Regulation of Gonococcal Sialyltransferase, Lipooligosaccharide, and Serum Resistance by Glucose, Pyruvate, and Lactate

DAVID J. MCGEE AND RICHARD F. REST*

Department of Microbiology and Immunology, MCP-Hahnemann School of Medicine, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania 19129

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Strain F62 of *Neisseria gonorrhoeae* gonococci (GC) is sensitive to normal human serum unless CMP-*N*-acetylneuraminic acid (CMP-NANA) is present. NANA is transferred primarily to a 4.5-kDa lipooligosaccharide (LOS) structure by a GC sialyltransferase (Stase). We investigated LOS and Stase expression and serum resistance in strain F62 grown in different carbon sources and growth conditions. Pyruvate-grown GC expressed 1.9- to 5.6-fold more Stase activity than did glucose-grown GC, whereas lactate-grown GC generally expressed intermediate Stase activities. Broth-grown GC expressed two- to fourfold more Stase activity than did plate-grown GC in all carbon sources. Pyruvate- or lactate-grown GC expressed significantly more of the sialylatable 4.5-kDa LOS species than did glucose-grown GC. Anaerobically, the 4.5-kDa LOS species was expressed in greater quantity than the 4.9-kDa *N*-acetyl galactosamine-terminating species in all carbon sources. Pyruvate-grown GC also incorporated up to threefold more radiolabelled CMP-NANA onto the 4.5-kDa LOS species than did glucose-grown GC. In serum resistance studies, pyruvate-grown GC were 6.5- to 16.1-fold more serum resistant than glucose-grown GC at limiting CMP-NANA concentrations (1.56 to 12.50 μ g/ml). Taken together, these results indicate that gonococcal expression of Stase activity is up-regulated by growth in pyruvate or lactate, which correlates with enhanced expression of the sialylatable 4.5-kDa LOS and, for growth in pyruvate, correlates with enhanced sialylation of gonococcal LOS and greater serum resistance. In different *in vivo* niches, gonococcal LOS sialylation, serum resistance, and interaction with host cells can be highly regulated.

Although a small number (~2%) of strains of *Neisseria gonorrhoeae* GC are stably resistant to the complement-mediated bactericidal action of NHS upon laboratory subculture (46), most strains remain NHS sensitive (39, 52) unless incubated with CMP-NANA (35, 42; also reviewed in references 24, 44, 47, 48). It has been confirmed that this sialylation occurs *in vivo* (1, 25). Additionally, there are at least two other serum resistance-inducing factors (10, 41). One of these was recently identified as lactate (40), which is utilized by GC as a carbon and energy source. In addition, GC use human neutrophil-derived lactate to increase their oxygen consumption, leading to resistance to the oxidative stress from neutrophils, as well as the creation of an anaerobic environment (7, 8, 16).

The mechanism of killing of serum-sensitive GC is due, in part, to immunoglobulin M antibodies, naturally present in NHS, against the 4.5-kDa galactose- β -1,4-*N*-acetylglucosamine- β -1,3-galactose- β -1,4-glucose-R (Gal-GlcNAc-Gal-Glc-R) LOS structure (2). GC synthesize a Stase to transfer NANA to the terminal Gal residue of this 4.5-kDa species, which results in an increase in M_r to 4.9 kDa (25) and in the observed serum resistance (24, 35, 39, 42). This 4.9-kDa species comigrates with the *N*-acetylglucosamine (GalNAc-Gal-GlcNAc-Gal-Glc-R)-terminating 4.9-kDa species, which is more apparent on LOS gels when GC are grown without CMP-NANA.

Previous work has indicated that anaerobically grown GC require less CMP-NANA to become serum resistant than do aerobically grown GC, because of both an increased expression

of the sialylatable 4.5-kDa epitope and an increased sialylation of this epitope (15). Recently, Emond et al. suggested that expression of Stase activity is increased by growth of GC with CMP-NANA itself, although they did not measure Stase activity directly (12). Regulation of Stase expression by other environmental stimuli has not been reported. Mutants defective in Stase activity have been isolated from GC (5, 27, 44) and *Neisseria meningitidis* (49) and should be useful tools for studying the Stase gene and potential regulators.

In addition to using lactate, GC utilize glucose and pyruvate as carbon and energy sources (30). GC are routinely grown in the laboratory aerobically in the presence of glucose. However, when GC are grown with both glucose and pyruvate (37), or under glucose-limiting conditions (33), LOS expression is altered. In this study, we asked whether LOS and Stase expression were altered when GC were grown separately with three carbon sources, pyruvate, lactate, or glucose, and under three different growth conditions, aerobic broth, aerobic plate, and anaerobic plate. We show that the expression of LOS and Stase activity is altered under certain conditions that can exist *in vivo*. We further show that enhanced Stase activity correlates with enhanced incorporation of radiolabelled CMP-NANA onto LOS and increased serum resistance of GC grown in pyruvate compared with that for GC grown in glucose.

