J. Microbiology. September 1996 p. 255-262 Vol. 34, No. 3

Lipopolysaccharide Yields from *Rhodobacter capsulatus* with indirect ELISA

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(Received March 25, 1996/Accepted June 10, 1996)

The lipopolysaccharide(LPS) yields were measured in *Rhodobacter capsulatus* under several conditions by the ELISA method. The purification of LPS was done by affinity chromatography of IgG coupled CNBr-activated sepharose-4B instead of ultra-centrifugation. The purity of the LPS didn't show much difference between affinity chromatography and ultra-centrifugation method, but affinity chromatography method required much fewer organisms and was more convenient. LPS yield was measured in ng units by the ELISA method. Mannitol was a better single carbon source than other sugars, but mixing two carbon sources resulted in greater LPS yields than any sugar alone. LPS yield was directly proportional to NH_4Cl concentration, with optimum yields at 0.05% nitrogen. In contrast to LPS yields, which decreased at 0.005% nitrogen concentration total protein was increased 16 times. Calcium influenced LPS yields. At 0.7 mM $CaCl_2$, the LPS yield was 16.5 μg/mg DW, five times the yield without calcium.

Key words: *Rhodobacter capsulatus*, LPS, ELISA method, affinity chromatography

Rhodobacter capsulatus is a gram-negative photosynthetic bacterium. A common feature of gram-negative organisms is the typically structured cell wall. Characteristic components of the outer layer are lipopolysaccharide(LPS,endotoxin). Almost 35 years ago, Salton(**24**)found "lipid-polysaccharide-protein" fraction in cell walls of *Rhodospirillum rubrum*. But the first complete analysis of a cell wall LPS in photosynthetic bacteria was accomplished with *R. capsulatus* 37b4(32). The LPS consists of lipid A, covalently linked to a core-polysaccharide to which the o-specific chains are attached. Lipid A constitutes the active site of bacterial endotoxin (LPS). It activates target cell functions, like cytokine production, probably by interaction with specific receptor(11).

The O-antigenic side chains consist of oligosaccharide repeating units and are species-specific. Chemical analysis of lipopolysaccharide (LPS) and electromicrographs of gram-negative photosynthetic bacteria were studied (8, 9, 10, 13, 27, 29, 30, 31). LPS can function as a receptor for phage attachment as well as for self protection, defense, and nitrogen fixation (8, 9, 17, 21, 28). Formation of slime, capsule, and membrane were studied under various environmental conditions (8, 18, 19, 22, 26). Omar *et al.* (19), suggested that under such conditions LPS yields should be changed. Ellwood *et al.* (7), also proposed that lipopolysaccharide and exopolymers varied in quantity and composition with growth conditions. LPS can be purified more easily and with greater purity when affinity chromatography is used (35) instead of ultracentrifugation (33, 34). Enzyme-Linked Immunosorbent Assay (ELISA) would be a more convenient way for measurement of LPS quantity (35). The aim of this work was to study LPS quantity under various environmental conditions with ELISA method. *Rhodobacter capsulatus* was cultured under various environmental conditions: carbon sources, concentrations of carbon, nitrogen, calcium, EDTA, light conditions, and chemoorganotrophic conditions.

Materials and Methods

Organism and culture conditions

Rhodobacter capsulatus DSM1710 were grown photoheterotrophically and anaerobically in R8ÄH medium (6) containing malate(0.25%) and yeast extract(0.1%), at 28~30℃ in the light(2000Lux). Instead of using malate as a carbon source in R8ÄH, several other carbon com-

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pounds including succinate, pyruvate, mannitol, butyrate, D-glutamate, fructose, and glucose were used. Bacteria were cultivated Chemoorganotrophically in the dark and shaken at 500~600 rpm by a rotatory shaker. The cells were harvested at the early stationary phase and washed with distilled water and lyophillized. The yield of LPS was measured at several concentrations of calcium and EDTA.

Dry weight of bateria

Dry weight of bacteria was measured by Hillmer *et al.* (12). Protein was determined by the method of Lowry (15) using bovine serum albumine as standard.

Isolation and purification of lipopolysaccharide(LPS)

LPS was extracted by the hot phenol-water method (33,34) and purified by centrifugation (four times at 105,000×g) in a Beckman ultracentrifuge. RNA was removed by treating with 2% cetavlon and RNase 50 μg/ml. CNBr-Activated Sepharose 4B Affinity chromatography(2, 25) coupled with IgG was used as an alternative method of LPS purification. Purification of LPS was estimated by absorption spectrum of 190~700 nm with UV visible-spectrophotpmeter.

