been identified as a 2-amino-6-deoxyhexose (dhexN). The mass spectrum of the O-persilylated (but not reduced or N-derivatized) sugar obtained from acid hydrolysates is very similar (but not identical) to that of glucosamine and galactosamine; all feature an ion with an *m/z* of 131 $[H_2N\overset{+}{C}HCHOSi(CH_3)_3]\cdot$

that is characteristic of 2-amino sugars and contributes about 40% of the total ions (*m/z*, 50 to 550) in the spectrum. The mass spectrum of the dhexN, when it chromatographed as the reduced (alditol), N-acetylated and O-silylated derivative, was essentially identical to that reported previously (14) for the corresponding derivative of 2-fucosamine and featured ions with an *m/z* of 174 [$Si(CH_3)_3OCH_2\overset{+}{C}HNHCOCH_3$], 117 [$H_3\overset{+}{C}HOSi(CH_3)_3$], and 480 [$M^+$-$CH_3$], all consistent with a dhexN. Evidently, this sugar is peculiar to the HMPS, and we have made use of the abundant ion with an *m/z* of 131 to simplify the analysis of crude extracts by displaying and integrating the chromatograms representing this single ion.

As shown in Fig. 2, the dhexN that was found in the HMPS and detected as ions with an *m/z* of 131 at retention times of 6.96 and 7.35 min (Fig. 2a; the two peaks represent anomeric trimethylsilylglycosides) was also abundant in hydrolysates of the crude extracts from the parent strain (Fig. 2b) but, when comparable amounts of material were chromatographed, was virtually absent from mutant NG-B (Fig. 2c). Similar results were obtained for the other mutants. Though this cannot be seen in Fig. 2, when the chromatograms were examined on a smaller abundance scale, statistically significant numbers of ions with an *m/z* of 131 were found at the retention times characteristic of the dhexN. The results were quantified as follows. The internal standard, *m*-inositol, was added in proportion to the amount of nucleic acid in the crude extracts and thus represents the amount of cell mass equivalent to the material injected onto the column. The content of dhexN was taken to be the number of ions with an *m/z* of 131 in the chromatographic peaks at 6.96 and 7.35 min (from integration of data such as is shown in Fig. 2) divided by the total ions (*m/z*, 50 to 500) in the *m*-inositol peak from the same chromatogram. The values obtained for the HMPS mutants were expressed as a percentage of that obtained for the parent strain (which was the mean of three determinations that agreed to within 20%) and ranged from 0.11 to 0.61%. As further evidence of sensitivity and accuracy, when a preparation from strain NG-C was mixed with a sample from the parent strain in the ratio of 50:1, the value obtained in the analysis was 2.3%. The mutants, then, contained less than 1% as much HMPS as did the parent strain.

The HMPS also contained galactosamine (Fig. 2a), and this was similarly present in the crude extracts of the parent strain (Fig. 2b) and absent from the mutants (Fig. 2c); in contrast, glucosamine, which, as we describe below, is characteristic of the lipopolysaccharidelike (9) LMPS found in these organisms, was retained by the mutants (Fig. 2c).

Colony spreading. As shown in Fig. 3, mean colony diameters of the HMPS mutants at two different times of incubation were significantly less than that of the parent strain and never exceeded about 40% of the latter value. Nonetheless, diameters for the selected mutants were significantly greater than that for strain NS-1, which has a colony morphology typical of nonmotile bacteria (and also shows no raft formation, much less movement); values for this strain thus represent the background resulting from growth alone. If one were to subtract the values for NS-1 from all the others, the other mutants would then give values from 10 to 27% of that for the parent strain.

Colony morphology of the five HMPS mutants differed from that of the parent strain and that of NS-1. Some of the differences, which include greater irregularity of the edges of the HMPS mutant colonies as well as the difference in colony diameters, can be seen in Fig. 4. Additional features

