Influence of Nutrient Limitation and Growth Rate on the Outer Membrane Proteins of *Klebsiella aerogenes* NCTC 418

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Four major proteins with molecular weights of 78 000, 37 000, 34 000 and 20 000 were present in the envelope of *Klebsiella aerogenes* when cultured at a high specific growth rate. However, at lower growth rates, the protein content and composition of the envelope depended on the imposed nutrient limitation. Under potassium-, carbon-, sulphur- and phosphorus-limited conditions, derepression of synthesis of limitation-specific proteins was observed, their apparent molecular weights being 90 000, 48 000, 41 000 and 36 000, respectively. Nitrogen-limited cells had no additional proteins. For a particular limiting nutrient, expression of the limitation-specific proteins was independent of the chemical or physical form in which the nutrient was supplied. Under potassium or sulphur limitation the specific proteins were present maximally at the lowest imposed growth rate, whereas under carbon limitation a maximum expression of these proteins was found at moderate growth rates. It is concluded that limitation-specific proteins which are associated with the outer membrane function in the uptake of limiting nutrients or, possibly, limitation-releasing compounds.

INTRODUCTION

The outer membrane of Gram-negative bacteria separates the growth environment from the periplasmic space and forms a selective barrier for permeation of solutes. This is accomplished by a class of trans-membrane proteins, called porins, that form aqueous pores through which nutrients may pass (Di Rienzo *et al.*, 1978; Nikaido & Nakae, 1979). In *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*, outer-membrane proteins (OMPs) form non-specific channels through the outer membrane, selecting only on size and hydrophilicity the molecule that can be transported (Nikaido *et al.*, 1980; Nakae, 1976; Hancock *et al.*, 1979). Other OMPs are involved in a more specific uptake of nucleosides (Hantke, 1976), amino acids (Heuzenroeder & Reeves, 1981) and vitamin B₁₂ (Di Masi *et al.*, 1973), whereas permeation of maltose and maltodextrins in *E. coli* preferably occurs through the MalB protein (Szmecman & Hofnung, 1976; Nakae & Ishii, 1980). Furthermore, a number of proteins are related to the uptake of chelated iron in *E. coli* (Hancock *et al.*, 1976) and in *Vibrio cholerae* (Sigel & Payne, 1982). When growth was restricted by the supply of the phosphorus source, expression of the PhoE protein was observed in *E. coli* (Argast & Boos, 1980; Overbeeke & Lugtenberg, 1980) and of protein P in *P. aeruginosa* (Hancock *et al.*, 1982). Both proteins appeared to form channels through the outer membrane with affinity for phosphorus-containing compounds and anionic solutes (Hancock *et al.*, 1982; Overbeeke & Lugtenberg, 1982; Nikaido *et al.*, 1983). Physiologically, this was a remarkable observation, since the substrate that was actually growth-limiting could be adequately transported through the general aqueous pores present in the outer membrane of both species (Nikaido *et al.*, 1980; Hancock *et al.*, 1979; Benz & Hancock, 1981).

Abbreviation: OMP, outer-membrane protein.

In this study we investigated whether synthesis of limitation-specific OMPs is a more general phenomenon. In addition, we were interested in the effect of growth rate on the expression of the OMPs. In order to obtain steady-state nutrient-limited growth, we used continuous cultures of *Klebsiella aerogenes*. This species was chosen because a pronounced phenotypic response with respect to the functioning of uptake systems located within the cytoplasmic membrane had already been demonstrated in this organism (Tempest *et al.*, 1970; Neijssel *et al.*, 1975; Sterkenburg & Wouters, 1981). Furthermore, earlier studies of Robinson & Tempest (1973) demonstrated that this organism can easily alter the protein composition of its cell envelope as a response to growth conditions.

METHODS

Organism. *Klebsiella aerogenes* NCTC 418 was maintained by monthly subculture on nutrient agar.

