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INDUCTION OF IRON REGULATED PROTEINS DURING NORMAL GROWTH OF Neisseria meningitidis IN A CHEMICALLY DEFINED MEDIUM

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SUMMARY

The expression of iron regulated proteins (IRPs) in vitro has been obtained in the past by adding iron chelators to the culture after bacterial growth, in the presence of an organic iron source. We have investigated aspects concerning full expression of the meningococcal IRPs during normal growth, in defined conditions using Catlin medium, Mueller Hinton and Tryptic Soy Broth (TSB). The expression of IRPs varied between different strains with respect to Ethylenediamine Di-ortho-Hidroxy-phenyl-acetic acid (EDDA) concentrations, and according to culture medium, and also between different lots of TSB. For each strain, a specific set of IRPs were expressed and higher EDDA concentrations, or addition of glucose, or use of different culture media did not resulted in a differential expression of IRPs. We were not able to grow *N. meningitidis* under normal growth conditions using Desferal. We looked for a good yield of outer membrane vesicles (OMVs) expressing IRPs in iron-deficient Catlin medium containing EDDA and Hemin. Culture for 32 h at 30°C after growing for 16 h at 37°C supported good bacterial growth. Bacterial lysis was noted after additional 24 h at 30°C. Approximately 4 times more OMVs was recoverable from a culture supernatant after 24 h at 30°C than from the cells after 16 h at 37°C. The IRP were as well expressed in OMVs from culture supernatant obtained after 24 h at 30°C as from the cells after 16 h at 37°C.

KEYWORDS: *Neisseria meningitidis;* Iron regulated proteins.

INTRODUCTION

Iron is an essential nutrient for bacterial growth and the ability of different bacteria to obtain iron (Fe) from the host during infection can define their potential for virulence[4,7,9,16,21,31]. In the extracellular space of the host, the concentration of free Fe is extremely low, a limiting factor for microbial growth. A major Fe source in the serum and extracellular compartment is transferrin[31], whereas on the mucosal surface it is lactoferrin[13]. Intracellular hemoglobin is generally unavailable to microorganisms.

Bacterial pathogens such as *Escherichia coli, Salmonella* sp. and *Pseudomonas aeruginosa* have evolved a system of Fe acquisition from the host, based on elaboration of soluble siderophores to

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compete with Fe binding to transferrin and lactoferrin[18,21]. The Fe-siderophore complex binds to surface siderophore receptors with internalization into the bacterial cell. In contrast, the Human restricted bacterial pathogens *Neisseria meningitidis, Neisseria gonorrhoeae* and *Haemophilus influenzae* do not produce siderophores, but are able to elaborate surface receptors for transferrin and lactoferrin under Fe-limiting conditions[6,15,16,19,23,26,32]. These receptors are glycoproteins termed iron regulated proteins (IRPs). In *Neisseria* there are 5 to 6 major IRPs and the molecular weights of the best characterized ones are generally 37, 70, and 85-105 KDa[8]. Removal of Fe from transferrin or lactoferrin by IRPs requires direct contact with the bacterial cell surface, and there is no uptake of these proteins into the cell. It is, however, not clear how the Fe is internalized[1,6,14,29].

The host specificity of the IRPs for human transferrin and lactoferrin has been demonstrated in vitro[1,25,27,28] and in vivo[24]. Meningococci are not able of obtain Fe bound to bovine transferrin[5,24,28] or lactoferrin[24,27]. This can explain in part the inability of human restricted pathogens to cause infection in other hosts. Bovine hemoglobin has, however, been used as an Fe source by meningococcal strains[2,24].

The antibody response of meningococcal patients to IRPs indicate that these proteins are expressed in vivo[3]. Simulated in vivo meningococcal growth conditions have shown that IRPs are probably induced during invasion[2]. Meningococcal IRPs have been suggested as possible vaccine antigens since they are antigenic and induce antibodies that may block Fe uptake. In addition, some monoclonal antibodies to the 70 KDa IRP have bactericidal activity[22].

In vitro, expression of IRPs requires growth under Fe starvation conditions[18,19,28,29]. This has been obtained by using ferric Fe chelators such as Desferrioxamine mesylate (Desferal) or Ethylenediamine Di-ortho-Hidroxy-phenyl-acetic acid (EDDA) in the presence of an organic Fe source in the form of transferrin, lactoferrin, hemin or hemoglobin in various culture media. A number of aspects concerning full expression of these outer membrane proteins under defined conditions requires further investigation. We therefore present in this paper optimized conditions for elaboration of meningococcal IRPs in a chemically defined medium[5] and present some qualitative and quantitative aspects of meningococcal growth in this medium.