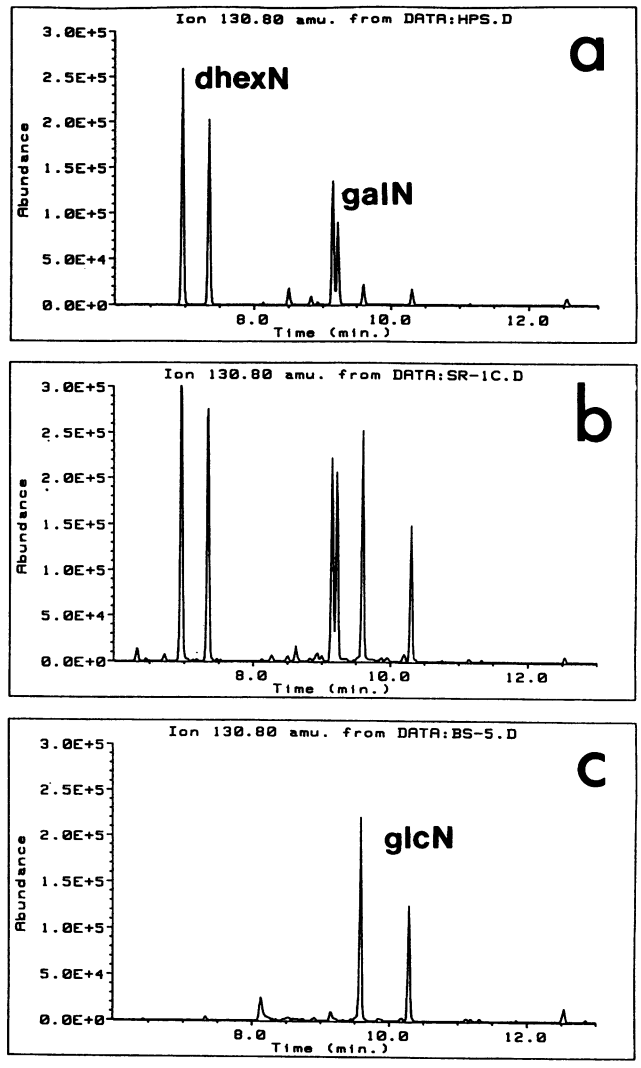

FIG. 2. Sugar analysis by GC-MS. The sugars, from acid hydrolysates, were in the hemiacetal ring forms, trimethylsilylated at all but the ring oxygen; amino groups were not derivatized. Though mass spectra (*m/z*, 50 to 500) were recorded, the figure displays only the abundance of the ion with an *m/z* of 131 that is characteristic of 2-amino sugars (as described in the text). (a) Purified HMPS; (b) the crude extract from strain SR-1; (c) the crude extract from strain NG-B. The *m*-inositol internal standard yields few ions of the mass displayed and appears as small peaks at 12.55 min. The total ion (*m/z*, 50 to 500) integrals for these peaks, from the same chromatograms shown here, were 2.3×10^7 for strain SR-1 (b) and 4.6×10^7 for strain NG-B (c). galN, galactosamine; glcN, glucosamine.

were apparent in unstained colonies viewed under oblique lighting. The colonies of SR-1 had a slightly thickened central area surrounded by a veil, an extremely thin film of cells. Those of the HMPS mutants were uniformly thick over their entire surfaces and had irregular surfaces. Those of NS-1 were smooth, domed, and much thicker at their centers than those of the other strains, an appearance typical of nonmotile bacteria.

The generation times of the mutants (which ranged from 86 to 103 min in liquid medium) did not differ significantly from that of the parent strain (95 min); hence the differences in colony size could not have resulted from growth defects.

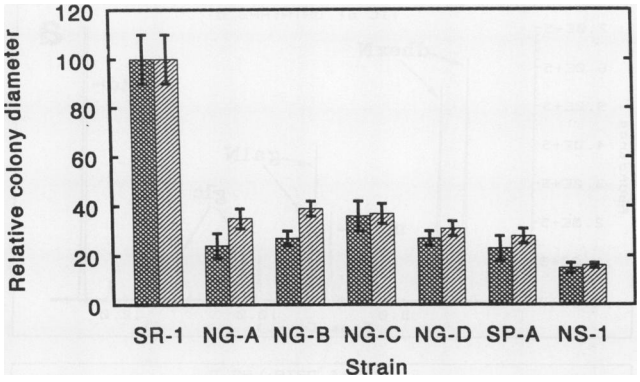

FIG. 3. Colony spreading in HMPS-deficient mutants. Cells were spread on LTY medium at appropriate dilutions, and the diameters of 20 colonies were measured after incubation for 43 to 46 h (▩) and 67 to 70 h (▨). For noncircular colonies, the average of the largest and smallest dimension was recorded. The values (mean ± standard deviation as shown by the error bars) were expressed as a percentage of the mean for the parent strain incubated on the same batch of medium, in the same incubator, for the same length of time. The standard deviation values shown for the parent strain are typical ones; this value never exceeded 12% of the mean. Typical colony diameters for SR-1 were 5 to 7 mm at the earlier time and 16 to 18 mm at the later. Shown for comparison are values for strain NS-1, in which the increase in diameter evidently resulted from growth alone.