Antiserum preparation

Whole cell antigen was prepared anaerobically cultured cells. Cells were harvested and washed in 0.85% saline solution three times. The final sample contained 3×10^9 cellls/ml. The cells were placed in a boiling water bath for 2.5 hours. New Zealand White rabbits(2.4 Kg) received intravenous injection of 0.25, 0.5, and 1 ml of heat killed cells at days 1,2, and 3 respectively. After the third day, they were injected with 1 ml at three day intervals for 88 days. On day 93, 15 ml of blood was removed from the animals by cardiac puncture. The blood was allowed to clot at room temperature for 30 min, and at 4°C for 10 h, and was then centrifuged and stored at -40°C. Pre-immune samples were collected before immunization.

Measurement of antibodies

Antiserum activity against whole cell antigens was determined by the method of Ouchterlony (20) and by indirect ELISA using LPS-BSA (Bovine serum albumin, Fraction V; sigma) complex as antigen (1).

Preparation of anti-rabbit IgG conjugates for ELISA

Rabbit IgG was prepared by affinity chromatography with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals). First, 1.5 g of Protein A-sepharose CL-4B was swollen in 0.01 M phosphate buffer at pH 7.0 and washed with the same buffer. Then 2 ml of antiserum diluted in 8 ml of 0.01 M phosphate buffer was adsorbed to the column by a peristaltic pump(at 4°C, overnight). Each 1.5 ml solution of antibodies in 0.01 M phosphate buffer was fractionated completely until no more absorbance was observed at 280 nm. Elution of IgG from protein A-sepharose CL-4B was achieved using 1 M acetate(pH 4.0). Each 1.5 ml was fractionated, and absorbance was determined at 280 nm. Fractions with absorbance of 0.5 or greater were collected and dialysed in a mixture of buffer solution (0.01 M phosphate buffer, pH 7.0, gelatin(1 g/l, and NaN_3 0.5 g/l) for 48 hrs at 4°C in a cold room.

LPS preparation with CNBr-activated sepharose-4B

LPS was extracted by affinity chromatography of CNBr-activated sepharose-4B liganded IgG(2, 25).

Standard curve by indirect ELISA

R. capsulatus LPS was used as antigen for the standard curve according to indirect competitive ELISA (4, 5).

Results

Purity of LPS

The purity of LPS was measured by absorbance at wavelength 220~300 nm and relatedness was measured between 260 nm and 280 nm. We obtained better purity of LPS by adding 2% cetavlon and RNase than we did by using ultracentrifugation or gel filtration (Fig. 1).

IgG purification by rabbit anti-LPS antibody

Fractions of rabbit IgG were collected on protein A-Sepharose CL-4B. If pooled aliquots(4~6) showed an absorbance greater than 0.5 at 280 nm, they were used as ligands to CNBr-activated Sepharose-4B (Fig. 2).

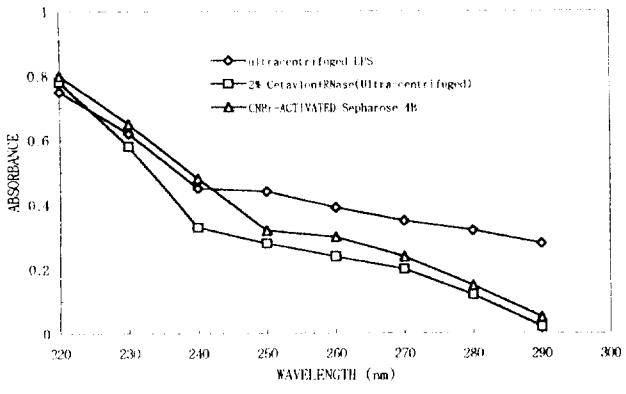

Fig. 1. Absorption spectrum of *Rhodobacter capsulatus* DSM 1710 lipopolysaccharides.

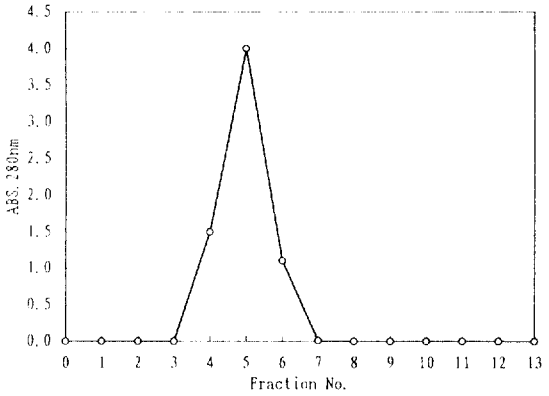

Fig. 2. Fractionation of rabbit IgG on Protein A-Sepharose CL-4B. Rabbit serum was applied to the column in 0.01 M phosphate buffer, pH 7.0.

