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Alphen, W. van; Lugtenberg, E.J.J.

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# Influence of Osmolarity of the Growth Medium on the Outer Membrane Protein Pattern of *Escherichia coli*

WIM VAN ALPHEN\* AND BEN LUGTENBERG

Department of Molecular Cell Biology, Section of Microbiology,\* and Institute for Molecular Biology, State University, Transitorium 3, Padualaan 8, Utrecht, The Netherlands

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Supplementation of the growth medium with high concentrations of NaCl, KCl, or sucrose caused a drastic change in the ratio of the two peptidoglycan-associated major outer membrane proteins of *Escherichia coli* K-12 in that the amounts of proteins b and c present in cell envelope preparations decreased and increased, respectively. Kinetic studies showed that, after the osmolarity of the medium was changed, one protein was hardly incorporated into the membrane, whereas the other was incorporated with an increased rate. After about 1.5 to 2 generations, the cell envelopes obtained the b/c ratio characteristic for the new medium, and both proteins were subsequently incorporated with rates that ensured this new ratio. Once proteins b and c were incorporated in the cell envelope, they were not converted into each other by changes in osmolarity of the growth medium

The outer membrane of *Escherichia coli* K-12 contains a family of major outer membrane proteins that can be resolved into four protein bands: a, b, c, and d (12). Protein a might be identical to Schnaitman's protein 3b (22, 23; P. Manning and P. Reeves, personal communication). Protein bands b and c are identical to Henning's bands Ia and Ib, respectively (9, 21). Proteins b and c together correspond to Schnaitman's protein 1 (12, 22, 23). Protein d is identical to Schnaitman's protein 3a (12, 22, 23) and to Henning's protein II\* (12, 13, 21). The functions of these proteins are largely unknown. The relative amounts of proteins b and c are dependent on strain, growth medium, growth temperature, and growth phase such that a small amount of protein b is more or less compensated for by an increased amount of protein c and vice versa (13). These two proteins are the only proteins that are strongly, but not covalently, linked to peptidoglycan (13, 20, 21). Moreover, Schmitges and Henning (21) reported that b and c represent modifications of the same polypeptide, which differ from each other in only one cyanogen bromide fragment that does not correspond with the C- or N-terminal of the protein molecule.

In this paper we describe the influence of osmolarity of the growth medium on the composition of the major outer membrane proteins, especially on the relative amounts of the peptidoglycan-associated outer membrane proteins b and c. This effect was studied in some detail to contribute to understanding the functions of these proteins.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Sources, origins, and relevant characteristics of *E. coli* K-12 strains PC0205, JC7620 (previously designated as PC1349), JF404, PC0668, P400, and its protein d-deficient derivative strain, P460, were described previously (13). Strain CE1036 (lacking protein c) is a derivative of strain AB1859 (13). Mutants resistant to bacteriophage M<sub>el</sub> were obtained as described earlier (27).

Except where noted, cells were grown under vigorous aeration at 37°C. The compositions of brain heart medium and of glucose minimal medium were described earlier (13). If required, the leucine concentration in the latter medium was 45 µg/ml, except when radioactive leucine was used as a precursor. In these cases, the leucine concentration used will be described in the text. The composition of yeast broth was as described previously (13), except that NaCl (85 mM) was omitted. The various concentrations of NaCl, KCl, and sucrose used will be given below.

**Isolation and characterization of cell envelopes.** Exponentially growing cells were disintegrated by sonic treatment, and a sample was taken to determine total cell protein. Cell envelopes were isolated by differential centrifugation as described previously (12). 2-Keto-3-deoxyoctulosonic acid was determined as described earlier (1). Peptidoglycan-associated proteins were isolated by the method of Rosenbusch (20) as modified by Lugtenberg et al. (13). Protein was determined by the method of Lowry et al. (11). Separation of cell envelope proteins by polyacrylamide gel electrophoresis and staining of the protein bands was carried out as described earlier (12), except that shorter staining and destaining procedures were used. For fixing and staining, the gel was incubated for 15 min at room