The mutants all exhibited luxuriant growth on the glucose-salts medium and therefore were not auxotrophs.

Raft movement. As shown in Fig. 5, the mutants were defective in raft movement to about the same extent as they were defective in colony spreading; they gave values for raft speed about one-third as large as those obtained for the parent strain. The rafts formed by the mutants were also smaller than those formed by the parent strain under the same conditions.

Cell cohesion. Cells deficient in a surface polysaccharide might well be expected to have altered surface properties, and indeed, we noticed that when cells of the HMPS mutants (grown in the liquid medium) were placed in solutions of low ionic strength (such as 10 mM sodium HEPES buffer) they formed huge aggregates (up to 100 μm in diameter and containing thousands of cells); no such behavior was exhibited by the parent strain. The aggregation could be reversed by the addition of 0.2 mM Na_2EDTA and then reestablished by the addition of 0.5 mM Mg^{2+} or Ca^{2+}. The aggregation could also be reversed by addition of NaCl (60 mM); the inclusion of 30 mM NaCl in the media used for raft movement studies (as described in Materials and Methods) was necessitated by the fact that without it, the mutant cells aggregated to the point at which the measurements were impossible. (Thirty millimolar NaCl was the highest concentration that did not interfere with raft movement by the parent strain and served to prevent most of the aggregation of the mutants. Rigorous exclusion of divalent cations could not be used to prevent aggregation in this assay; such conditions inhibited movement by the parent strain.)