MATERIALS AND METHODS

Strains: The strains of *N. meningitidis* used in this study were N.44/89 (B:4:P1.15), N.735/90 (B:8:P1.6), N.337/90 (B:NT:P1.14) and N.1002/90 (C:2b:P1.3,6) all from the Collection of the Bacteriology Branch, Adolfo Lutz Institute. Many of the experiments were performed using strain N.44/89.

Catlin Medium: The Catlin meningococcal defined medium was prepared according to the published formulation[5] using acid washed glassware and glass distilled water.For some batchs no Fe was added (incomplete Catlin, IC). A stock solution of 30 μM FeC13.6H2O was made in 10 μM sodium citrate solution and stored at 4 °C. Fe was added (10μM) to obtain complete Catlin (CC) medium. Stock solutions of 30 μM EDDA, 30 μM Desferal, 200 μM bovine hemin, 200 μM human hemoglobin and 200 μM bovine hemoglobin were prepared using chemicals obtained from Sigma Chemical Co., St. Louis, Mo, and stored at 4 °C. Tryptic Soy (TSB) and Mueller Hinton (MHB) broths were from Difco, Detroit, Mich.

For each experiment, the bacteria were first grown overnight in a candle jar on Tryptic Soy Agar (Difco) supplemented with 1% horse serum. Cells from the plate were inoculated to 50 ml of IC, TSB or MHB which were incubated for 4 h, 37°C at 100 rpm on gyrotory shaker. A volume of 10 ml of the 4 h culture was then used to inoculate 100 ml of IC, TSB or MHB which were incubated for the next 4 h under the same conditions. These last cultures were used as the inoculum for the various experimental growth conditions. In this manner any residual levels of inorganic iron were diluted out.

Addition of EDDA: A volume of 10 ml of 4 h subcultures of the 4 strains in IC and TSB were

inoculated into 100 ml of CC or TSB suplemented with 10, 20, 30, 40 or 50 μM EDDA. Also, 10 ml volumes of the 4 h subculture of the N.44/89 strain were inoculated into IC containing 3, 6, 9, 12, 18, 24 or 30 μM EDDA. The cultures were incubated overnight at 37 °C on the shaker at 100 rpm. Outer membrane vesicles (OMVs) were then isolated and purified (see below). Bacterial growth in IC was measured by absorbance at 540 nm (OD_{540}).

Evaluation of hemin and hemoglobin: A volume of 10 ml of an overnight culture of strain N.44/89 in IC was inoculated into 100 ml of CC or IC containing 30 or 60 μM of EDDA plus 5 μM of bovine hemin or 4 μM of human or bovine hemoglobin. These cultures were grown for 8 h, 37°C shaking at 100 rpm. From these 8 h cultures, 25-30 ml were inoculated into 400 ml of the same media formulation. These last cultures were grown overnight as before and harvested for preparation of OMVs.

Comparison of Fe chelators: A volume of 10 ml of a 4 h subculture of N.44/89 in IC was inoculated to 100 ml of CC or IC containing 5 μM of human hemin and 0, 15, 25, 35 or 50 μM EDDA or Desferal. The cultures were incubated as before, for 16 h. The OD_{540} of cultures was read at 0 and 16 h.

Release of OMVs containing IRPs during growth: A volume of 30 ml of a 4 h subculture of N.44/89 in IC was inoculated to each of 4 flasks containing 400 ml of CC, CC containing 30 (30μM EDDA CC) or 40 μM EDDA (40 μM EDDA CC) and IC containing 5 μM human hemin and 50 μM EDDA (50 μM EDDA IC + Hm). The cultures were incubated for 16 h at 37 °C, then an additional 32 h at 30°C, on the shaker at 100 rpm. The OD_{540} was measured and the OMVs were isolated from the cells and the culture supernatant after 16 h at 37 °C (T1), and after an additional 8 h (T2), 24 h (T3) and 32 h (T4) at 30 °C. The protein and keto-deoxy-octulonic acid (KDO) concentrations in OMVs recovered from the cells and culture supernatant at each time point were determined by LOWRY et al.[12] and thiobarbituric acid[20] methods, respectively. This experiment was also performed using the other 3 strains in CC containing the concentration of EDDA best correlated in earlier experiments to each strain for expression of IRPs. TSB and MHB with and without 40 μM EDDA (40 μM EDDA TSB, 40 μM EDDA MHB) were also used. To investigate the effect of glucose on growth and IRP expression, the 48 h growth experiment, using N.44/89, was made in CC and 40 μM EDDA CC and to both media were added 2.5 g/l glucose (CC + gl, 40 μM EDDA CC + gl), the same amount as contained in TSB.