MATERIALS AND METHODS

Abbreviations. GC, *N. gonorrhoeae* gonococci; NANA, *N*-acetylneuraminic acid; Stase, sialyltransferase; LOS, lipooligosaccharides; PBSGCM, phosphate-buffered saline containing 0.1% (wt/vol) gelatin plus CaCl_2 and MgCl_2 (0.01% [wt/vol] each); GCB, GC broth; NHS, normal human serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Bacteria, carbon sources, and growth conditions. Unless otherwise indicated, all growth conditions were aerobic. Nonpilated, transparent (Pil^- Opa^-) variants of GC strain F62 were used. GC were clonally transferred every evening onto GC agar (Difco) plates (15 ml per plate) containing supplements. Plates

* Corresponding author. Mailing address: Allegheny University of the Health Sciences, MCP-Hahnemann School of Medicine, 2900 Queen Ln., Department of Microbiology and Immunology, MS 410, Philadelphia, PA 19129. Phone: (215) 991-8363. Fax: (215) 848-2271. Electronic mail address: restr@allegheny.edu.

were air dried for 1 to 2 days and used within 2 weeks. GCB was prepared with the individual components of GC agar, minus the agar. The supplement added following autoclaving of supplement-free media contained the following compounds at the final concentrations indicated in parentheses: cocarboxylase (200 ng/ml), $\text{Fe}(\text{NO}_3)_3 \cdot 9 \text{H}_2\text{O}$ (20.8 $\mu\text{g}/\text{ml}$), L-glutamine (100 $\mu\text{g}/\text{ml}$), and a carbon source (D-glucose, lactate, or pyruvate [20 mM, unless otherwise noted]). DL-Lactate was used unless otherwise noted. Stocks of CMP-NANA (Sigma) were at a concentration of 5.0 mg/ml in sterile water and were added onto the surfaces of plates or to broth at the concentrations described in the text and figure legends. For long-term storage, GC strains were swabbed from GCA plates <18 h old into GC Freeze Medium (42.5% GCB, 42.5% [vol/vol] heat-inactivated horse serum [Gibco], and 15% glycerol) and frozen at -70°C . Fresh cultures were struck from frozen stocks about once a week to maintain strain continuity.

For growth on plates, GC were suspended in GCB to an A_{550} of ~ 1.0 and 100 μl was spread onto pyruvate, lactate, or glucose plates and incubated at 36°C in a 5% CO_2 environment. Plate-grown GC were 14 to 18 h old in all experiments.

For anaerobic growth, GC were suspended as they were for aerobic plates. In addition, 35 μl of a 16% (wt/vol) NaNO_2 solution was added to a central filter paper disc to serve as a terminal electron acceptor as reported by Knapp and Clark (22). Plates were incubated in an anaerobic jar containing a BBL GasPak Plus system. In some instances, nitrite-containing discs were added on aerobic plates in the same way as a control. Unless otherwise noted, GC from plates containing nitrite were removed from the heaviest growth area nearest the nitrite disc.

For growth in broth, GC from plates were inoculated into 3 to 4 ml of GCB (pH 7.15) with supplements containing the same carbon sources as the plates from which they originated (initial A_{550} , 0.050 to 0.090). GC were grown for 3 h at 37°C in a shaking water bath. On a given day, all cultures were started at the same initial A_{550} and grew to about the same A_{550} .

Preparation of detergent extracts and measurement of Stase activity. GC grown under various conditions were harvested by centrifugation and washed once with PBSGCM. Following centrifugation, GC (3×10^8) were resuspended by pipetting up and down at least 15 times with 60 μl of 0.5% ultrapure Triton X-100 (Sigma) to extract Stase. Following centrifugation ($10,000 \times g$, 3 min) at 4°C , supernatants were transferred to new microcentrifuge tubes and stored on ice. Extracts were assayed the same day for Stase activity (see below) or were stored at -20°C .

Stase activity in detergent extracts was measured by the incorporation of radiolabelled NANA from CMP-[sialic-4,5,6,7,8,9- ^{14}C]NANA (Dupont NEN) onto purified LOS from strain F62 as described by Mandrell et al. (26), with sodium phosphate buffer (0.5 M, pH 6.8) and 2% bovine serum albumin. The reaction mixture was modified as follows: purified LOS (1 μl ; 10 mg/ml), radiolabelled CMP-NANA (0.030 μCi ; 100 pmol; specific activity, 5.8×10^{-2} $\mu\text{Ci}/\mu\text{g}$), and 0.5% Triton X-100 were added to a final volume of 50 μl . The assay was started by the addition of 2 μl of undiluted or 10 μl of a 10-fold-diluted detergent extract; 0.5% Triton X-100 was the diluent. The assay was linear with respect to time and concentration of extract (3). The assay was conducted at 37°C for 15 min and was stopped by acid precipitation of the bovine serum albumin-LOS-[^{14}C]NANA complex as described previously (26). The precipitate was washed once, solubilized with Tissue Solvable (Packard Instrument Company, Meriden, Conn.), added to 5 ml of scintillation fluid (Scintisafe; Fisher Scientific), and counted in a liquid scintillation counter (26). The counts in negative controls, i.e., those containing 0.5% Triton X-100, were subtracted from counts of all samples. All Stase activity measurements are given as counts per minute per 2 μl of extract.

To look at the direct effect of various compounds on the Stase assay, we added pyruvate, lactate, or glucose (5 mM), nitrite (20 mM), EDTA (10 mM), or a known inhibitor of Stase activity (26), CMP (4 mM), to Stase assays consisting of an extract made from glucose or pyruvate broth-grown GC.