temperature in 50% methanol-10% acetic acid and further incubated for 1 h at 60°C in a solution of 0.1% fast green FCF in 50% methanol-10% acetic acid. This procedure allows interpretation of the results on the same day electrophoresis is carried out. Samples were always applied on the gel in at least two different concentrations. To estimate the relative amount of protein in each band, the gel was scanned with a Vitatron TLD 100 densitometer at a rate of 1.0 cm/min. For autoradiography, the gels were further soaked in 50% methanol-5% glycerol with gentle shaking at 37°C for at least 1.5 h and subsequently dried (12). Autoradiography was carried out for 5 to 10 days at 4°C with Kodak Rapid Processing Royal X-Omat medical X-ray film (RP/R-14). The relative amount of radioactivity in each protein band was determined by scanning the autoradiogram, taking into account that the results were only used when the surface area was proportional to the protein concentration.

**[<sup>14</sup>C]leucine incorporation.** An overnight culture of the leucine auxotrophic strain JC7620 in glucose minimal medium was diluted 1:10 in glucose minimal medium (without NaCl) containing [<sup>14</sup>C]leucine (Radiochemical Centre, Amersham, England) (10 µg/ml; specific activity, 6 mCi/mmol) and incubated under aeration at 37°C. After three generations, judged from absorbance measurements with a Unicam SP 600 spectrophotometer at a 660-nm wavelength, a sample of 50 ml was taken for isolation of cell envelopes, and the remainder of the culture was centrifuged at 37°C. The cells were suspended into 5 volumes of nonradioactive glucose minimal medium (45 µg of leucine per ml; 300 mM NaCl) and incubated at 37°C. During at least two generations, samples were taken at various times for the determination of total cell protein and for the isolation of cell envelopes.

In an analogous experiment, cells of strain JC7620 were labeled in the presence of a high NaCl concentration (glucose minimal medium; 10 µg of leucine per ml; 300 mM NaCl) and, after centrifugation, further incubated in nonradioactive medium without NaCl (glucose minimal medium; 45 µg of leucine per ml).

**Lipopolysaccharide analysis.** <sup>32</sup>P-labeled lipopolysaccharide (LPS) was isolated from cells grown in low-phosphate medium (2) supplemented with tryptophan (20 µg/ml) and Casamino Acids (0.2%). Samples containing 10,000 to 15,000 cpm were applied to Whatman 3 MM chromatography paper (40 by 35 cm) and chromatographed in isobutyric acid-1 M ammonium hydroxide (7:3, vol/vol). After drying, the chromatogram was exposed for 2 to 7 days to X-ray film (Kodak X-Omat R film/XR-1).

## RESULTS

**Effect of osmolarity of the medium on the relative amounts of major outer membrane proteins.** During studies on the membrane protein pattern of a temperature-sensitive *fabB* mutant, which can grow at the restrictive temperature without exogenous unsaturated fatty acid provided that the growth medium is sup-

plemented with high concentrations of NaCl, KCl, or sucrose (3, 4), we found that these additions to the medium influenced the pattern of the major outer membrane proteins. This influence was found to be independent of the *fabB* mutation. The addition of 300 mM NaCl or KCl or 600 mM sucrose to yeast broth caused a decrease in the amount of protein b, which was accompanied by an increase in the amount of protein c (Fig. 1). That, in addition to NaCl and KCl, sucrose also caused this effect shows that it was probably caused by the osmolarity of the medium rather than a specific ion. No other significant changes in the pattern of cell envelope proteins were detected (Fig. 1). The ratios of cell envelope protein to total cell protein and of the major outer membrane proteins (a plus b