OMV extraction: OMVs were isolated from cells and from culture broth using modifications of published methods[17,30]. The cultures were centrifugated at 5,000g for 30 min at 4°C. The cells were resuspended in 3-5 ml of Tris EDTA buffer (TE) pH 8.5, followed by 5 min ultrasonic pulses in a sonicating wather-bath, and centrifugated 10,000g for 25 min at 4°C. The cell pellet was discarded. The OMVs were pelleted at 100,000g for 2 h at 4 °C and resuspended in 100 to 500 ul of TE buffer.The OMVs from the culture supernatant were obtained by centrifugation at 100,000 g for 2 h. Aliquots in 1% glycerol were frozen at - 20°C.

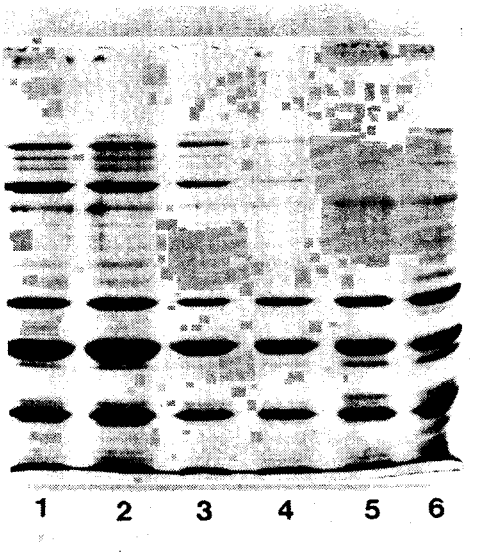

FIGURE 1. Expression of iron regulated proteins (IRPs) in Meningococcal strain N.735/90, in membranes obtained from cells grown in CC for 16 h at 37°C. Lane 1, with 50 μM EDDA; lane 2, with 40 μM EDDA; lane 3, with 30 μM EDDA, lane 4, with 20 μM EDDA; lane 5, with 10 μM EDDA; lane 6, no EDDA. Molecular weight standards are indicate in kilodaltons.

SDS-PAGE analysis: The OMVs were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) using the discontinuous buffer as described by LAEMMLI and 12% acrylamide[10]. After electrophoresis, the gels were stained by Coomassie Brilliant blue R-250.

RESULTS

Strain N.44/89, after growing on serum agar, was able to grow in 2 successive subsultures in IC for 4 h each, but did not grow in the next IC culture containing 3 to 30 μM EDDA. We therefore initially grow the bacteria in successive cultures in IC to remove the iron contamination from the inoculum. Using CC, the different strains all grew to some extent in all concentrations of EDDA, from 10 to 50 μM (Table 1). It is seen that the higher the EDDA concentration the lower the bacterial growth; the decreases in bacterial growth were greater with concentrations of EDDA higher than 30 μM. Expression of IRPs varied from strain to strain with respect to EDDA concentration. Addition of 10 or 20 μM EDDA was not enough to reduce levels of free Fe sufficiently to induce maximal IRPs expression. In figure 1, all of the high molecular weight IRPs characteristic of strain N.735/90 are seen and with EDDA concentrations as low as 20 μM EDDA the IRPs can be weakly seen. The concentration of EDDA needed for maximal IRP expression without making reduction in growth was strain dependent. Approximately 40 μM was optimal for strains N.44/89 and N.735/90, and 30 μM for N.337/90 and N.1002/90. Experiments in TSB demonstrated that the EDDA concentration necessary for IRP expression varies according to the production lot of media. Some TSB lots required much higher EDDA concentrations than others for inducement of IRPs.

We evaluated bacterial growth and IRP expression using an organic Fe source in Fe deficient medium containing EDDA. Addition of 5 μM bovine hemin, or 4 μM human or bovine hemoglobin to IC containing 30 or 60 μM EDDA improved considerably the growth of N.44/89. However, IRPs were expressed only when hemin was used as an Fe source in IC. The hemoglobin precipitated when added to the Catlin medium, and other experiments suggested that the precipitation was due to the salt concentration in IC.