Protein concentration of detergent extracts. Protein concentrations in detergent extracts were measured by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) following the manufacturer's protocol. Dilutions of bovine serum albumin (Sigma) were used to create the standard curve. Specific activity is defined as follows: average Stase activity (in counts per minute)/average amount of protein in extract (in nanograms).

Isolation and purification of LOS from whole GC. LOS from strain F62 was purified by the ethanol-acetone protocol (11) and resuspended in sterile distilled water to a concentration of 10 mg/ml. For comparison of LOS phenotypes, LOS was isolated directly from whole GC by the proteinase K method of Hitchcock (19), using 3×10^8 GC grown under various conditions. These preparations were stored at -20°C . One microliter was analyzed by tricine-SDS-PAGE in a Mini-Protein apparatus (Bio-Rad) with a 4% stacking and 14% resolving gel (average length, 4.2 cm) containing urea as described previously (45) with the modifications of Lesse et al. (23). Electrophoresis was conducted at 85 mA for 60 min and then 35 mA for 90 min. The gel was fixed overnight with 40% ethanol-5% acetic acid and silver stained according to Tsai and Frasch (50), except the first series of washes with water were increased to 30 min each.

LOS structure was confirmed by probing a colony blot with the monoclonal antibody 1B2-1B7 which recognizes the 4.5-kDa Gal β -1,4-GlcNAc LOS epitope. This assay is described elsewhere (27).

Incorporation of radiolabelled NANA into GC. Strain F62 was suspended in 1 ml of GCB to an A_{550} of ~ 1.0 , and 100 μl was spread onto pyruvate or glucose

plates. These plates also contained CMP-9-[^3H]NANA (1.56 to 12.50 $\mu\text{g}/\text{ml}$; 0.1 mCi/ml stock; specific activity, 2.1×10^{-2} $\mu\text{Ci}/\mu\text{g}$, Dupont NEN). Plates lacking CMP-NANA served as negative controls. After overnight growth, GC were suspended to an A_{550} of 0.40 in PBSGCM. About 7×10^8 GC were pelleted and resuspended in 500 μl of 1.0% Triton X-100, added to 5 ml of scintillation fluid, and counted. The results reported represent averages of duplicates from two separate experiments. In a third experiment, CMP-9-[^3H]NANA was replaced with CMP-[sialic-4,5,6,7,8,9- ^{14}C]NANA with comparable results.

Incorporation of radiolabelled NANA into LOS. LOS was isolated by proteinase K digestion from GC grown on pyruvate or glucose plates containing various concentrations of CMP-[sialic-4,5,6,7,8,9- ^{14}C]NANA (specific activity, 4.3×10^{-3} $\mu\text{Ci}/\mu\text{g}$). LOS (10 μl) was analyzed by SDS-PAGE. After being silver stained, the gel was sprayed with En 3 Hance (Dupont NEN), dried, and exposed for 5 days to the Molecular Imager Imaging Screen Cassette-CS (Bio-Rad) or for 30 days at -70°C on X-Omat AR film (Eastman Kodak). The image was analyzed on the Molecular Imaging PC system (model GS-363; Bio-Rad).

Serum bactericidal assay. The preparation of NHS was described previously (15). GC grown on plates or in broth in the presence (1.56 to 25.0 $\mu\text{g}/\text{ml}$ for plates, 50.0 $\mu\text{g}/\text{ml}$ for broth) or absence of CMP-NANA were suspended in prewarmed PBSGCM. GC (5×10^6 to 10×10^6 in 2.5 or 5.0 μl) were added to reaction mixtures containing 20% (vol/vol) NHS and PBSGCM to a final volume of 250 μl . After 30 min of incubation at 37°C without agitation, GC were diluted in supplement-free GCB and grown on glucose plates. After overnight incubation, the colonies were quantified and the values for the replicates were averaged. Results are shown as numbers of CFU surviving NHS treatment.

RESULTS

We grew GC in different carbon sources and under different growth conditions and analyzed Stase activity, LOS phenotype, and serum resistance. We made the following general observations: (i) GC grew anaerobically with pyruvate or lactate, using nitrite as a terminal electron acceptor; (ii) GC grown on anaerobic or aerobic lactate plates yielded a zone of inhibition around the nitrite disc that was about twice that seen for GC grown on glucose plates (i.e., they were more sensitive to nitrite inhibition [zone diameters for glucose, pyruvate, and lactate, 30, 46, and 58 mm, respectively]); (iii) GC grown aerobically or anaerobically on lactate plates grew more slowly than GC grown on pyruvate or glucose plates; (iv) growth rates in broth in all three carbon sources were similar; (v) no appreciable growth of GC on any carbon source occurred anaerobically when nitrite was omitted; (vi) no differences in the number of CFU per optical density unit were observed in GC grown on plates or broth containing various carbon sources; (vii) Gram staining revealed no differences in morphology in GC obtained from different carbon sources; (viii) Stase activity in detergent extracts from GC grown in the presence of CMP-NANA (25 to 50 $\mu\text{g}/\text{ml}$) in different carbon sources or under different growth conditions was not significantly different from those from GC grown in the absence of CMP-NANA; and (ix) addition of EDTA (26), pyruvate, glucose, lactate, or nitrite directly to Stase enzyme assays had no effect on Stase activity, whereas addition of CMP caused >95% decrease in Stase activity, as expected (26).