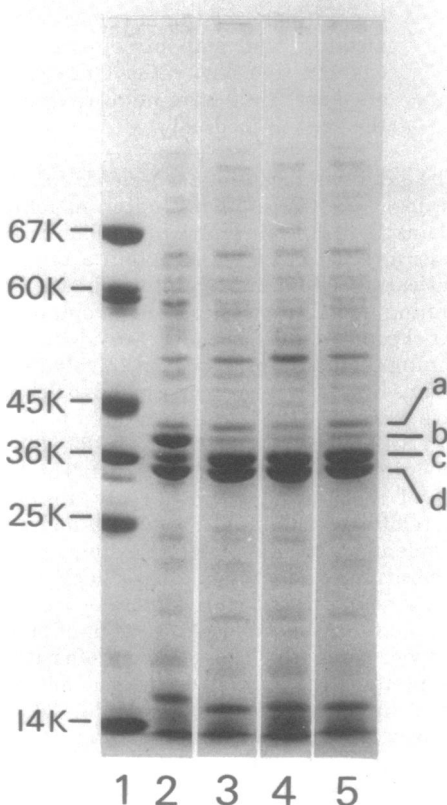


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of (1) molecular weight standards; the other slots contain samples of cell envelopes of strain JC7620 grown in (2) yeast broth, (3) yeast broth with 300 mM NaCl, (4) yeast broth with 300 mM KCl, and (5) yeast broth with 600 mM sucrose. The standard protein bands are indicated at the left by their molecular weights (e.g., 67 K = 67,000 molecular weight). The positions of proteins a, b, c, and d are indicated at the right.

plus c plus d) to total cell envelope protein were not influenced by increased osmolarity of the growth medium, as was calculated from protein determinations and scanning of gels.

The osmotic effect on the amounts of proteins b and c was general for all strains tested that were wild type with respect to these proteins (Table 1). In strains that contain a high amount of protein b in their cell envelopes after growth in yeast broth, the addition of NaCl reduced the amount of this protein (e.g., strain PC0205), whereas in strains containing a small amount of protein b after growth in yeast broth, supplementation with NaCl led to the absence of protein b (e.g., strain AB1859). It seems, therefore, that by supplementation of yeast broth with NaCl the amount of protein b in cell envelopes can be reduced by a certain amount and that the strain can phenotypically lack protein b in yeast broth (300 mM NaCl) only when the amount of protein b is already low after growth in yeast broth (no NaCl). The decrease in protein b was more or less compensated for by an increase in protein band c. The amount of protein a was not significantly influenced by the addition of NaCl. The amount of protein d seemed to increase in strain PC0205 and to decrease in strain P400, whereas it was hardly influenced in the other strains (Table 1).

The question arose whether the increase in protein band c was due either to a real increase of protein c or to the synthesis of a new protein with the same electrophoretic mobility as protein c. Two experiments showed that the increase was due to a real increase of the amount of protein c; namely, (i) after growth in both yeast broth with 0 mM NaCl and yeast broth with 300 mM NaCl, more than 90% of protein band c could be recovered associated with peptidoglycan, and (ii) growth of the protein c-deficient strain, CE1036, in yeast broth with 300 mM NaCl resulted in the absence of protein b

but not in the appearance of a protein c band (Table 1). The same result was obtained with another protein c-deficient mutant, isolated as a bacteriophage Me1-resistant derivative of strain PC0205, which possesses a high relative amount of protein b in its outer membrane after growth in yeast broth.

Figure 2A shows the amounts of four major outer membrane proteins (a, b, c, and d) of strain JC7620 relative to total cell envelope protein as a function of the NaCl concentration in yeast broth. The amount of protein b decreased with increasing NaCl concentration up to about 200 mM and was rather constant at higher concentrations. No evidence for excretion of protein b into the growth medium could be obtained, as was measured in the acid-precipitable material of the supernatant obtained after centrifugation of the cells. The amount of protein c increased more or less in parallel with the decrease in protein b, whereas the amount of protein d decreased gradually. The amount of protein a was rather constant except for a slight tendency to decrease at higher NaCl concentrations. The influence of the NaCl concentration on the generation time is given in Fig. 2B.