Cells that adhere tightly to each other might form a thick

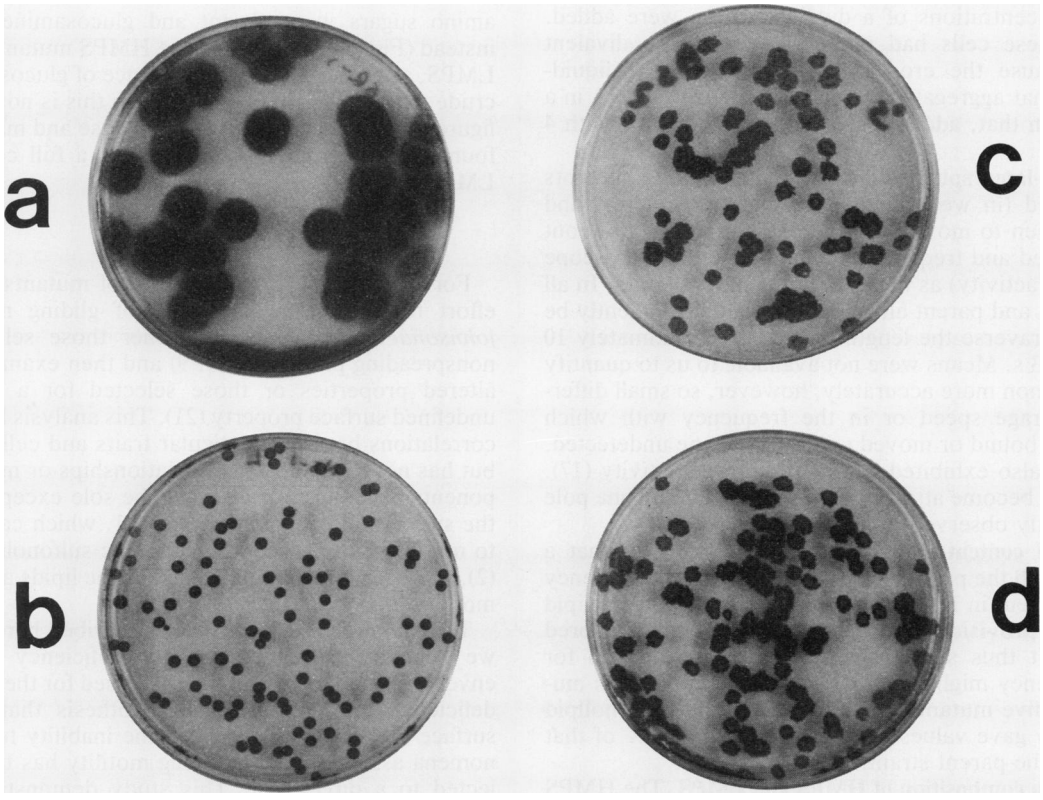

FIG. 4. Colony morphology of HMPS-deficient mutants. The plates were incubated for 68 h and were stained by being sprayed with a solution of nitroblue tetrazolium; conditions were otherwise as described in the legend to Fig. 3. (a) Parent strain SR-1; (b) strain NS-1 (totally nonspreading); (c) strain NG-A; (d) strain NG-B. The petri dishes are 85 mm in diameter.

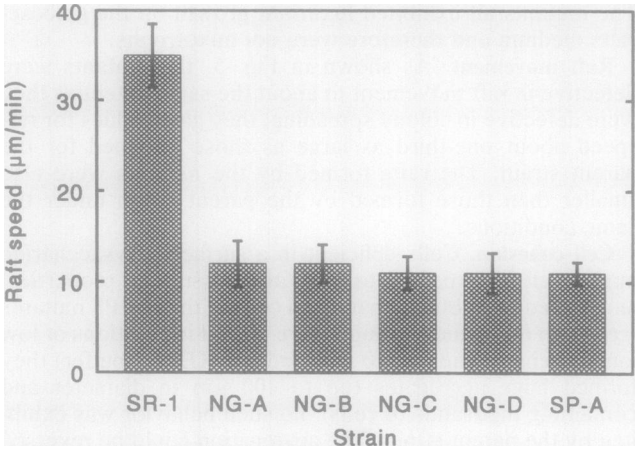

FIG. 5. Raft movement in HMPS-deficient mutants. The bars and error bars represent, respectively, the mean ± standard deviation of at least 15 measurements.

mat rather than spread over the agar surface, and we considered the possibility that this cohesiveness alone might account for the mutants' defect in colony spreading. Three observations suggest that this is not the case. The addition of 30 mM NaCl to the LTY agar neither affected spreading of the parent strain nor cured the defect in the mutants. Conductivity measurements indicated that the medium (without added salt) was not, in fact, of very low ionic strength; its conductivity was equivalent to that of 33 mM NaCl. Finally, mutant cells grown on LTY agar and washed off the surface with 10 mM HEPES buffer did not aggregate until low concentrations of a divalent cation were added. Evidently, these cells had not acquired enough divalent cations to cause the cross-bridging reaction; the liquid-grown cells that aggregated spontaneously were grown in a richer medium that, additionally, was supplemented with 4 mM $MgCl_2$.

Polystyrene-latex sphere movement. The HMPS mutants were observed (in wet mounts) to bind the spheres, and these were seen to move over the cell surfaces with about the same speed and frequency (many cells in a microscope field showing activity) as they did in the parent strain. In all cases, mutant and parent alike, spheres could frequently be observed to traverse the length of a cell (approximately 10 μm) in about 3 s. Means were not available to us to quantify this phenomenon more accurately, however, so small differences in average speed or in the frequency with which spheres were bound or moved might have gone undetected. The mutants also exhibited another wild-type activity (17). Cells that had become attached to the glass slide by one pole were frequently observed to spin about that point.

Sulfonolipid content. We previously reported (9) that a mutant in which the primary lesion is sulfonolipid deficiency was also deficient in HMPS and that curing the sulfonolipid deficiency by provision of a specific precursor also restored the HMPS. It thus seemed reasonable that selection for HMPS deficiency might coselect some sulfonolipidless mutants, but all five mutants were normal in total sulfonolipid content. They gave values ranging from 93 to 108% of that obtained for the parent strain.