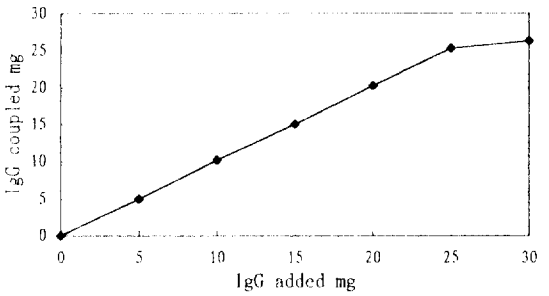

Fig. 3. Effect of protein concentration on amount of protein coupled. Protein was coupled to 5.2 ml CNBr-activated Sepharose 4B in $NaHCO_3$/NaCl solution, pH 8.3.

LPS purified CNBr-activated Sepharose 4B

LPS cultured under various conditions was purified by CNBr-activated Sepharose 4B coupled ligand IgG of rabbit. CNBr-actiated Sepharose 4B(1.5 g) liganded about 25 mg of IgG (Fig. 3). Aliquots of 2 ml of antibody-bound antigen were measured by indirect ELISA at 492 nm. Usually, 20 aliquots were collected.

Serological activity

Serological activity was tested by immunoprecipitation according to the Ouchterlony test or indirect ELISA. In the Ouchterlony test, precipitation lines were observed clearly in the 1/8 dilution of antiserum, but weakly in the 1/16 dilution (Fig. 4). According to indirect ELISA, LPS-BSA complex could be measured until the 1 : 51,200 dilution and free LPS was observed at the 1 : 102,400 dilution (Fig. 5). Normal serum showed less than 0.08 absorbance.

Standard curve

A standard curve was developed from the results of our experiments on indirect competitive ELISA. Fig. 6 shows responses ranging from 12.5 g/ml to 391 ng/ml.

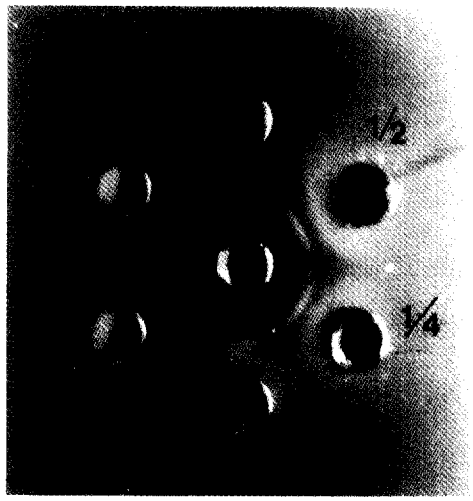

Fig. 4. Ouchterlony immunodiffusion patterns of LPs from *R. Capsulatus*.

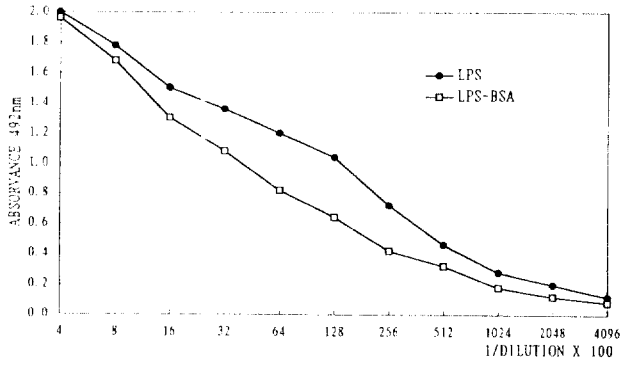

Fig. 5. Titration of rabbit anti-LPS antibody by indirect ELISA.

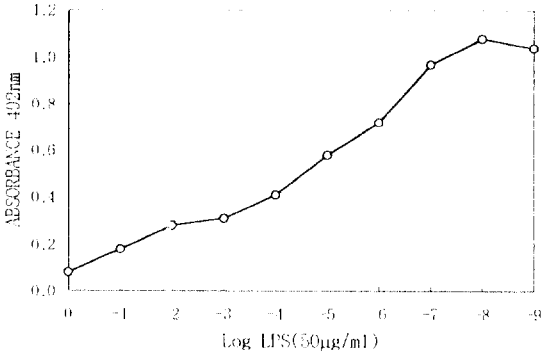

Fig. 6. Indirect competitive ELISA standard curve to lipopolysaccharides.