It has been reported that the b/c ratio is dependent on the composition of the growth medium and on the growth temperature (13). We tested the effect of NaCl in glucose minimal medium, yeast broth, and brain heart medium on strain JC7620 at temperatures between 30 and 42°C. The results showed that the presence of NaCl under all conditions caused a decrease in protein b accompanied by an increase in protein c. The highest relative amount of protein b was found in cells grown at 30°C in glucose minimal medium (b/c ratio, 2.5); the lowest occurred in cells grown at 42°C in brain heart medium supplemented with 300 mM NaCl. Under the latter growth conditions, protein b could not be detected anymore.

TABLE 1. Effect of NaCl in the growth medium on the content of major outer membrane proteins

Strain	Protein deficiency	Relative amt of individual major outer membrane proteins (% of total major outer membrane protein) <sup>a</sup>							
		Yeast broth (0 mM NaCl)				Yeast broth (300 mM NaCl)			
		a	b	c	d	a	b	c	d
PC0205	None	6	39	22	33	4	10	32	54
JC7620	None	9	27	20	44	10	5	41	44
AB1859	None	10	21	28	41	7	0	51	42
P400	None	3	16	15	66	5	0	43	52
JF404	None	7	10	40	43	4	0	47	49
PC0668	None	6	4	49	41	6	0	53	41
CE1036	c	2	15	0	83	2	0	0	98

<sup>a</sup> Cells were grown in the indicated growth medium for at least 10 generations. Cell envelope proteins were separated by gel electrophoresis. The data were calculated from scans of stained gels.

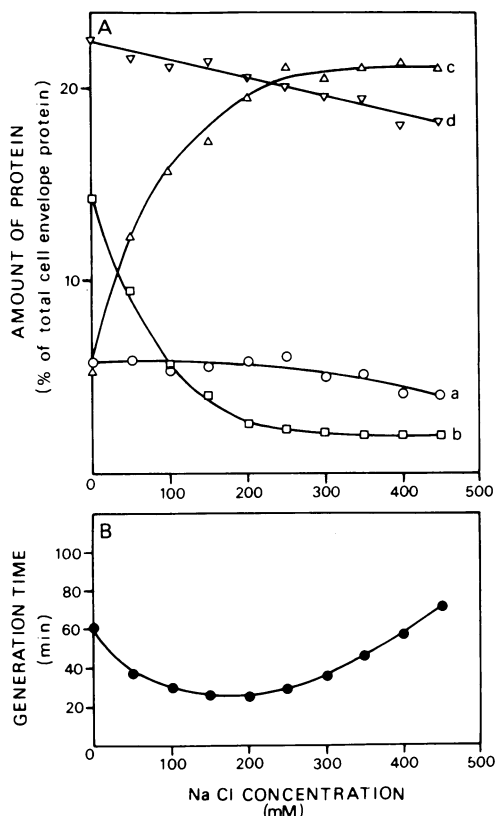


FIG. 2. (A) Relative amounts of the major outer membrane proteins a, b, c, and d as percentages of total cell envelope protein of cells of strain JC7620 grown at 37°C in yeast broth supplemented with various NaCl concentrations. Data were calculated from scans of stained gels. Symbols: ○, protein a; □, protein b; △, protein c; ▽, protein d. (B) Generation times of cells of strain JC7620 grown at 37°C in yeast broth supplemented with various NaCl concentrations.

**Kinetics of the change in the protein pattern.** To study the effect of a change in osmolarity of the growth medium on the kinetics of changes in amounts of major outer membrane proteins, a series of experiments was carried out in which cells were labeled with [<sup>14</sup>C]leucine in a medium with a low or high NaCl concentration, respectively, and subsequently shifted to a nonradioactive medium containing a high or low NaCl concentration, respectively. The change in NaCl concentration hardly influenced the generation time. No significant increase in the amount of acid-precipitable radioactivity of cells or cell envelopes could be measured after the shift to the nonradioactive medium.