Difference in composition of HMPS and LMPS. The HMPS contains the dhexN and galactosamine (as noted above) as well as glucose and mannose (Fig. 6a); these two neutral sugars were also found in the LMPS, but the aforementioned

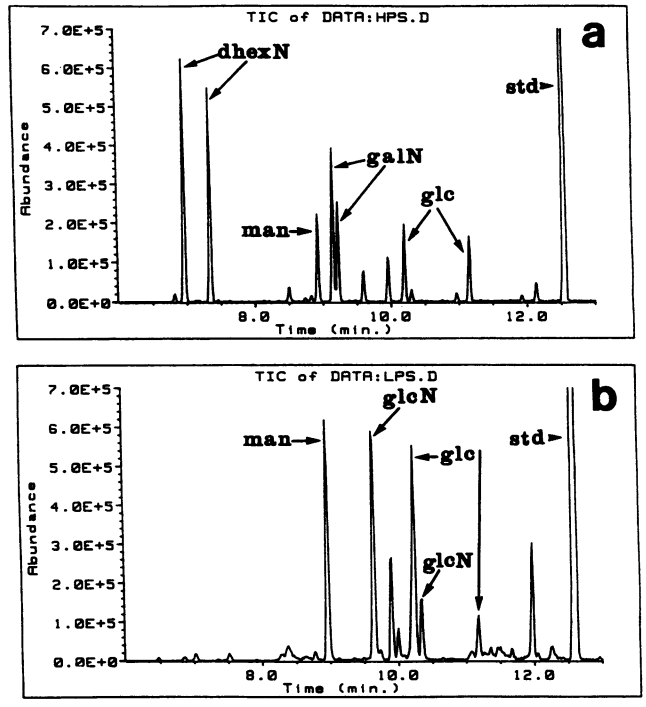

FIG. 6. Sugar composition of purified HMPS (a) and LMPS (b). Conditions were as described in the legend to Fig. 2, except that the ordinate represents the total number of ions from *m/z* 50 to 550. glc, glucose; man, mannose; std, the *m*-inositol added as an internal standard; other abbreviations are explained in the legend to Fig. 2.

amino sugars were absent and glucosamine was present instead (Fig. 6b). Evidently, the HMPS mutants retained the LMPS, as evidenced by the presence of glucosamine in their crude extracts (Fig. 2c) and (though this is not shown in the figure) the presence of as much glucose and mannose as was found in strain NS-1, which retains a full complement of LMPS (9).

DISCUSSION

For the most part, examination of mutants as part of an effort to understand the basis of gliding motility in *C. johnsonae* has focused on either those selected for the nonspreading phenotype (4, 9) and then examined for other altered properties or those selected for a biochemically undefined surface property (21). This analysis has resulted in correlations between particular traits and cell translocation but has not revealed causal relationships or molecular components necessary for gliding. The sole exception has been the sulfonolipid-deficient mutant 21, which can be restored to motility by provision of a specific sulfonolipid precursor (2), an observation that establishes the lipids as essential for motility in cytophagas.

The mutants of *C. johnsonae* described here are the first, we believe, to be selected for deficiency of a specific envelope component and then assessed for the impact of this deficiency on motility. The hypothesis that lack of cell surface HMPS would result in the inability to display phenomena associated with gliding motility has thus been subjected to a direct test. This study demonstrates that the mutants examined retain the ability to move polystyrene-latex spheres and also exhibit raft movement and colony spreading, though at rates much lower than those observed

in the wild type. Though nitrosoguanidine is often considered as tending to produce multiple lesions, this does not seem a matter for concern in the present study, since four independently induced mutants (as well as one spontaneous mutant) all had precisely the same phenotype and none exhibited any auxotrophy or growth defects. In addition, we have never experienced such problems with *C. johnsonae*. Earlier, several other NG-induced mutants were studied (2); none had any new nutritional requirements or growth defects and one (strain 21) was shown, by virtue of being curable, to have only a single primary lesion that affected motility.

Nature of mutants 21 and NS-1. Both of these strains were selected as nongliders, both are deficient in HMPS (9), and both differ from the HMPS mutants described here in being devoid of motility-dependent colony spreading, raft movement, and polystyrene-latex sphere movement. Hence, our original suggestion (9) that the HMPS deficiency in strains 21 and NS-1 was the lesion actually responsible for their lack of all manifestations of motility cannot be correct. In strain 21, the sulfonolipid deficiency that is clearly the primary lesion (curing it cures all the others) must affect gliding in ways unrelated to the HMPS deficiency. The same must be true for strain NS-1, which must have either a second unrelated lesion that affects gliding or a lesion that affects both gliding and HMPS content independently.

