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Polyamines as Constituents of the Outer Membranes of *Escherichia coli* and *Salmonella typhimurium*

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Extraction of whole cells of *Salmonella typhimurium* and *Escherichia coli* with 1 M NaCl released 8 to 13% of their total cellular polyamines (putrescine, cadaverine, and spermidine). This extraction did not cause significant cell lysis, release of outer membrane (OM) constituents, or leakage of periplasmic β-lactamase. The extraction released nearly equal amounts of polyamines from *mdo* (membrane-derived oligosaccharide) mutants and wild type. These findings suggest that the released polyamines are apparently bound to the cell envelope. NaCl (1 M) was as effective as trichloroacetic acid in releasing polyamines from isolated OM and lipopolysaccharide (LPS). Isolated OM contained four times more polyamines than the cytoplasmic membrane. The increased binding to the OM is apparently due to the association of polyamines with the polyanionic LPS. Nearly identical amounts of polyamines were found in the OM and LPS preparations (as quantified per milligram of LPS). These amounts are equal to those released from the intact cells by 1 M NaCl (quantitation as above). However, redistribution of polyamines took place after cell disruption, because the relative proportions of different polyamines varied in the OM and LPS preparations. These results indicate that polyamines released from intact cells during 1 M NaCl extraction are preferentially derived from the OM.

Polyamines, ubiquitous polycationic compounds in prokaryotic and eukaryotic cells, have been implicated in a wide variety of biological reactions, including nucleic acid and protein synthesis (27, 37, 38). Studies with polyamine-auxotrophic *Escherichia coli* strains have shown that polyamines are essential for normal cell growth (7). Polyamines are known to bind to isolated negatively charged cellular macromolecules such as nucleic acids (29) and cellular organelles such as ribosomes (35), probably because of their amino groups protonated at physiological pH. Exogenous polyamines are proposed to bind to membranes and stabilize *E. coli* spheroplasts and protoplasts (19, 33), as well as halophilic organisms (39), against osmotic shock. Souza (31) has shown that preliminary exposure of logarithmic-phase *E. coli* to polyamines protects the cells from reduction of viability during freeze-thawing.

The outer membrane (OM) forms the outermost layer in the cell envelope of gram-negative bacteria. It is a highly asymmetric lipid bilayer in which the outer layer is covered by lipopolysaccharide (LPS) and the inner layer is occupied by phospholipids. Nearly half of the mass of the OM is protein (18, 25). The OM is a very effective permeability barrier against hydrophobic compounds and is virtually resistant to neutral or anionic detergents. These properties are proposed to result from the presence of anionic LPS (17, 23). In the OM, LPS associates very tightly with protein and with adjacent LPS via electrostatic interactions mediated by divalent cations (Mg^{2+} and Ca^{2+}). Thus, inorganic divalent cations play a crucial role in maintaining the organization of the OM. On the other hand, very high concentrations of both divalent and monovalent cations are known to disrupt the OM at cold temperatures (2, 10). Furthermore, many other cations (such as polymyxin B and its derivatives, polylysine and protamine), when added exogenously, bind to LPS and effectively disorganize the OM (8, 11, 42, 43). Polyamines are abundant in isolated LPS preparations (5) and are known to bind to purified LPS (28). However, they were inactive as both permeability-increasing agents and antibacterial agents (cadaverine, spermidine, spermine studied and tested at concentrations up to 30 μg/ml [41]).

It would be tempting to suppose that not only inorganic divalent cations but also polyamines or other cationic agents such as cationic proteins could mediate interactions between OM constituents. In this study, we have tried to answer the question whether polyamines are present in the normal intact OM. This is a very interesting question since, to the best of our knowledge, no other reports have appeared on this topic. This is also expected to be a very difficult question because polyamines could be thought to bind artificially to the OM during its isolation. We will show that extraction of whole cells with 1 M NaCl releases polyamines from *Salmonella typhimurium* and *E. coli* without causing cell lysis or membrane damage. This suggests that the released polyamines are apparently bound to the cell envelope. Analysis of isolated membranes reveals that polyamines are more abundant in the OM than in the cytoplasmic membrane (CM). Thus, we believe that most of the polyamines released from intact cells during extraction with NaCl are located in the OM.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Most of the experiments were done with *S. typhimurium* SH5014 strain (LT2 line, LPS chemotype Rb_2) (32). Its derivative, SH9178 (LT2 line, LPS chemotype Rb_2) (24), carrying pR471a-encoded β-lactamase, was used in β-lactamase assays. *S. typhimurium* SH6482 (LT2 line, LPS chemotype Rb_2) (32) was used as one source of LPS. It is a direct derivative of SH5014. *mdo* (membrane-derived oligosaccharide) mutant strains *E. coli* NFB200 (*mdoA* [1]) and NFB80 (*mdoA1* [1]) and their parent MA1008 (1) were kind gifts of E. Kennedy.