In the first type of experiment, cells of strain

JC7620 were labeled with [<sup>14</sup>C]leucine in glucose minimal medium (10 μg of leucine per ml; 0 mM NaCl). After about three generations, 85% of the radioactivity was incorporated into the cells. After centrifugation, the cells were incubated (zero time) at 37°C in nonradioactive high-salt medium (glucose minimal medium; 45 μg of leucine per ml; 300 mM NaCl). Cell envelopes, isolated from samples taken at various times during at least two generations, were analyzed by polyacrylamide gel electrophoresis. Stained gels as well as autoradiograms were scanned. With respect to both protein content and radioactivity, the ratios of cell envelope protein to total cell protein and of total major outer membrane protein (a plus b plus c plus d) to total cell envelope protein did not change during the experiment. In Fig. 3A and B the amounts of radioactivity and protein, respectively, of the individual major outer membrane proteins a, b, c, and d are expressed as percentages of the amount of total major outer membrane protein. The relative amounts of radioactivity in the four proteins in cell envelopes did not change significantly after the shift to nonradioactive medium without NaCl (Fig. 3A). The relative amounts of protein a and d remained constant (Fig. 3B), whereas the amount of protein b decreased and that of protein c increased. Figure 3C shows specific activity in the individual major outer membrane proteins as a function of the number of cell divisions after the shift to medium supplemented with NaCl. The specific activities of proteins a and d decreased at equal rates, which hardly differ from the rate expected from a protein that neither incorporates radioactivity after the shift, nor is subject to turnover (see theoretical line in Fig. 3C). The specific activity of protein b remained constant during about 1.5 generations and subsequently decreased with about the same slope as the theoretical line. During the first 1.5 generations, the specific activity of protein c strongly decreased and subsequently diminished at about the same rate as the specific activities of the other three proteins.

The results discussed above are explained as follows. The relative amounts of proteins a and d in cell envelopes were hardly, or not at all, influenced by the presence of NaCl in the growth medium. Immediately after the shift, new protein b was incorporated into the membrane at a strongly decreased rate (Fig. 3A). Protein c was then incorporated at a strongly increased rate. After 1.5 generations, the amounts of proteins b and c reached levels characteristic for the new growth medium. Subse-