Expression of Stase activity by GC grown in broth or on plates containing pyruvate, lactate, or glucose. Detergent extracts of GC grown on pyruvate, lactate, or glucose plates or in pyruvate, lactate, or glucose broth were assayed for Stase activity as described in Materials and Methods. GC grown in pyruvate broth expressed an average of 2.4-fold more Stase activity than those grown in glucose broth (21,144 versus 8,759 cpm [Fig. 1]). When grown on pyruvate plates, GC expressed an average of 5.6-fold more Stase activity than those grown on glucose plates (11,811 versus 2,106 cpm [Fig. 1]). The level of Stase activity from lactate-grown GC was either slightly lower than that from pyruvate-grown GC or slightly higher than that from glucose-grown GC. Also, two- to fourfold more Stase activity was obtained from broth-grown GC than from plate-grown GC, irrespective of carbon source (Fig. 1). We also measured Stase activity from GC on plates containing both

FIG. 1. Stase activity of GC strain F62 grown aerobically on plates or in broth containing 20 mM pyruvate, lactate, or glucose. Detergent extracts were made and measured for Stase activity as described in Materials and Methods. Background levels have been subtracted. Numbers in parentheses represent numbers of experiments performed (each assayed in duplicate). Data shown are means $\pm$ standard deviations (bars). Abbreviations: Pyr, pyruvate; Lact, lactate; Glc, glucose; pl, plate.

pyruvate and glucose (20 mM each); this level of Stase activity was identical to that obtained from GC grown in glucose plates (data not shown), suggesting that the repressive effect of glucose on Stase activity is dominant over the induction effect of pyruvate. Additionally, decreasing the carbon source (glucose, pyruvate, or lactate) by 50- to 100-fold did not alter Stase activity, despite slower growth of GC under these carbon-limiting conditions (data not shown). Stase activities from GC grown in DL-lactate, D-lactate, or L-lactate were identical.

Expression of Stase activity by GC grown on aerobic or anaerobic plates containing pyruvate, lactate, or glucose. GC were grown on plates aerobically or anaerobically and detergent extracts were assayed for Stase activity as described in Materials and Methods. GC from anaerobic pyruvate plates expressed an average of 1.9-fold more Stase activity than did GC from anaerobic glucose plates (14,023 versus 7,317 cpm [Fig. 2]). Stase activities from GC grown on anaerobic lactate or glucose plates were similar. Under aerobic conditions, GC from pyruvate plates expressed an average of 2.5-fold more Stase activity than did GC from glucose plates (13,075 versus 5,178 cpm [Fig. 2]). Together, the results in Fig. 1 and 2 indicate that pyruvate-grown GC, regardless of growth conditions, express more Stase activity than do glucose-grown GC. In addition, Stase activities from GC grown on aerobic and anaerobic plates were similar for any given carbon source (Fig. 2), suggesting that anaerobiosis does not increase Stase activity, despite increases in the 4.5-kDa LOS (see below and reference 15) and increased sialylation under anaerobic conditions (15).

Expression of Stase activity by GC grown on pyruvate, lactate, or glucose plates with or without nitrite. Although nitrite is unnecessary for the aerobic growth of GC, we included nitrite to see if it affected expression of Stase activity. To our surprise, nitrite appeared to enhance expression of Stase activity by GC grown on glucose but not pyruvate plates: glucose-grown GC expressed an average of 2.5-fold more Stase activity when grown with nitrite than without nitrite (5,178 versus 2,106 cpm [Fig. 3]), whereas pyruvate-grown GC expressed similar levels of Stase activity when grown with or without nitrite (13,075 versus 11,811 [Fig. 3]). Thus, nitrite can induce the

FIG. 2. Stase activity of GC strain F62 grown on aerobic or anaerobic plates containing pyruvate, lactate, or glucose. Detergent extracts were made and Stase activity was measured as described in Materials and Methods. Abbreviations: aer, aerobic; ana, anaerobic. Other details are as explained in the legend to Fig. 1.

expression of Stase activity when GC are grown on glucose plates.

Higher levels of Stase activity in pyruvate-grown GC could be due to several things, including more protein being synthesized, higher-level specific activity, or larger organism size. We made detergent extracts from GC grown under different conditions and found that extracts of pyruvate-grown GC had slightly higher protein concentrations than those of glucose-grown GC (343 versus 254 μ g/ml in broth; 293 versus 177 μ g/ml on plates [Table 1]). Extracts of broth-grown GC had higher protein concentrations than those of plate-grown GC. When these values were used to calculate specific activity (Table 1), pyruvate-grown GC had more Stase-specific activity than did glucose-grown GC in broth (30.82 versus 17.20 cpm/ng of protein) or on plates (22.54 versus 5.95 cpm/ng of protein). Additionally, the Stase-specific activity of GC grown in broth was greater than that of GC grown on plates (30.82 versus 22.54 cpm/ng of protein for pyruvate; 17.20 versus 5.95

FIG. 3. Stase activity of GC strain F62 grown on plates containing pyruvate, lactate, or glucose, with (+) or without (-) nitrite. Detergent extracts were made and Stase activity was measured as described in Materials and Methods. Other details are as explained in the legend to Fig. 1.