Growth of N.44/89 in IC containing 5, 1, or 0,5 μM Fe or 5 μM human hemin was similar among them. The IRP were expressed weakly with 5 μM Fe in IC, being more evident using the 1, or 0,5 μM Fe or with 5 μM hemin.

The experiment to compare Fe chelators showed that strain N.44/89 was not able to grow in IC in presence of 15 to 50 μM of Desferal supplemented with 5 μM hemin as a source of Fe, while good growth occurred even with 50 μM EDDA (Table 2). Desferal appeared to chelate iron too avidly.

The yield of OMV recovered from the cells and from the broth following growth in CC, IC, TSB or MHB were compared. The bacteria were grown in the different media for 48 hs with and without 40 μM EDDA (Fig. 2). The CC and IC media supported a good level of growth of N.44/89 for approximately 24 h at 30°C (T3),

TABLE 1

Growth[a] of *N. meningitidis* in CC containing different concentrations of EDDA[b].

CC + EDDA (uM)	Change in OD_{540}[c]			
	Strains			
	N. 44/89	N. 735/90	N. 337/90	N. 1002/90
CC + 0	1.03	1.19	1.34	1.04
CC + 10	1.03	1.16	1.22	1.04
CC + 20	0.94	1.13	1.14	0.92
CC + 30	0.89	1.14	0.99	0.50
CC + 40	0.69	0.94	0.62	0.36
CC + 50	0.37	0.94	0.63	0.15

a Growth for 24 h at 37°C on gyrotory shaker at 100 rpm

b EDDA - Ethylenediamine
Di-ortho-Hidroxy-phenylacetic acid

c OD540 - Final absorbance minus initial absorbance

and after this period, bacterial lysis increased. As expected, addition of EDDA to CC partially inhibited bacterial growth. Using 40 μM EDDA, we noted early bacterial lysis after 8 h at 30°C (T2).

The protein and LPS concentrations were measured in OMVs isolated from cells and from culture supernatant in IC containing 5 μM hemin and 50 μM EDDA. The protein and LPS concentrations from cells decreased with culture time probably due to bacterial lysis, whereas they increased in the culture supernatant, showing that meningococci can release large amounts of OMVs during stationary growth at 30°C (Table 3). Thus, aproximately 4 times more OMVs were recoverable from the culture supernatant at 48 h than from the cells at 16 h. Analyses by SDS-PAGE showed that IRPs were well expressed in OMVs recovered from OMV obtained from cells, after 16 h at 37°C and up to 48 h from the culture supernatant. The amount of IRPs relative to other outer membrane proteins did not change over the 48 h period (Fig. 3). Similar results were seen when the three other strains were grown in the same manner (Fig. 4). OMVs recovered from the culture supernatant after an additional 32 h at 30°C have well expressed IRPs.In response to Fe-starvation, 3 new proteins between 70 and 108 KDA were expressed by all 4 strains under various growth conditions and culture media. The corresponding IRPs expressed for each strain were shown to differ somewhat in their apparent molecular weights. When the intensity of Coomassie blue-stained bands on SDS-PAGE gels are compared, the 70-71 KDa and 107-108 KDa proteins are expressed in larger quantities while proteins between 83-92 KDa are weakly expressed. Smaller quantities of the 37 KDa IRP, could be observed only in the N.44/89 strain.

TABLE 2

Growth[a] of *N. meningitidis* strain N. 44/89 in IC[b] supplemented with 5 uM hemin and different concentrations of EDDA[c] or Desferal (DF)

Addition(s) to culture media	Change in OD_{540}[d]
10 uM Fe[e]	1.01
5 uM hemin	0.83
5 uM hemin + 15 uM EDDA	0.64
5 uM hemin + 25 uM EDDA	0.63
5 uM hemin + 35 uM EDDA	0.68
5 uM hemin + 50 uM EDDA	0.61
5 uM hemin + 15 uM DF	- 0.02
5 uM hemin + 25 uM DF	- 0.08
5 uM hemin + 35 uM DF	- 0.09
5 uM hemin + 50 uM DF	- 0.08

a Growth for 24 h at 37°C on gyrotory shaker at 100 rpm
b Fe deficient Catlin medium
c EDDA - Ethylenediamine Di-ortho-Hidroxy-phenylacetic acid
d OD540 - Initial absorbance minus final absorbance
e Same as complete Catlin medium