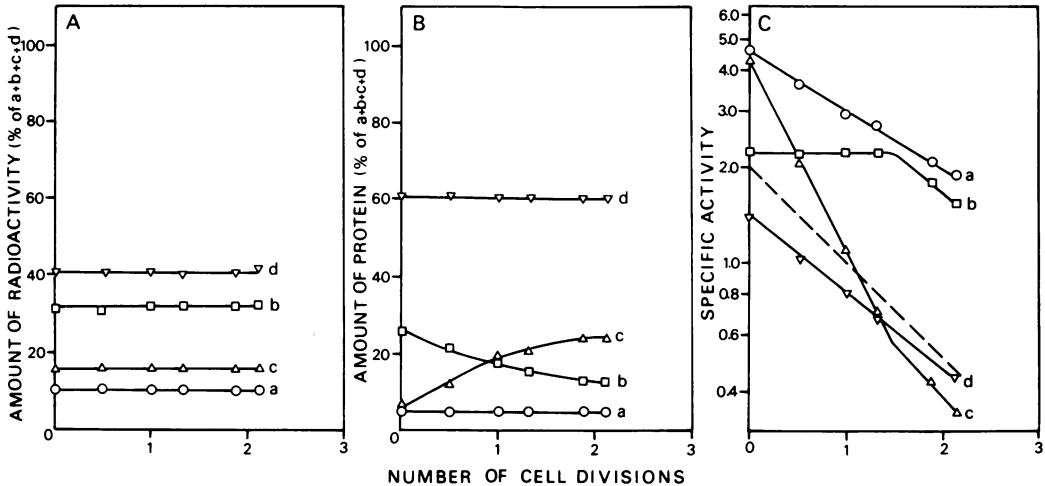


FIG. 3. Kinetics of changes in relative amounts of proteins *a*, *b*, *c*, and *d* after growth of strain JC7620 cells in glucose minimal medium (10  $\mu\text{g}$  of [ $^{14}\text{C}$ ]leucine per ml, 0 mM NaCl) at 37°C and a shift at zero time to glucose minimal medium (45  $\mu\text{g}$  of unlabeled leucine per ml, 300 mM NaCl). (A) Relative amounts of radioactivity in the individual proteins as percentages of the total radioactivity in *a* plus *b* plus *c* plus *d*. As explained in the text, the ratio (*a* + *b* + *c* + *d*)/total cell protein remained constant during the experiment. (B) Relative amounts of protein in the individual proteins as percentages of the total amount of protein in *a* plus *b* plus *c* plus *d*. (C) Specific activity (arbitrary) units of the individual proteins. Note the logarithmic scale of the y axis in (C). The broken line indicates the curve expected for a protein that is neither synthesized nor subject to turnover in the course of the experiment. Symbols:  $\circ$ , protein *a*;  $\square$ , protein *b*;  $\triangle$ , protein *c*;  $\nabla$ , protein *d*.

quently, these proteins were incorporated at new rates specific for this medium.

In the second type of experiment, strain JC7620 cells were labeled with [ $^{14}\text{C}$ ]leucine in glucose minimal medium (10  $\mu\text{g}$  of leucine per ml, 300 mM NaCl). After centrifugation, the cells were incubated at 37°C in nonradioactive medium without NaCl (glucose minimal medium, 45  $\mu\text{g}$  of leucine/ml, 0 mM NaCl). The latter part of the experiment was carried out in a manner similar to that for the former. Again it was found that, with respect to both protein content and radioactivity, the ratios of total cell envelope protein to total cell protein and of total major outer membrane protein (*a* plus *b* plus *c* plus *d*) to total cell envelope protein did not change during the experiment. The relative amounts of radioactivity in the proteins (*a*, *b*, *c*, and *d*) in cell envelopes did not change after the shift (Fig. 4A). The relative amounts of proteins *a* and *d* in cell envelopes did not significantly change after the shift, whereas the amount of protein *b* strongly increased during about the first 1.5 generations after the shift (Fig. 4B). The relative amount of protein *c* strongly decreased during this period. The specific activities of proteins *a* and *d* decreased at equal rates, which correspond rather well with a simple dilution of their radioactivities (Fig. 4C). The specific activity of protein *b* strongly decreased

during the first 1.5 generations and subsequently followed the kinetics of dilution. The results of the experiment plotted in Fig. 4 can be explained in essentially the same way as those of Fig. 3, except that *b* and *c* should be reversed.

Interference contrast microscopy on exponentially growing cells of strain JC7620 did not indicate a major influence of the osmolality of the growth medium on the shape and size of the cells, except that some wrinkling of the cell surface was observed for cells grown in a medium with a high osmolality. An increase of the osmolality of the growth medium did not change the cell size but resulted in slightly irregularly shaped plasmolyzed cells with a slight tendency to form clusters.

**Influence of the osmolality on LPS.** The existence of a relationship between the structure of LPS and the relative amounts of the major outer membrane proteins has been described previously (1, 7, 10, 13, 26). To test whether a changed *b/c* ratio, caused by the presence of NaCl in the growth medium, was accompanied by a change in the LPS structure,  $^{32}\text{P}$ -labeled LPS was isolated from cells grown in the absence and presence of 300 mM NaCl. After paper chromatography, equal  $R_f$  values were obtained for the two preparations. It is unlikely, therefore, that the NaCl concentra-