TABLE 1. Comparison of Stase activities, protein concentrations, and specific activities from GC grown in pyruvate or glucose^a

Growth condition ^b	Stase activity (cpm)	Stase activity (% Glc plate ^c)	Mean protein concn (ng/μl ± SD) (n ^d)	Protein concn (% Glc plate)	Sp act (cpm/ng)	Sp act (% Glc plate)
Glc plate	2,106	100	177 ± 76 (5)	100	5.95	100
Pyr plate	11,811	561	262 ± 42 (4)	148	22.54	379
Glc broth	8,759	416	254 ± 11 (5)	144	17.20	289
Pyr broth	21,144	1,004	343 ± 36 (5)	194	30.82	518

^a GC were grown aerobically in pyruvate or glucose broth or on glucose or pyruvate plates. The data for Stase activity are from Fig. 1. Protein concentrations were measured as described in Materials and Methods. Specific activity of Stase in extract is defined as Stase activity (in counts per minute/amount of protein in extract (in nanograms)).

^b Abbreviations are as in the legend to Fig. 1.

^c % glc plate, comparison of data to those obtained under glucose plate conditions. The values for glucose plate conditions were arbitrarily set to 100%.

^d n, number of experiments.

cpm/ng of protein for glucose). The specific activity data correlate with the differences we obtained by comparing Stase activities per CFU. When we compared the most extreme conditions (Table 1), we found that for GC grown in pyruvate broth Stase activity was increased 10-fold, protein was increased 2-fold, and specific activity was increased 5-fold compared with GC grown on glucose plates.

LOS analysis of GC grown in different carbon sources and under different growth conditions with or without CMP-NANA. LOS from GC grown with pyruvate, lactate, or glucose was analyzed under three different growth conditions, aerobic broth, aerobic plate, and anaerobic plate, and in the presence (25.0 μg/ml for plates, 50.0 μg/ml for broth) or absence of CMP-NANA. The LOS profiles of GC grown with pyruvate and lactate were identical, irrespective of growth condition (representative profiles shown in Fig. 4, lanes 1, 3, and 5). Compared with glucose-grown GC, two additional lower-molecular-mass LOS species (4.1 and 3.6 kDa) (Fig. 4, lanes 1 to 6), similar to those previously reported for GC grown simultaneously in glucose and pyruvate (37), were detected. These LOS species were barely detectable in glucose-grown organisms (Fig. 4, lanes 7 to 12), even when the lanes were overloaded with sample (data not shown). Additionally, GC grown in pyruvate or lactate constitutively express the 4.5-kDa Gal-GlcNAc-Gal-Glc-R LOS species in quantities greater than the 4.9-kDa GalNAc-Gal-GlcNAc-Gal-Glc-R species, under all growth conditions (Fig. 4, lanes 1, 3, and 5 [open and closed

arrows, respectively]). Under aerobic conditions, glucose-grown GC express the 4.9-kDa GalNAc species in quantities greater than that of the 4.5-kDa species (Fig. 4, lanes 7 and 9) whereas under anaerobic conditions the quantity of the 4.5-kDa species is equal to or greater than that of the 4.9-kDa GalNAc species (Fig. 4, lane 11), as has been shown previously (15).

When GC are grown with nonlimiting concentrations of CMP-NANA, the NANA moiety is covalently transferred to the 4.5-kDa LOS species, causing a complete shift upward in migration to 4.9 kDa, comigrating with the 4.9-kDa GalNAc species (25). This occurred under all growth conditions and with all carbon sources we tested (Fig. 4, even-numbered lanes). Additionally, in GC grown with pyruvate or lactate there was no evidence that the smaller LOS species were sialylated, since they remained at their respective *M_r*s. Thus, under all the conditions we tested sialylation of only the 4.5-kDa LOS epitope occurred.

Since pyruvate-grown GC express more Stase activity than do glucose-grown GC, we asked whether GC grown on pyruvate plates would sialylate the 4.5-kDa LOS species at a lower CMP-NANA concentration than would GC grown on glucose plates. GC were grown on pyruvate or glucose plates containing 1.56 to 12.50 μg of CMP-NANA per ml, and LOS was isolated and analyzed. Whereas GC grown on pyruvate plates sialylated their LOS at 3.13 μg of CMP-NANA per ml, the same degree of sialylation by GC grown on glucose plates was

FIG. 4. LOS phenotypes of strain F62 of GC grown in different carbon sources and under different growth conditions in the presence (+) or absence (-) of CMP-NANA. LOS was isolated from whole cells by proteinase K digestions and was analyzed on Tricine-SDS-polyacrylamide gels. Gels were stained with silver nitrate. In the odd-numbered lanes, the 4.9-kDa LOS is GalNAc-Gal-GlcNAc-Gal-Glc-R and the 4.5-kDa LOS is Gal-GlcNAc-Gal-Glc-R. In the even-numbered lanes, the 4.9 kDa LOS is a combination of GalNAc-Gal-GlcNAc-Gal-Glc-R and NANA-Gal-GlcNAc-Gal-Glc-R. There is no 4.5-kDa LOS in the even-numbered lanes, br, broth. Other abbreviations are as in the legends to Fig. 1 and 2.