Comparing TSB and MHB with CC for long term culture at 30°C, we found that bacterial growth was greatest in TSB (Fig. 5). Addition of 40 μM EDDA to TSB reduced the bacterial growth, but growth reduction was less than when EDDA was added to MHB or CC. Addition of EDDA to TSB or to MHB did not increase bacterial lysis after 24 h, as occurred in CC.There was no difference in the expression of major outer membrane proteins and IRPs in membranes of N.44/89 grow in TSB, MHB or CC containing

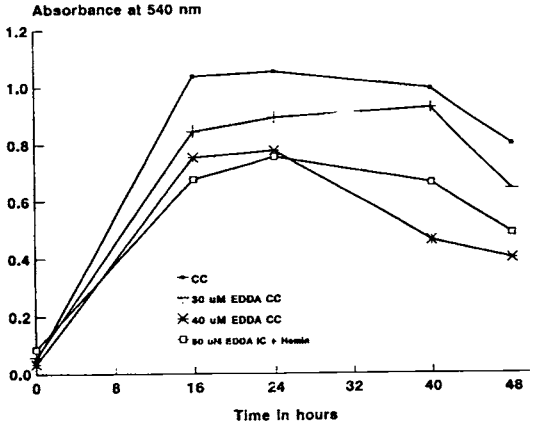

FIGURE 2. Growth of Meningococcal strain N.44/89 in complete (CC) and incomplete (IC) Catlin media at 37°C for 16 h then at 30°C for up to 32 h.

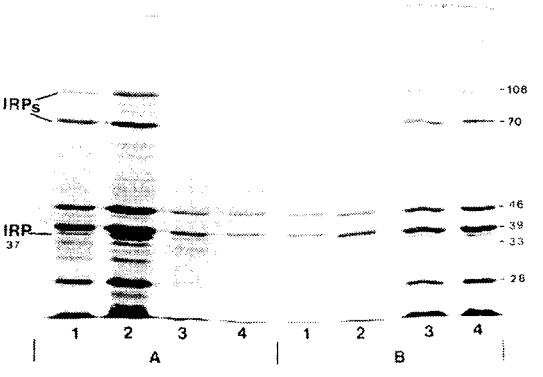

FIGURE 3. Membranes from cells (A) and culture supernatant (B) of Meningococcal strain N.44/89 grown in incomplete Catlin containing 5 μM hemin and 50 μM EDDA. The growth times at temperature are: Lane 1, 16 h at 37°C; lane 2, + 8 h at 30°C; lane 3, + 24 h at 30°C; lane 4, + 32 h at 30°C. Molecular weight standards are indicate in kilodaltons.

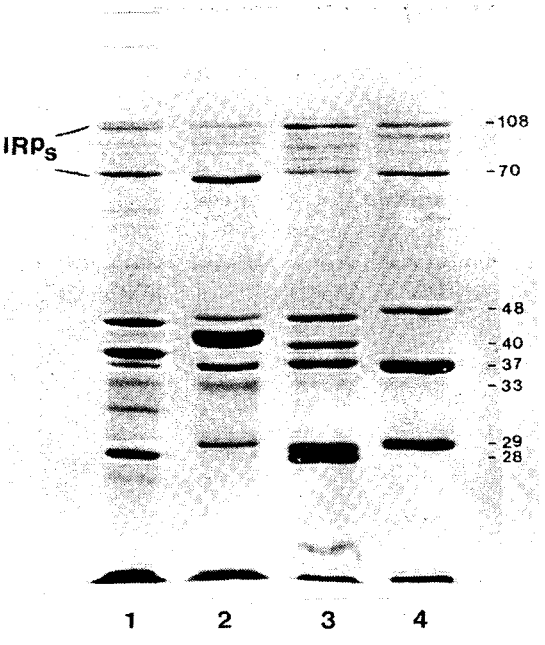

FIGURE 4. Expression of IRPs on membranes recovered from the culture supernatant of CC containing optimized amounts of EDDA, for 16 h at 37°C then 32 h at 30°C. Lane 1, N.44/89 (40 μM EDDA); lane 2, N.1002/90 (30μM EDDA); lane 3, N.337/90 (30μM EDDA); lane 4, N.735/90 (40 μM EDDA). Molecular weight standards are indicated in kilodaltons.

EDDA. The protein yield from the culture supernatant in these media at T4 were 11.4, 8.0 and 4.32 mg/l respectivelly.