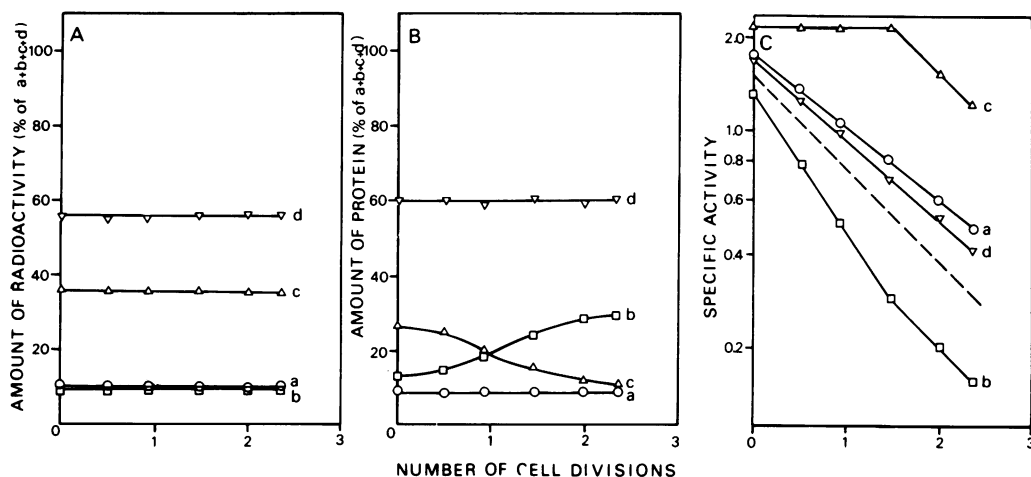


FIG. 4. Kinetics of changes in the relative amounts of proteins a, b, c and d after growth in glucose minimal medium ( $10 \mu\text{g}$  of [ $^{14}\text{C}$ ]leucine per ml,  $300 \text{ mM NaCl}$ ) at  $37^\circ\text{C}$  and a shift at zero time to glucose minimal medium ( $45 \mu\text{g}$  of unlabeled leucine per ml,  $300 \text{ mM NaCl}$ ). (A) Relative amounts of radioactivity in the individual proteins as percentages of the total radioactivity in a plus b plus c plus d. (B) Relative amounts of protein in the individual proteins as percentages of the total amount of protein in a plus b plus c plus d. (C) Specific activity of the individual proteins. For further details, see the legend of Fig. 3. Symbols:  $\circ$ , protein a;  $\square$ , protein b;  $\triangle$ , protein c;  $\nabla$ , protein d.

tion in the growth medium affected the LPS structure.

The ratios of 2-keto-3-deoxyoctulosonic acid to total cell protein and of 2-keto-3-deoxyoctulosonic acid to total cell envelope protein decreased by about 15 to 25% when the cells were grown in the presence of  $300 \text{ mM NaCl}$ .

## DISCUSSION

The results presented in this paper have shown that supplementation of the growth medium of *E. coli* K-12 strains with high concentrations of  $\text{NaCl}$ ,  $\text{KCl}$ , or sucrose results in a strong decrease of outer membrane protein band b, accompanied by a roughly equal increase in outer membrane protein band c (Fig. 1, Table 1). Since protein c was not found in the cell envelope of protein c-deficient mutants after growth under these conditions, the increase in protein band c must be due to a real increase in the amount of protein c and not to production of a new protein with the same electrophoretic mobility as protein c.

The b/c ratio differs strongly for various *E. coli* K-12 strains (13). In addition to the osmolarity of the growth medium, the b/c ratio of a particular strain can also be influenced by the nutrient composition of the growth medium and by the growth temperature (13). Thus, the b/c ratio can be changed dramatically, whereas the total amount of these two proteins remains about constant. A change in the amount(s) of

protein(s) b and/or c can also influence the amount of protein d (23; Fig. 2). The results of this paper show, as was reported earlier (8, 13, 23, 26), that the composition of the outer membrane, with respect to the amounts of various major outer membrane proteins, is extremely flexible.