Growth conditions. Organisms were grown aerobically in a 300 ml chemostat (Bioflo, New Brunswick Scientific). Media were based on the following compositions: 5 mM- NaH_2PO_4 , 50 mM- NH_4Cl , 5 mM-KCl, 1 mM- Na_2SO_4 , 1 mM-citric acid, 0.63 mM- MgCl_2 , 0.01 mM- CaCl_2 , 1.5% (w/v) glucose and trace elements as described by Evans *et al.* (1970). In carbon-limited media, the amount of the carbon source (glucose, glycerol or maltose) was lowered to 0.1% (w/v); phosphorus-limited media contained 0.2 mM- NaH_2PO_4 or 0.2 mM-*sn*-glycerol 3-phosphate; for nitrogen limitation 3 mM- NH_4Cl (or 3 mM- NaNO_3 , or 3 mM-sodium glutamate) was present in the medium and 47 mM-NaCl was added for adjustment of medium osmolarity; sulphur limitation was obtained with 0.04 mM- Na_2SO_4 or 0.04 mM-methionine, and potassium limitation with 0.04 mM-KCl in the medium. Growth temperature was 37°C; the culture pH was monitored *in situ* and was automatically maintained at 6.8 by addition of 1 M-NaOH (phosphorus limitation) or 4 M-NaOH (other limitations). Samples were withdrawn when the culture had reached a steady state. In batch culture, organisms were grown in Nutrient Broth (Difco) at 37°C under vigorous aeration.

Isolation of cell envelopes. Culture samples (50 ml, containing approximately 20 mg dry weight of bacteria) were washed with 0.9% (w/v) NaCl; cells were resuspended in 10 ml buffer containing 50 mM-Tris/HCl and 2 mM-EDTA (pH 8.5) and subjected to ultrasound (80 W; Sonifier, Branson Instruments, Danbury, Conn., USA) for periods of 15 s with cooling intervals on ice. A cell-free extract was obtained by centrifugation at 1000 *g* for 20 min (4°C) in a Sorvall RC2-B centrifuge and crude envelopes were collected from the resulting supernatant by subsequent centrifugation (12000 *g*, 1 h, 4°C) and resuspended in 2 mM-Tris/HCl buffer, pH 7.8, to a final concentration of 3–10 mg protein ml^{-1} .

Extraction of envelope proteins. Envelopes were washed twice with 2 mM-Tris/HCl, pH 7.8, and resuspended in 10 mM-HEPES, pH 7.4, containing 2% (w/v) Triton X-100 (Schnaitman, 1971). After 15 min at room temperature, samples were cooled on ice, and the insoluble fraction was collected by centrifugation (49000 *g*, 1 h, 4°C) and resuspended in 2 mM-Tris/HCl, pH 7.8, to a final concentration of 40 mg protein ml^{-1} .

Equilibrium sucrose density gradient centrifugation. Crude envelopes (approximately 20 mg protein in 0.5 ml) were layered on top of a discontinuous 35–55% (w/w) sucrose gradient in 10 mM-HEPES, pH 7.4 (5% concentration intervals) and centrifuged for 48 h at 131000 *g* (4°C) in a MSE Super Speed 75 ultracentrifuge. The gradient was fractionated into 0.5 ml samples with continuous recording of A_{260} (Uvicord, LKB). Samples were diluted fivefold with 10 mM-HEPES, pH 7.4, concentrated by centrifugation at 49000 *g* (2 h, 4°C) and resuspended in 2 mM-Tris/HCl, pH 7.8.

SDS-polyacrylamide gel electrophoresis. This was performed as described by Lugtenberg *et al.* (1975). Crude envelopes were boiled for 5 min in gel sample buffer and samples were applied to a 11% (w/v) polyacrylamide gel. Gels were run at 30 mA constant current. Protein bands were stained with an aqueous solution of 0.04% (w/v) Coomassie Blue G250 containing 25% (v/v) 2-propanol and 7% (v/v) acetic acid and destained in a 12.5% (v/v) 2-propanol/7% (v/v) acetic acid mixture. Molecular weights were determined from relative mobilities when compared with high-molecular weight protein standards (Bio-Rad).

Densitometric analysis of gels. To obtain quantitative densitometric data, three different amounts of total envelope proteins were applied to the gels (approximately 10, 20 and 40 µg protein). Protein patterns were analysed by densitometric tracing (densitometer model DD2, Kipp & Zonen, Delft, The Netherlands) and the peaks were integrated (model 602 Minigrator, Spectra Physics, Santa Clara, Calif., USA). Integration values obtained from the three samples of each envelope preparation were compared, and for each individual OMP band a correction was made to allow for differing saturation kinetics of staining. Corrected integration values were correlated with values found for three standard amounts (2.5, 5 and 10 µg, respectively) of bovine serum albumin, that had also been applied to each gel. Intensities of OMP bands were converted to equivalents of the albumin standard and then expressed as percentages of the total number of OMP albumin-equivalents present in a sample.