TABLE 2. Incorporation of radiolabelled NANA into GC grown on plates containing pyruvate or glucose^a

CMP-NANA concn (μg/ml)	Avg cpm incorporated during growth on ^b :		Ratio ^c
	Pyruvate	Glucose	
12.50	14,448	11,305	1.28
6.25	10,413	6,400	1.63
3.13	5,808	3,872	1.50
1.56	4,243	1,792	2.37
0	45	98	0.46

^a Strain F62 was grown aerobically on pyruvate or glucose plates with increasing concentrations of CMP-NANA and CMP-9-[³H]NANA, solubilized in 1% Triton X-100, and prepared for liquid scintillation counting as described in Materials and Methods.

^b The average (in counts per minute) of duplicates from two separate experiments are shown.

^c The ratio was determined as follows: counts per minute measured in pyruvate-grown GC/counts per minute measured in glucose-grown GC.

not detected until a concentration of 6.25 μg of CMP-NANA per ml was reached (data not shown). Thus, GC grown with glucose required twice as much CMP-NANA as GC grown with pyruvate did to completely sialylate their LOS. These results correlate with the enhanced Stase activity observed for GC grown in pyruvate compared with glucose. Further confirmation of this was obtained by using radiolabelled CMP-NANA (see below) (see Table 2 and Fig. 5).

GC grown on pyruvate plates incorporate more radiolabelled NANA onto their LOS than those grown on glucose plates. We investigated whether GC grown in pyruvate would incorporate more radiolabelled NANA than those grown in glucose. GC were grown on pyruvate or glucose plates containing increasing concentrations of radiolabelled CMP-NANA and prepared for liquid scintillation counting as described in Materials and Methods. At all concentrations of CMP-NANA, GC grown in pyruvate incorporated 1.3- to 2.4-fold more radiolabelled NANA than did GC grown with glucose (Table 2). Comparable results were obtained with CMP-9-[³H]NANA or CMP-[sialic-4,5,6,7,8,9-¹⁴C]NANA. These results indicate that enhanced Stase activity from GC grown in pyruvate results in enhanced incorporation of radiolabelled sialic acid into whole GC.

To confirm that the enhanced incorporation was onto the 4.5-kDa LOS species, GC were grown on pyruvate or glucose plates containing radiolabelled CMP-NANA. LOS was exposed to film as described in Materials and Methods (Fig. 5). Densitometric analysis of the image by PhosphorImager confirmed that pyruvate-grown GC incorporate 1.5- to 2.8-fold more radiolabelled NANA onto their LOS than do glucose-

FIG. 5. Incorporation of radiolabelled NANA onto LOS in GC grown on pyruvate or glucose plates. LOS was isolated by proteinase K digestion, analyzed on a Tricine-SDS-polyacrylamide gel, and exposed to film. Abbreviations: P, pyruvate; G, glucose.

TABLE 3. Serum resistance of GC grown aerobically on pyruvate or glucose plates with increasing concentrations of CMP-NANA^a

CMP-NANA concn (μg/ml)	Resistant (10 ³ CFU) ^b after growth on:		Ratio ^c
	Pyruvate	Glucose	
25.00	17,719	24,883	0.7
12.50	12,740	790	16.1
6.25	3,850	310	12.4
3.13	1,430	173	8.3
1.56	324	50	6.5
0	22	21	1

^a Strain F62 was grown aerobically on pyruvate or glucose plates with increasing concentrations of CMP-NANA and 10⁷ GC were added to 20% NHS as described in Materials and Methods. After 30 min of incubation at 37°C, survivors were diluted and plated.

^b Average number of CFU. The average represents three separate experiments, with two to four replicates per experiment.

^c The ratio was calculated by the following formula: number of serum-resistant CFU from pyruvate-grown GC/number of serum-resistant CFU from glucose-grown GC.

grown GC (Fig. 5). There was no evidence for sialylation of other LOS species in these experiments.

GC grown on pyruvate plates are more serum resistant than those grown on glucose plates. To determine whether there was biological relevance to the enhanced Stase activity and enhanced sialylation of LOS seen with GC grown in pyruvate, we grew GC under various carbon and growth conditions in the absence or presence of varying concentrations of CMP-NANA and subjected them to NHS bactericidal assays as described in Materials and Methods. Regardless of carbon source or growth conditions, at nonlimiting CMP-NANA concentrations (25.0 μg/ml for plates, 50.0 μg/ml for broth) GC were completely protected from killing by NHS (2 to 4 log units of protection compared with that for GC grown without CMP-NANA). For example, when GC were incubated on pyruvate or glucose plates containing CMP-NANA (25.0 μg/ml) there was complete protection from NHS: 1 × 10⁷ GC were added to NHS, and 1.8 × 10⁷ (pyruvate) or 2.5 × 10⁷ (glucose) serum-resistant GC were recovered (Table 3). Thus, when not killed by NHS, GC grow (15).