Standard deviation was below 0.01.

LPS obtained by different carbon sources and concentrations

All experiments were done by late log phase. There are no large differences between most of the six carbon sources, but mannitol resulted in a slightly higher LPS

Table 1. Quantitative value of LPS dependent on carbon sources and growth phase using indirect competitive ELISA

Carbon source (0.25%)	growth phase	LPS(μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
Succinate	stationary phase	16.01±0.45	19.87±0.04	0.084±0.005
Malate	stationary phase	17.54±0.69	18.86±0.01	0.083±0.004
Pyuvate	stationary phase	18.56±0.85	18.45±0.01	0.086±0.005
Mannitol	stationary phase	19.76±0.67	18.28±0.02	0.086±0.003
D-Glutamate	stationary phase	18.78±0.46	18.96±0.02	0.084±0.004
Butyrate	stationary phase	15.09±0.22	28.92±0.03	0.083±0.003

Table 2. Quantitative value of LPS dependent on carbon sources and growth phase using indirect competitive ELISA

Carbon source	growth phase	LPS (μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
Malate (0.25%)	stationary phase	18.01±0.10	17.32±0.04	0.087±0.004
Fructose(0.3%)	stationary phase	18.39±0.11	14.34±0.02	0.087±0.006
Glucose(0.3%)	stationary phase	18.36±0.14	15.28±0.01	0.086±0.007
Malate(0.15%)+Glucose(0.15%)	stationary phase	23.21±0.51	15.14±0.01	0.090±0.004
Glucose(0.15%)+Fructose(0.15%)	stationary phase	22.41±0.79	14.96±0.01	0.089±0.005

Table 3. Quantitative values of LPS dependent on carbon concentration using indirect competitive ELISA.

carbon conc.	carbon source	LPS(μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
0.1%	malate	14.41±0.36	41.49±0.05	0.83±0.005
	fructose	12.71±0.21	39.46±0.02	0.082±0.004
	glucose	12.89±0.11	38.27±0.03	0.082±0.004
	malate	17.90±0.11	21.46±0.02	0.084±0.003
0.2%	fructose	16.09±0.20	23.59±0.01	0.084±0.005
	glucose	16.06±0.12	24.28±0.04	0.083±0.003
	malate	18.01±0.10	17.32±0.04	0.087±0.004
0.3%	fructose	18.39±0.11	14.36±0.02	0.087±0.006
	glucose	18.36±0.14	16.28±0.01	0.086±0.007
	malate	19.15±0.16	9.85±0.02	0.088±0.004
0.4%	fructose	18.08±0.15	9.04±0.03	0.087±0.006
	glucose	17.84±0.16	10.36±0.03	0.088±0.006
	malate	11.15±0.15	27.78±0.02	0.080±0.004
4.0%	fructose	8.11±0.134	27.25±0.02	0.081±0.006
	glucose	8.19±0.13	28.31±0.04	0.080±0.005

Table 4. Yied of LPS dependent on nitrogen concentration(NH_4Cl) and growth phase using indirect competitive ELISA

nitrogen conc. (NH_4Cl)	growth phase	LPS(μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
0.2%	stationary phase	13.89±0.51	23.95±0.01	0.087±0.005
0.1%	stationary phase	17.61±0.84	22.69±0.03	0.086±0.004
0.05%	stationary phase	20.31±0.28	5.71±0.01	0.087±0.005
0.005%	stationary phase	7.45±0.39	84.20±0.06	0.087±0.006

yield than did butyrate. In contrast, butyrate resulted in higher total protein than did mannitol (Table 1). Tables 2, 3 and 4 show LPS yields from different concentrations of carbon and nitrogen sources. LPS increased according to sugar concentration up to 0.3%. At 0.4% sugar concentration LPS decreased, except when malate was the carbon source. At 4.0% of all sugars, LPS yield decreased. Protein yields were always inversely proportional to LPS yield. When two carbon sources were added together, LPS yield was much greater than that