Nature of the HMPS mutants' motility defect. Why these mutants exhibit defective colony spreading and raft movement is not clear, but it seems clear that the basic machinery remains functional and the defects result from loss of a surface component that somehow facilitates translocation. There is general agreement (for a review, see reference 16) that cell surface properties must have some importance in gliding, and since HMPS deficiency demonstrably alters those properties it is, in a sense, not surprising that gliding should be affected. The tendency of the mutants to aggregate in the presence of low concentrations of divalent cations, in solutions of low ionic strength, presumably reflects the presence of a surface negative charge which can form cell-to-cell cross bridges involving the divalent cations. This behavior is prevented in the parent strain by the presence of the long-chain HMPS which, perhaps, prevents an apposition of the cells close enough for the cross bridging to occur. (The possibility that the HMPS itself is ionic cannot be ruled out, though the amino sugars in it evidently are N-acetylated, as they are recovered mostly in that form after acid methanolysis of the HMPS [8]). As we note above, cohesion per se cannot account for the motility defects of the mutants, which persist under conditions that do not promote cohesion; rather, the absence of the HMPS may interfere with some cell-cell or cell-substratum interaction involved in gliding.

Many workers believe that gliding is accompanied by the deposition of slime trails. Cells of certain species move faster when gliding on a preexisting trail than when traversing virgin territory (for a review, see reference 3); hence the slime is supposed to have something to do with the interaction of the cell with the substratum. As we have pointed out (9), the putative slime has never been isolated and characterized chemically. We have considered the possibility that the HMPS, which is firmly anchored to the outer membrane (9), might be a precursor of this putative slime and have examined liquid-culture supernatants and supernatants from cells washed off solid media, intending to use the dhexN as a probe for secretion products related to HMPS. Thus far, we have not found any alcohol-precipitable carbohydrate that amounts to more than a small fraction of the amount of HMPS that was present in the cells that were removed from the preparation (8). The possibilities remain, however, that slime (i) exists and (ii) is not produced when HMPS is not present, and these possibilities could account for the deficiency in spreading. The antibodies we have obtained (together with fluorescence or other labelling techniques) may provide an additional, immunochemical means to detect slime trails that are chemically related to the HMPS.

Whatever the basis, it seems clear that the HMPS may play a significant ancillary role in gliding motility, for it appears to permit the locomotory machinery to propel the cells much faster than is the case when it is absent. It will be of interest to determine whether other cytophagas, including strain U-67, which moves so rapidly (13), have a similar surface polysaccharide present in similar amounts.

The present study enlarges our understanding of the polysaccharides in the cell envelope of *C. johnsonae*. Earlier, we noted (9) that this organism contains, in addition to the HMPS, the LMPS that is evidently a lipopolysaccharide (or some related substance), being relatively rich in ketodeoxyoctonate, phosphorus, fatty acids (9), and glucosamine (Fig. 6). This fraction gave multiple bands by SDS-PAGE (9), presumably representing chains with different numbers of repeating units (O-antigen analogs), but contained neither of the two amino sugars found in the HMPS (Fig. 6). The HMPS, then, is not simply a longer version of the lipopolysaccharidelike LMPS fraction with the same repeating units; either it is different entirely or it represents LMPS to which additional and unique polymeric material has been added. As we have noted earlier (9), the HMPS is so large (eluting at or near the void volume from Sephadex G-200) that studying the end groups that anchor the polymer in the outer membrane and distinguishing them from contamination by LMPS would be difficult.

It is thus clear that, in several ways, the findings we describe here differ from those reported for another gliding bacterium that is quite unrelated (15, 18) and whose cells are typified by quite different traits. In their study of *M. xanthus*, Fink and Zissler (7) noted that their rough mutants (deficient in the O-antigen analog of their lipopolysaccharide) were defective in one type of motility (adventurous, the movement of isolated cells) but not in another (social, movement by groups, or rafts, of cells). Clearly, the HMPS mutants of *C. johnsonae* are not missing the O-antigen analog, but instead are deficient in a cell surface polysaccharide of different structure. Furthermore, cytophagas are not known to display the two distinct types of motility characteristic of myxobacters; hence the lesion in raft movement (which might be perceived as an analog of social motility) exhibited by our mutants may be more an apparent, rather than a real, contradiction of the results obtained with *M. xanthus*.

Continued application of biochemical analysis of the components of the cell envelope, combined with specific selection of mutants deficient in one or more components, will lead to the identification of the key participants and the ways in which they function and will thus serve to unravel the 100-year-old mystery of the mechanism of gliding motility.

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