Since CMP-NANA concentrations can be limiting for GC in serum, we grew GC on pyruvate or glucose plates containing 1.6 to 12.5 μg of CMP-NANA per ml. GC grown on pyruvate plates were 6.5- to 16.1-fold more resistant to 20% NHS than those grown on glucose plates under these conditions (Table 3). No differences in serum resistance between pyruvate- and glucose-grown GC were observed when CMP-NANA was omitted (Table 3). The number of pyruvate-grown GC survivors was 3 log units higher with CMP-NANA (12.50 μg/ml) than without CMP-NANA, whereas in glucose-grown GC, it was only 1.6 log units higher. These results demonstrate that enhanced Stase activity in pyruvate-grown GC increases their serum resistance at limiting CMP-NANA concentrations and thus could have biological relevance.

DISCUSSION

Most investigators grow GC in vitro under aerobic conditions in glucose broth or on glucose plates lacking CMP-NANA. GC encounter a myriad of changes in their in vivo environments, including changes in iron level, carbon source, nutrient limitation, pH, anaerobiosis, etc. These environments alter the expression of numerous genes and can be simulated by altering the environment(s) in vitro. In a previous report, a

strong correlation among enhanced sialylation of LOS, radio-labelled incorporation of NANA onto gonococcal LOS, and enhanced serum resistance when GC were grown anaerobically instead of aerobically was demonstrated (15). In this work, we show a strong correlation among enhanced Stase activity, enhanced expression of the 4.5-kDa sialylatable LOS epitope, enhanced incorporation of NANA onto LOS, and increased serum resistance when GC are grown in pyruvate compared with GC grown in glucose *in vitro*.

When GC were grown with pyruvate, they expressed an average of 1.9- to 5.6-fold more Stase activity than when grown in glucose (Fig. 1 and 2). The differences varied depending on the growth condition from which the organisms were obtained, with aerobic plate-grown GC exhibiting the greatest difference in Stase activity. GC grown in broth expressed the highest Stase activity, irrespective of carbon source (Fig. 1). Thus, Stase activity in GC can be regulated by variation both in carbon source and in growth condition. That the Stase level from broth-grown GC is higher than that for plate-grown GC could be due to differences in physiologic state between plate-grown GC (late log to early stationary phase) and broth-grown GC (mid-log phase) used in our experiments; active growth is required for Stase expression (12, 15, 51).

Anaerobic GC do not express more Stase activity than aerobic GC. However, previous work has indicated that GC express more 4.5-kDa LOS and are more sialylated under anaerobic conditions (15). Additional sialylatable 4.5-kDa LOS substrate could account for increased serum resistance under anaerobic conditions when CMP-NANA is present. Similarly, the increase in the 4.5-kDa LOS species under certain conditions studied here (Fig. 4; also see below) could also account for increased serum resistance of pyruvate-grown GC versus glucose-grown GC when CMP-NANA is present (Table 3).

Regulation of the expression of gonococcal Stase activity and/or LOS expression under different conditions could be due to a two-component regulatory system (reviewed in reference 38), the use of alternate sigma factors (21), or catabolite repression (reviewed in reference 4). We have been unable to completely repress Stase activity by growth in different environmental conditions, suggesting that basal production of Stase occurs (13, 28). When we grew GC in the presence of both glucose and pyruvate, Stase activity was the same as that for GC grown in glucose alone, supporting the argument for catabolite repression. It is possible that in media containing glucose and pyruvate glucose is selectively metabolized, resulting in Stase activity characteristic of glucose-grown GC. However, the presence of cyclic AMP remains controversial (29, 31) and it is not known whether GC use glucose preferentially over pyruvate when both carbon sources are present. Cloning of the Stase gene should provide more insight into its regulation by carbon and growth conditions.

That the level of Stase activity observed in GC grown on glucose plates containing nitrite was higher than that in GC grown without nitrite could be due to variations in the growth rate in medium containing nitrite or due to a mechanism not yet determined. When GC are grown anaerobically with nitrite, they grow as a heavy ring when the nitrite concentration is optimal (22). When GC are grown aerobically, this ring is less pronounced. When GC were grown on pyruvate plates, nitrite did not affect expression of Stase activity, possibly because less nitrite is needed by these GC, or perhaps because Stase activity is already maximally expressed when GC are grown in pyruvate and thus the induction by nitrite would be masked.

GC oxidize glucose via the Entner-Doudoroff and pentose phosphate pathways to acetate (34). GC oxidize lactate to

pyruvate by using lactate dehydrogenases (14). GC convert pyruvate to acetate or metabolize pyruvate via tricarboxylic acid cycle enzymes. The oxidation of acetate through the tricarboxylic acid cycle is more likely, since GC convert acetate into acetyl coenzyme A when glucose is exhausted (18, 20, 32). An alternate metabolic pathway is that in which pyruvate or lactate is converted into glucose-6-phosphate via gluconeogenesis (34). Gluconeogenesis occurs when ATP levels are high. GC grown in pyruvate or lactate can bypass numerous Entner-Doudoroff and pentose phosphate enzymes, which undoubtedly alters the intracellular ratio of ATP to AMP. Perhaps ATP/AMP ratios are sensed directly at the enzyme level by Stase; the addition of ATP to Stase assays does not affect Stase activity, but AMP inhibits Stase by 25% (26).