In our laboratory we have applied this knowledge of the influence of the growth conditions on mutants deficient in one or two outer membrane proteins (b, c, and d) for the purification of these proteins. Strain CE1034 (13), grown in yeast broth supplemented with  $300 \text{ mM NaCl}$ , lacks proteins b and d and contains large amounts of protein c. Strain CE1036, which lacks protein c (13) and contains small amounts of protein b after growth in brain heart medium, was found to be an excellent source of protein d (25a). Strain CE1041 (13), grown in yeast broth, lacked proteins c and d and contained large amounts of protein b. The flexibility of the composition of the outer membrane can be shown extremely well by growing the latter strain in brain heart medium supplemented with  $300 \text{ mM NaCl}$ , resulting in the absence of all three outer membrane proteins (b, c, and d).

Both proteins b and c are noncovalently associated with the peptidoglycan layer (6, 9, 13, 20, 21). Schmitges and Henning (21) reported that these two proteins are almost chemically identical. The only difference is a cyanogen bromide fragment that corresponds neither with

the N-terminal part nor with the C-terminal part of the protein molecule. Based on this striking chemical similarity, it was suggested that both proteins b and c might be products of one structural gene and that the difference is introduced by post-translational modification (21). Our kinetic data (Fig. 3 and 4) show that, once these proteins are incorporated into the outer membrane, a change of osmolarity of the growth medium does not result in conversion of the bulk of one protein into the other. So if post-translational modification occurs, it most likely takes place before the proteins become inserted into the outer membrane.

Another common property of proteins b (13, 17) and c (17, 21) is their interaction with LPS. However, this is not an exclusive property of these two proteins, since it was recently shown that protein d also has this attribute (25a). The affinity for LPS might even be the reason why these proteins are located in the outer membrane and not in the cytoplasmic membrane.

Nakae has shown that the incorporation of certain outer membrane proteins of *Salmonella typhimurium* (14, 15) and of the peptidoglycan-associated matrix protein b of *E. coli* B (16, 20) into phospholipid-LPS vesicles loaded with dextran and sucrose results in leakage of sucrose but not of dextran. This system mimics the aquaous pores of the outer membrane that are supposed to be responsible for the extremely high permeability of the outer membrane for hydrophilic low-molecular-weight substances (18). Lugtenberg et al. showed that the outer membrane proteins of *Salmonella typhimurium*, which produce aquaous pores in phospholipid-LPS vesicles (15), are also peptidoglycan associated and proposed that the formation of aquaous pores might be a property of peptidoglycan-associated proteins in general (11a). The affinity of both peptidoglycan-associated proteins of *E. coli* K-12 for LPS and the presence of LPS in the vesicles described by Nakae suggest that LPS is also required for the formation of aquaous pores (13a) and also that protein c is involved in pore formation. One might even speculate that (part of) the particles that can be seen by freeze-fracture electron microscopy on the outer fracture face of the outer membrane (5, 24, 25, 28, 29) are identical to aquaous pores, since strong evidence was presented for the idea that these particles are composed of LPS aggregates stabilized by divalent cations and possibly also containing protein and/or phospholipid (29). Since the presence of NaCl in the growth medium has no influence on the density of particles at the outer fracture face of wild-type cells (29), one can expect that in the presence of NaCl the density

of pores or particles that contain protein b would decrease, whereas the density of those containing protein c would increase.

We are planning now to test whether both proteins b and c of *E. coli* K-12 are indeed active in pore formation and, if so, to see whether the two proteins differ in specificity towards various components. The results might answer the question of whether the observed influence of the osmolarity on the b/c ratio is either "accidental" or meant to protect the cell against such a high osmolarity. An accidental change in the b/c ratio can be the result of influence of osmolarity on the conformation of a structural or regulatory protein (19). This could result in a strong decrease in the incorporation of protein b or c into the outer membrane. The observed compensation effect then can be explained by assuming that the total amount of b plus c is regulated either by the space available in the outer membrane or on the peptidoglycan or by the amount of LPS available. If the b/c ratio changes to protect the cell, this protection could be accomplished either to decrease the influx of harmful components or waste products or to facilitate the influx of certain nutrients.

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