Addition of glucose to CC and 40 μM EDDA CC improved growth of N.44/89 during the first 16 h at 37°C, but it also increased bacterial lysis after this period (Fig. 6). Recovery of protein from cells grown for 16 h at 37°C was approximately 6 times higher (6,17 mg/l) when glucose was added to the medium, however after 32 h at 30°C the recovery of proteins from broth decreased 4 fold (1,32 mg/l). There was no difference in expression of different outer membrane proteins when glucose was added to CC.

DISCUSSION

Addition of Fe-chelators only to culture media used for expression of IRPs inhibits bacterial growth, making it necessary to add Fe to enable growth. Previous studies have shown that meningococci can assimilate a variety of iron salts added to the culture media, as well as organic Fe such as hemin and hemoglobin[15,16].

TABLE 3

Protein and LPS concentrations from OMVs isolated from cells and culture supernatant of strain N. 44/89, following growth in incomplete Catlin medium supplemented with 50 uM[a] EDDA and 5 uM hemin.

Time in culture (h)	Temperature (°C)	OMVs recovered from			
		Cells		Supernatant	
		Protein[b]	LPS[c]	Protein	LPS
16 (T1)	37	1.12	1.15	-[d]	0.88
24 (T2)	30	0.76	0.45	0.96	1.64
40 (T3)	30	0.32	0.24	3.68	3.04
48 (T4)	30	0.04	-	4.32	4.32

a EDDA - Ethylenediamine Di-ortho-Hidroxy-phenylacetic

b Lowry method, mg/ml

c estimative from KDO by thiobarbituric acid method, mg/ml KDO x 20

d - not determinated

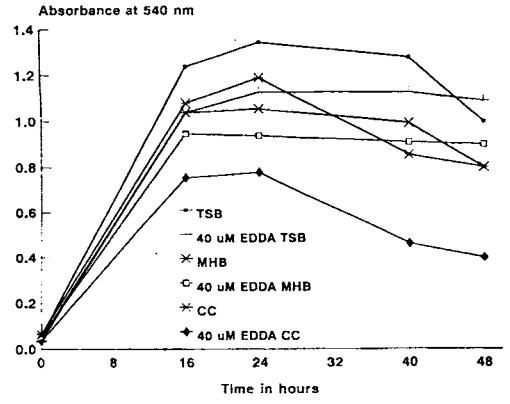

FIGURE 5. Comparative growth of strain N.44/89 grown in different media at 30°C, after an initial 16h at 37°C.

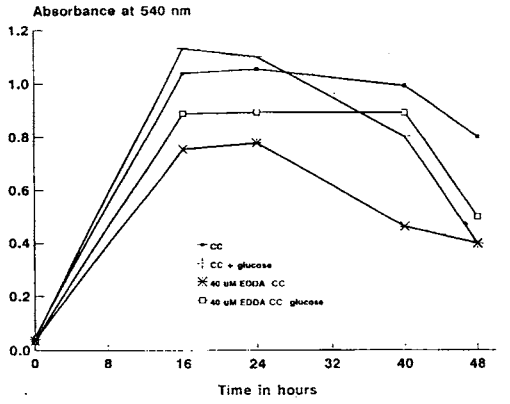

FIGURE 6. Effect of glucose (2.5 g/l) on prolonged growth of strain N.44/89 in Catlin media at 30°C.

We found that meningococci could be subcultured twice in IC with unrestricted growth, due probably to small amounts of Fe contamination present in culture media and because the bacteria were initialy grown in a rich serum containing medium. The amount of EDDA needed to be added to different growth media to obtain optimal expression of IRPs varied from medium to medium and also between different lots of TSB medium. In a chemically defined media, such as Catlin medium, the available Fe is more controllable, and in Catlin medium we were able to observe that the level of EDDA necessary for maximal expression of IRPs varied depending on the nutritional requirements of the strain. For each strain a specific set of IRPs were all expressed and higher EDDA concentrations resulted in enhanced expression of these IRPs, but not in differential expression, as would have been expected if expression of each IRP was under separate genetic control.