Pyruvate- or lactate-grown GC expressed two additional LOS species with lower M_r s not observed in GC grown with glucose. The identity of these bands has not been established by us, although they probably represent the 3.6-kDa Gal-Glc-R and the 4.1-kDa GlcNAc-Gal-Glc-R truncated LOS species. These smaller LOS species are presumably the same ones observed previously (36). The smaller LOS species are observed when sulfite is added to fresh medium (36). Although the effect on LOS expression by lactate was not previously studied, it is not surprising that lactate had the same effect as pyruvate, since GC possess at least two membrane-bound lactate dehydrogenases that readily convert lactate into pyruvate (14). When GC were grown in pyruvate or lactate, there was greater expression of the 4.5-kDa sialylatable LOS epitope than when GC were grown in glucose (Fig. 4). Additionally, when GC were grown in pyruvate or lactate, the 4.5-kDa LOS species was expressed in greater quantity than the 4.9-kDa GalNAc species under all growth conditions. In contrast, when grown in glucose, GC express more 4.9-kDa GalNAc LOS than 4.5-kDa LOS under aerobic conditions, but express equal or greater quantities of the 4.5 kDa than the 4.9 kDa GalNAc LOS under anaerobic conditions (Fig. 4) (see also reference 15 and this study). Since there is both greater expression of Stase and the 4.5-kDa LOS (a substrate for Stase) when GC are grown in pyruvate or lactate than when they are grown in glucose, this suggests that the expression of Stase and the 4.5-kDa LOS species can be coregulated under certain conditions (see above).

Alteration of LOS structure by growing GC *in vitro* at different pHs (43) and by using glucose-limiting conditions in continuous culture (33) have been reported. Thus, the expression of LOS appears to be altered by numerous conditions (anaerobiosis, limiting or nonlimiting carbon source, pH). Further analysis of the upstream region of the cloned *lgt* operon is necessary (17).

When GC are grown with nonlimiting concentrations of CMP-NANA, only the 4.5-kDa LOS is sialylated, with a concomitant increase to 4.9 kDa. We did not detect sialylation of any other LOS species under any condition tested, using two different experimental methods (Fig. 4 and 5), suggesting that the 4.5-kDa epitope is the major sialylatable structure, although other sialylatable epitopes exist (48). At limiting concentrations of CMP-NANA, GC grown in pyruvate incorporated more radiolabelled sialic acid onto whole cells (Table 2) and specifically onto the 4.5-kDa LOS (Fig. 5) than did GC grown in glucose, which was expected since there was enhanced Stase activity in GC grown in pyruvate (Fig. 1 and 2).

In serum resistance studies, we demonstrated that GC grown on pyruvate plates were 6.5- to 16.1-fold more serum resistance than GC grown on glucose plates when limiting concentrations of CMP-NANA are used (Table 3). The serum resistance data strongly suggest that elevated Stase levels (Fig. 1 and 2) cor-

relate with enhanced expression of the sialylatable 4.5-kDa LOS species (Fig. 4) and enhanced sialylation of LOS (Table 2 and Fig. 5). At limiting CMP-NANA concentrations, only some of the LOS molecules on the GC surface are sialylated. On a single GC, a certain percentage of LOS molecules apparently must be sialylated for protection of that bacterium from NHS.

Increased serum resistance is dose dependent with respect to CMP-NANA (references 12, 15, 47, and 48; also this study [Table 3]). Additionally, CMP-NANA concentrations in blood cell extracts are around 50 ng/ml (35, 47), amounts which can be limiting for GC sialylation. Some GC strains require lower concentrations of CMP-NANA to become serum resistant (12), and the amount of NANA on the surface of in vivo-obtained GC strains can vary 20-fold (53). These differences might be due to altered amounts of Stase among different strains. At 25 µg of CMP-NANA per ml, we did not observe any significant differences in serum resistance between GC grown in pyruvate and those grown in glucose. This is not surprising, since GC probably do not encounter these high CMP-NANA concentrations in vivo.

CMP-NANA was the first heat-labile compound in NHS that was found to induce serum resistance in GC (35, 42). Additional work to identify the other heat-labile serum resistance-inducing factors in NHS is being conducted (10, 41). One of these components was recently identified as lactate (40), which we show increases expression of Stase activity in GC compared with growth in glucose under conditions of aerobic broth or aerobic plate lacking nitrite. Pyruvate might also be a serum resistance-inducing factor in that it causes enhanced Stase expression and increased serum resistance in GC (this study). It has been reported that a heat-stable component with a relative molecular weight of less than 1,000 induces an increase in metabolic rate (oxygen consumption) in GC (6, 9). In subsequent studies, GC were shown to use human neutrophil-derived lactate as a carbon source, which GC subsequently use to enhance their resistance to oxidative stress by creating an anaerobic environment (7, 8, 16). Whether GC can use pyruvate from human neutrophils remains unanswered.

Sialylation of gonococcal LOS by Stase creates a "pseudo-capsule"; this sialylation could be modulated by different environmental conditions encountered by GC in vivo, e.g., in the male or female genital tract, and thus alter the pathogenic potential of GC.

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