Chemicals. SDS (90% pure) was from Merck; SDS (95% pure) was from Sigma, a 1:1 (w/w) mixture was used; bovine serum albumin and Coomassie blue G250 were from Sigma. All other reagents were analytical grade.

Fig. 1. Densitometric tracings of the membrane protein pattern of batch-grown *K. aerogenes*. Organisms were cultured in nutrient broth and samples were withdrawn from the early exponential phase (a) and the stationary phase (b). Proteins of crude envelopes from these samples were separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue G250. The obtained protein pattern was analysed by densitometry. Apparent molecular weights of the major peaks are indicated.

RESULTS

Envelope proteins in batch culture

In batch culture, growth occurs at an optimal rate until it becomes restricted, and subsequently stopped, by exhaustion of some nutrient(s) or accumulation of excreted products. We examined the effect on the envelope proteins of the large differences in growth conditions between the early exponential phase and the stationary phase. The cell envelope contained four major proteins with apparent molecular weights of 78 000 (78 kDal), 37 kDal, 34 kDal and 20 kDal. The protein composition was not markedly dependent on the growth phase from which cells had been harvested. Only minor differences in relative amounts of the 34 kDal and the 37 kDal proteins could be detected (Fig. 1 *a, b*). Treatment of envelope samples with Triton X-100 yielded an insoluble fraction that was enriched with these major proteins.

OMPs in continuous culture

Effect of nutrient limitation on the OMPs. In the chemostat, the protein composition of the cell envelopes depended on the imposed growth conditions. Under nitrogen-limited conditions the protein profile was similar to the pattern found in batch culture (Fig. 2 *a, d*). The 44 kDal protein that appeared in these samples presumably was mainly situated in the cytoplasmic membrane, since in sucrose density gradients only a small amount of this protein was found in the outer membrane fraction (Fig. 3 *b*). This protein was solubilized by treatment of the envelopes with Triton X-100. The protein profile was independent of the nature of the nitrogen source because it was found not only under ammonia limitation, but also under nitrate- and glutamate-limited conditions. When phosphorus (inorganic phosphate as well as *sn*-glycerol 3-phosphate) was the growth-limiting nutrient, the 37 kDal protein was present to a much lesser extent. Instead, a protein that moved slightly faster was found with an apparent molecular weight of 36 kDal (Fig. 2 *b*). Under sulphur (sulphate, methionine) limitation the 34 and 37 kDal proteins were present, and relatively high amounts of a 41 kDal protein were found (Fig. 2 *d*). Glucose-limited cultures had a membrane protein profile that was rather comparable with the pattern found under nitrogen limitation; however, in these cultures large amounts of a 48 kDal protein were detected (Fig. 2 *f*). This protein was also present in comparable amounts in the envelopes of glycerol- or maltose-limited cultures.

In the high molecular weight range, significant alterations in the protein pattern were found under potassium limitation. Under these conditions a protein band with apparent molecular

Fig. 2. Membrane proteins from chemostat cultures of *K. aerogenes*, grown under (a,c) ammonia-, (b) phosphate-, (d) sulphur-, (e) potassium- and (f) glucose-limitation. Molecular weights of proteins in the reference standard (MW) are indicated.

weight of 90 kDal was present to a much higher extent (Fig. 2e) than in cultures grown with excess of potassium.

Crude envelopes from chemostat-grown cells were subjected to sucrose density gradient centrifugation. Samples separated into two fractions (Fig. 3a), with the major proteins being associated for the most part with the high-density membrane fraction (Fig. 3b). Envelope samples from glucose-limited cultures were also treated with Triton X-100. The major proteins shown in Fig. 2(f) were recovered in the insoluble fraction (Fig. 4). Similar experiments were done on envelopes from phosphate-, sulphate- and potassium-limited cells, and these demonstrated that the 36 kDal, 41 kDal and 90 kDal proteins, respectively, were also Triton X-100-insoluble (data not shown). Thus, this procedure gave an insoluble fraction enriched with the proteins that were associated with the high-density fraction obtained after sucrose density centrifugation of the envelopes (see Fig. 3). It was therefore concluded that the additional limitation-specific, Triton X-100-insoluble proteins present in the crude envelope preparations were mainly located within, or closely associated with the outer membrane.