Previously, WEST & SPARLING[32] found that expression of IRPs in gonococci varied depending upon the strain and the source of Fe. In our experiments, using different sources of organic Fe, we verified that IRPs were expressed with hemin. In contrast, addition of bovine or human hemoglobin reversed the growth limitation imposed by high levels of EDDA, but increased expression of IRPs was not detected. Expression of IRPs using human or bovine hemoglobin has been demonstrated by some investigators[2,32]. They, however, used high concentrations of Desferal as an Fe chelator. Desferal appears to be a stronger chelator than EDDA, since addition of as little as 15 μM Desferal to IC containing 5 μM hemin blocked all growth. MICKELSEN & SPARLING[15] were unable to grow *Neisseria* species in a chemically defined medium containing Desferal and an excess of inorganic Fe. Also, NORQUIST et al.[19] demonstrated that gonococci are unable to utilize Desferal-bound iron. For these reasons Desferal is added to culture medium after bacterial growth to induce IRP expression[2,32].

BANERJEE-BHATNAGAR & FRASCH[2] show that expression of IRPs can be induced without cell division when added during stationary phase of growth, but were unable to have both normal meningococcal growth and high levels of expression of IRPs. We therefore utilized a less avid Fe chelator and prolonged growth to allow release of large amounts of OMV expressing IRPs. Since autolysis must be avoided, the temperature was reduced to 30°C after growth for 16 h at 37°C. For all three media studied, the best growth time was 32 h at 30°C following 16 h at 37°C. Under those culture conditions, it was possible to obtain large amounts of OMV containing IRPs from the culture supernatant.

It was not clear which was the best medium to be used for IRP expression. Although TSB containing EDDA provided better bacterial growth

than MHB, and the meningococci growing in both released large amounts of OMVs containing IRPs. However, TSB and MHB contain high molecular weight complexes, which should be removed from the media by dialysis or ultrafiltration because they may interfer with the large scale OMV purification processes. These considerations are particularly important when the OMV are to be used in developing a vaccine. Controlable culture conditions are required, and a defined medium is important. Bacterial growth in Catlin medium containing EDDA allowed release into the culture medium of large amounts of OMVs expressing IRPs. Use of an inorganic Fe source instead of hemin facilitated preparation of the medium and avoided variations in Fe concentration. The increased bacterial growth in exponencial growth phase by addition of glucose to CC could be usefull since greater yields of OMVs could be obtained after 32 h at 30°C, but less than 2.5 g/l could be used to reduce bacterial lysis.

Controlled culture conditions appropriate for obtaining good growth yields and high levels of IRP expression are important for studies of IRP regulation and expression as well as for vaccine development. We have demonstrated that large amount of OMVs containing IRPs can be obtained from the culture supernatant following near normal growth in presence of inorganic iron and EDDA.

RESUMO

Indução das proteínas reguladas pelo ferro durante o crescimento normal de *Neisseria meningitidis* em meio quimicamente definido.

A expressão das proteínas reguladas pelo ferro (IRPs), in vitro, tem sido obtida pela adição de quelantes de ferro ao meio de cultura, após o crescimento bacteriano, na presença de fonte de ferro orgânico. Neste estudo foram investigados aspectos da máxima expressão das IRPs de meningococo durante o crescimento normal, em condições de cultura definidas, utilizando-se o meio de Catlin e os caldos Mueller-Hinton e Tryptic Soja (TSB). Foram avaliadas as melhores condições para se obter vesículas de membrana externa (OMVs) contendo IRPs para uso em vacina de meningococo B. A expressão das IRPs variou entre as diferentes cepas com relação as diferentes concentrações de Etilenediamine Di-orto-Hidroxifenil-ácido acético (EDDA), de acordo com o meio de cultura e também entre os diferentes lotes de TSB. Para cada cepa, um específico padrão de IRPs foi expresso e altas concentrações de EDDA, adição de glicose ou o uso de diferentes meios de cultura não resultou em expressão diferencial das IRPs. Não foi possível cultivar meningococo em condições normais de crescimento utilizando-se Desferral. Foi investigado condições de crescimento para se obter bom rendimento de OMVs com expressão de IRPs em meio de Catlin deficiente em ferro, contendo EDDA e hemina. Cultura de 32 h a 30°C, após incubação por 16 h a 37°C, manteve bom crescimento, porém, lise da bactéria foi observada após 46 h a 30°C. Aproximadamente 4 vezes mais OMVs foram recuperadas do sobrenadante de cultura após 24 h a 30°C do que das células após 16 h a 37°C. As IRPs foram bem expressas nas OMVs obtidas do sobrenadante de cultura após 24 h a 30°C como também nas OMVs obtidas das células crescidas por 16 h a 37°C.

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