Table 5. Quantitative values of LPS dependent on calcium concentration ($CaCl_2$) and growth phase using indirect competitive ELISA

calcium conc. ($CaCl_2$)	growth phase	LPS(μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
0.7 mM	stationary phase	16.56±0.89	18.86±0.03	0.084±0.005
0.5 mM	stationary phase	13.35±0.25	18.58±0.03	0.083±0.004
0.3 mM	stationary phase	9.37±0.07	25.33±0.04	0.083±0.006
0.09 mM	stationary phase	8.36±0.10	26.21±0.04	0.080±0.004
0	stationary phase	0.25±0.01	56.28±0.03	0.079±0.005

Table 6. Quantitative values of LPS dependent on EDTA concentration and growth phase using indirect competitive ELISA

EDTA conc. (%)	growth phase	LPS(μg/mg, dry weight)	Total protein (%/g, dry wight)	growth rate (μ)
0	stationary phase	14.95±0.58	7.46±0.03	0.082±0.005
0.05 mM	stationary phase	16.67±0.17	18.86±0.03	0.084±0.004
0.22 mM	stationary phase	14.84±0.38	29.45±0.02	0.084±0.006
0.43 mM	stationary phase	8.78±0.84	29.32±0.02	0.084±0.005
0.86 mM	stationary phase	3.66±0.73	8.34±0.08	0.084±0.004

obtained with one carbon source alone (Table 2). On the other hand, LPS yields were decreased by increased amounts of nitrogen. At nitrogen concentration of 0.005%, LPS yields were noticeably decreased but protein was largely increased (Table 4).

LPS yields by different concentration of calcium and EDTA

The yield of LPS increased proportionally to calcium concentration, with a seven fold Ca^{2+} concentration (by 0.7 mM) increase resulting in a doubling of LPS yield (Table 5). Protein was inversely proportional to calcium concentration and increased greatly in the absence of calcium. Increases in EDTA concentration by 0.86 mM resulted in decreases in LPS yield, and 0.4 mM EDTA cut LPS yield to half of yield of LPS obtained at 0.05 mM EDTA (Table 6).

LPS yields on lenght and intensity of illumination and comparision of phototrophic and chemotrophic cells

Table 7. Quantitative values of LPS dependent on length of illumination using indirect competitive ELISA

length of illumination	carbon source (0.25%)	LPS(μg/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
50 hours	malate	17.64±0.79	17.96±0.05	0.084±0.005
38 hours	malate	10.02±0.50	13.78±0.07	0.080±0.004
32 hours	malate	9.59±0.52	10.54±0.03	0.079±0.004
28 hours	malate	3.97±0.29	5.20±0.02	0.074±0.006
20 hours	malate	3.26±0.15	4.48±0.03	0.071±0.005

Table 8. Yield of LPS dependent on light intensity and growth phase using indrict competitive ELISA

Light intensity	growth phase	LPS(μ/mg, dry weight)	Total protein (%/g, dry weight)	growth rate (μ)
700 Lux	stationary phase	3.07±0.57	4.58±0.03	0.080±0.005
2000 Lux	stationary phase	17.15±0.31	15.23±0.01	0.085±0.004
3500 Lux	stationary phase	21.36±0.14	57.45±0.02	0.088±0.004

(Anaerobic condition, 28±2℃)

Table 9. Comparision of phototrophic and chemotrophic cells of *Rb. capsulatus* DSM 1710

	Chemotrophic condition	Phototrophic condition
Length of cell	1.68±0.03 μm	2.13±0.02 μm
LPS(μg/mg)	9.98±0.57 μg	17.64±0.76 μg
Total protein(%/g)	12.77±0.01%	17.96±0.04%
Growth rate(μ)	0.080±0.005	0.084±0.003

(Dry weight 1 mg)

Tables 7 and 8 show that LPS increased according to the length of illumination and intensity of light. With 20~50 hours of illumination using malate as a carbon source, LPS increase from 3.26 ug/mg DW to 17.64 ug/mg DW, more than five times. At light intensities between 700 Lux and 3500 Lux, LPS yields increased from 3.07 ug/mg DW to 21.36 ug/mg DW, about seven times. Of course, growth rate and total protein also increased. Table 9 shows that cell length, LPS yield, and total protein under phototrophic condition has better efficiency than under chemoorganotrophic conditions. LPS yield of phototrophs was almost twice that of chemoor-

ganotrophs, 9.98 μg/mg to 17.64 μg/mg DW.