Effect of growth rate on the OMPs. The phenotypic variability of the OMP pattern shown in Fig. 2 was found in cultures with a low growth rate; that is, with an extremely low extracellular concentration of the growth-limiting nutrient. In order to study the relationship between this concentration and the OMP profile, the effect of the culture dilution rate on the protein composition of the other membrane was investigated.

In Fig. 5 the relationship between the intensity of the OMP bands and the growth rate is shown for potassium-limited cultures. At low growth rates the expression of the 90 kDal protein was very pronounced. The amount of this protein decreased with increasing growth rates and was present in only small amounts at a culture dilution rate of 0.7 h^{-1} .

To measure the relative amounts of OMPs, the output of the densitometric analysis was integrated. In Fig. 6 relative amounts of OMPs are expressed as percentages of the total amount

Fig. 3. Sucrose density centrifugation of crude envelopes from cells grown under nitrogen limitation at a growth rate of 0.2 h^{-1} . Envelopes were centrifuged on a discontinuous sucrose gradient, and eluted as described in Methods. (a) Elution of the gradient as recorded by its absorbance (in arbitrary units) at 260 nm; (b) SDS polyacrylamide gel electrophoresis of samples ($200 \mu\text{g}$ protein) from the fractions 6 (lane A) and 30 (lane B), respectively. MW, molecular weight standard.

Fig. 4. Extraction of proteins from crude envelopes of cells, grown under glucose limitation at a growth rate of 0.2 h^{-1} . Membrane samples were resuspended in buffer containing Triton X-100, left for 15 min at room temperature, and the insoluble fraction was collected by centrifugation. Proteins from crude envelopes (a) and the Triton X-100-insoluble fraction (b) were separated by SDS-polyacrylamide gel electrophoresis, stained and the protein pattern obtained analysed by densitometry.

of OMPs. Generally, the amount of limitation-specific OMPs decreased with increasing growth rates. Under potassium limitation (Fig. 6a) the 90 kDal protein amounted to 48% of the total OMP at a low culture dilution rate (0.1 h^{-1}) and was barely detectable at a growth rate as high as 0.7 h^{-1} . Likewise, under sulphur limitation (Fig. 6c) the highest amount of the 41 kDal protein (18%) was found at a low growth rate (0.2 h^{-1}) and its amount also decreased gradually at higher growth rates. This protein was still present in small amounts at a very high dilution rate (1.2 h^{-1}), which was near to the maximum specific growth rate of *K. aerogenes* under the imposed chemostat conditions. The amount of 48 kDal protein present under carbon limitation

Fig. 5. Effect of growth rate on the protein composition of membranes from potassium-limited cultures. Samples were withdrawn from steady state cultures with growth rate 0.1 h^{-1} (a), 0.4 h^{-1} (b), 0.5 h^{-1} (c), 0.6 h^{-1} (d) and 0.7 h^{-1} (e). Further experimental procedures were as described in the legend to Fig. 1.

(Fig. 6b) was maximal (34%) at a dilution rate of 0.3 h^{-1} , and decreased to barely detectable levels at a higher growth rate (1.1 h^{-1}).

DISCUSSION

Differences in protein localization within the cell envelope may result in differences in the extent of solubilization of individual proteins upon treatment of membrane samples with detergents. Both Triton X-100 and Sarkosyl NL-50 have been used extensively for the isolation of the OMPs (Schnaitman, 1971) and penicillin-binding proteins (Spratt, 1977) in *E. coli*. Differential solubility in Triton X-100 has been used as a basis for the isolation of the outer membrane and purification of OMPs in *Pseudomonas* (Hancock & Carey, 1980; Hancock *et al.*, 1982), *Proteus* and *Klebsiella* (Hofstra & Dankert, 1979; Hofstra *et al.*, 1980). In the *Klebsiella* strain studied in this paper, proteins associated with the outer membrane were selectively retained in the envelope fraction after treatment with Triton X-100 (Figs 1 and 2).