Discussion

Lipopolysaccharides (LPS) has always been purified with the hot phenol-water extraction following ultracentrifugation according to Westphal *et al.*(33). However LPS was purified by affinity chromatography of CNBr-activated sepharose-4B liganded IgG instead of ultracentrifugation. The antibody was isolated by protein A-sepharose CL-4B with organism of R-type LPS by Wu *et al.*(35). *Rhodobacter capsulatus* has an S-type LPS. In this study, affinity chromatography was applied to purification of LPS as in the experiments of Wu *et al.*(32) and Enzyme-Linked Immunosorbent Assay(ELISA) was used for measurement. LPS yields under several conditions were measured.

Appelmelk *et al.*(1), noticed that the advantage of this gel filtration method is that it didn't need large cultures of organism and repeated ultracentrifugation (3~4 h). And the convenience of the ELISA method is that it can measure LPS in nanogram amounts with a small amount of antigen (1 μg/test) and it has more sensitivity. *Rhodobacter capsulatus* DSM 1710 was measured also in nanogram units by LPS yields and cultured in milliliter instead of liter amounts. The two methods didn't show much difference in purity of LPS, but greater purity was obtained by ultracentrifugation of LPS with 2% cetavlon and RNase (Fig. 1). LPS yield by gel filtration was estimated at 1.69~1.9%/cell dry weight. Wu *et al.*(35), reported that gel filtration yielded more R-type LPS, but we didn't observe much difference between filtration and centrifugation.

Appelmelk *et al.*(1) proposed using LPS-BSA complexes for solid phase coating in ELISAs for rabbit anti-LPS antibody titration instead of LPS alone because of higher sensitivity. The use of BSA had no effect on the results in our experiments(Fig. 5.1 : 102400). This agreed with the observations of Riezu-Boj *et al.*(23), which noticed no difference between LPS-BSA and LPS alone.

Nucleic acid contamination was assesed according to Wu *et al.*(35). There was no absorption peak in the 260 nm region of the spectrum for the ultracentrifugation-purified LPS containing 2% cetavlon and RNase. Protein content was also 1% according to the Lowry method. Affinity chromatography had little effect on protein and nucleic acid contamination(Fig. 1).

Nikaido *et al.*(16), noticed that there were differences in lipopolysaccharide composition and yield according to the carbohydrate given. Linton *et al.*(14), showed the same phenomenon with exopolysaccharide. There was no large difference between individual carbon sources in the LPS yield of *Rhodobacter capsulatus* DSM 1710. LPS production was between 15 μg~19 μg/dry weight(DW) (Table 1). However, when two carbon sources were mixed, the LPS yields were increased (22~23 μg/DW). LPS yields increased with concentration of carbon sources up to 0.3% and decreased at 4.0%. In contrast, total protein content was the inverse of LPS yields. LPS was directly proportional to NH_4Cl concentration, with optimum yields at 0.05% nitrogen. In contrast to LPS yields, which decreased at 0.005% nitrogen concentration, total protein was increased 16 times (Table 4). Nikaido (16, 17) noticed calcium and EDTA influenced LPS production. The LPS yields by *Rhodobacter capsulatus* DSM 1710 increased linearly according to the concentration of $CaCl_2$ (0.09 mM~0.7 mM) (Table 5). Calcium salts play a crucial role in the organization of LPS, as indicated by Nikaido. At 0.05 mM EDTA concentration, there was maximum production of the LPS (16.7 μg/mg), but there wasn't a large difference without EDTA. Above this EDTA concentration (0.05 mM), LPS yields were reduced. At 0.86 mM EDTA, LPS yield was remarkably reduced(3 μg/mgDW) and total protein was also reduced. However, growth rate was not affected. There were effects of nutrition on exopolysaccharide production of *Aeromonas salmonicida* (3) and of *Butyrivibrio fibrisolvens* (26).

LPS yields were measured under several environmental variations: light intensity, varied illumination length, and chemotrophic culture. Increases in the light intensity (700, 2000, 3500 Lux) increased the LPS yields proportionally (3, 17, 21 μg/mg DW). Illumination time (20, 32, 38, 50 hr) also increased LPS proportionally (3, 9.5, 10, 17.6 μg/mgDW). The LPS yield under phototrophic conditions was almost twice (18 μg/mg DW) that under chemotrophic conditions(10 μg/mg DW). At this time, total protein was always proportional to LPS yields. We suppose active photosynthesis exhibites active LPS formation and protein synthesis.

According to the environmental conditions there were varied LPS yields.

It's more convenient to measure LPS yields in ng units with the ELISA method and to purify LPS with affinity chromatography instead of ultracentrifugation, but there isn't much difference in the purity and yield of the LPS between ultracentrifugation and affinity chromatography method.

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