The OMP profile of early exponential phase batch cultures in nutrient broth was comparable with the pattern found in chemostat cultures with a high growth rate. In stationary phase cells, no additional OMP was detected, suggesting that growth may have been halted by nitrogen starvation. However, it is possible that the changes in growth conditions between the late exponential and the stationary phases may have occurred over too short a period, thereby not allowing the culture enough time for its cells to adapt.

Differences in the protein composition of the outer membrane affected by alterations in

Fig. 6. Relationship between growth rate and relative intensity of the major OMPs of *K. aerogenes*, grown under (a) potassium-, (b) carbon- and (c) sulphur-limitation. Electrophoretic patterns were analysed by densitometry and integration of the peaks obtained. Intensities of the 90000 (▨), 48000 (■), 41000 (□) and 34000 (▤) molecular weight proteins were expressed as percentages of the total amount of OMPs as described in Methods. Data represent the mean values of components from traces of at least three different samples from at least two different chemostat runs. Individual values varied at most by 20% of the mean value.

growth conditions may give an indication of the function of the OMPs. The synthesis of the 36 kDa protein was specifically derepressed under phosphorus limitation, and this was also found for the PhoE protein in *E. coli* (Overbeeke & Lugtenberg, 1980) and protein P in *P. aeruginosa* (Hancock *et al.*, 1982). It is conceivable that the 36 kDa protein is functionally related to the PhoE pore protein, enabling phosphorus-containing compounds to penetrate the outer membrane efficiently. Similarly, the 48 kDa protein found under carbon limitation, and the 41 kDa protein found under sulphur limitation, might function in an efficient uptake of carbon- or sulphur-containing compounds, respectively. The protein composition of the cell envelope under nitrogen limitation indicates that under these conditions the outer membrane provides the means of uptake of nitrogen-containing compounds without an additional protein. Indeed, the relatively high amounts of the 44 kDa Triton X-100-soluble protein indicate that nitrogen-limited conditions rather affect the composition of the cytoplasmic membrane. The 48 kDa protein found under carbon limitation may be functionally related to the MalB protein in *E. coli* that forms pores for maltose and maltodextrins (Szmecman & Hofnung, 1976; Nakae & Ishii, 1980). The appearance of the limitation-specific OMPs was not influenced by the nature of the growth-limiting nutrient (organic; negatively or positively charged inorganic). Presumably these proteins do not form specific pores for the substrate actually present in limiting amounts but rather function in optimizing of the uptake of limitation-releasing compounds.

The 90 kDa protein was maximally expressed under potassium limitation at a low growth rate. Under these conditions the potassium gradient across the cell envelope is extremely large

(Huetting *et al.*, 1979; Tempest *et al.*, 1966) and the 90 kDal protein may serve to impede leakage of K^+ ions from the cell. The affinity of the PhoE and the MalB proteins in *E. coli* for their substrates has been related to the presence of a recognition site on the protein (Overbeeke & Lugtenberg, 1982; Ferenci *et al.*, 1980). By analogy, if the 90 kDal protein has an affinity for K^+ ions and, hence, K^+ -binding capacity, leakage of the ion from the cell would be reduced, thus making a contribution towards the maintenance of the K^+ gradient across the cytoplasmic membrane.

Limitation-specific OMPs were barely detectable at high growth rates. Under these conditions the extracellular concentration of the limiting nutrient is relatively high and, therefore, the cultural phenotypic response was less pronounced. In the case of potassium- or sulphur-limited growth, the limitation-specific OMPs were maximally present at the lowest imposed cultural dilution rate. Under carbon limitation, however, the maximum amount of limitation-specific OMPs was not found at the lowest growth rate. Presumably it was not only the imposed nutrient limitation that determined the composition of the outer membrane, but also the rate at which nutrients must cross the membrane in order to replenish the periplasmic concentration of nutrients required for the maintenance of the growth rate. Under carbon-limited conditions the initial rise in the amount of the limitation-specific OMP which accompanied increases in growth rates may have reflected this necessity of an increased flux of nutrients across the membrane.

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