For all experiments, the bacteria were grown in L broth (per liter, 10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of NaCl; pH 7.0)

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on a rotary shaker (220 rpm) at 37°C until they reached the logarithmic growth phase (120 Klett units on a Klett-Summerson colorimeter with a red filter). The bacteria were collected by centrifugation (8,000 × *g*, 10 min, 30°C) and washed twice with warm (approximately 37°C) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4). Bacterial suspensions were kept above room temperature to avoid the excretion of putrescine (34) and the acetylation of spermidine (34).

Analysis of total polyamines. Polyamines were extracted from the bacteria with ice-cold 10% trichloroacetic acid (TCA) as described earlier (36) and analyzed as their dabsyl derivatives in reversed-phase high-performance liquid chromatography by the method of Koski et al. (13), using diaminopropane dihydrochloride as the internal standard.

Polyamine analysis of NaCl and sucrose extracts of whole cells. Bacteria (500 mg [wet weight]) were suspended in 1.5 ml of 1 M NaCl or sucrose (both diluted in HEPES buffer, pH 7.4) and incubated with shaking at 37°C for 10 min. Whole cells were removed by three subsequent centrifugations (8,000 × *g*, 10 min at room temperature). The supernatant was diluted (1:4) with deionized water, and 75 μg of diaminopropane dihydrochloride was added per ml as the internal standard. Dabsylation and analysis of polyamines were performed as described above.

Isolation of the OM, CM, and LPS. The OM and the CM were isolated by the modified procedure of Schnaitman exactly as described in reference 14. Protein content was determined by the modified method of Lowry (20), using bovine serum albumin as the standard. The LPS content of membranes was estimated by measuring 2-keto-3-deoxyoctulosonic acid (9), using isolated *S. typhimurium* LPS (LPS chemotype Rb_2) as the standard. The isolated OM and CM contained 360 and 56 μg of LPS/mg of protein, respectively. LPS extraction from logarithmic-growth-phase cells of strains SH6482 and SH9178 and from SH5014 grown on L agar plates was carried out by the phenol-chloroform-petroleum ether method (6). The isolated membranes (2.5 mg of protein per ml) and LPS (3 mg/ml in deionized water) were extracted with ice-cold 10% TCA or 1 M NaCl, and the polyamine content of extracts was measured as described above.

Effect of sodium azide on polyamine release. Washed cells (500 mg [wet weight]) were suspended in 750 μl of 6 mM sodium azide (in 10 mM HEPES, pH 7.4) and incubated with shaking at 37°C for 5 min. An equal volume of 2 M NaCl was added, and the suspension was incubated for 10 min. In control extractions, 6 mM NaCl was used instead of sodium azide. Intact cells were removed by centrifugation, and polyamines in the supernatants were analyzed as described above.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and LPS were carried out in slab gels (0.8 mm thick) by the method of Laemmli (15). The acrylamide concentration in the separating gel was 15%. In the analysis of LPS, the gel contained 4 M urea. The protein bands were stained with Coomassie blue (4), and LPS was visualized with silver nitrate (40). In protein analyses, supernatants of 1 M NaCl extractions were concentrated by precipitation with 90% acetone and precipitates were dissolved in sample buffer. Protein and LPS content was estimated by using isolated OM and LPS as reference samples.

Enzymatic assays. The leakage of periplasmic β-lactamase was measured by using nitrocefin as the substrate (26). Cell lysis was determined as glucose-6-phosphate dehydrogenase (G6PDH) (intracellular enzyme) activity by the method described in reference 16. Total cellular activities of β-lactamase and G6PDH were determined from bacteria forced through a French press. NaCl (1 M) did not interfere with the enzymatic assays performed.

RESULTS

Polyamine contents of NaCl extracts of whole cells. It can be expected that during cell lysis and isolation of the OM, polyamines will artificially bind to the acidic constituents of the OM. On the other hand, if polyamines were present in the native OM, they could probably be released from it by employing sufficiently efficient extraction methods which, however, should not be too harsh and cause cellular lysis. We decided to try to extract polyamines from the cell surface by decreasing their ionic interactions. We thought that inorganic cations, when present in a high concentration, could compete for the binding sites with polyamines and displace them. Accordingly, we chose 1 M NaCl.

Incubating *S. typhimurium* SH5014 for 10 min at 37°C in the presence of 1 M NaCl released 9% of its total putrescine and cadaverine, as well as 5% of its spermidine (Table 1). An identical treatment of *E. coli* MA1009 released similar amounts of putrescine and spermidine but about three times more cadaverine. Polyamines released in 1 M NaCl extractions represented approximately 8% of the total polyamines of *S. typhimurium*. In *E. coli*, the corresponding proportion was 13%. Ionic buffer seems to be necessary to release polyamines from intact cells. Only trace amounts of polyamines were released in the control buffer (0.01 M HEPES alone) or in a buffer containing 34% (wt/wt) sucrose (1 M sucrose) (Table 1). NaCl treatment did not cause significant cell lysis. Less than 2% of the total activity of a cytoplasmic marker enzyme, G6PDH, was detected in the supernatant of the extraction with 1 M NaCl (Table 1).

Released OM constituents (LPS and proteins) were analyzed from supernatants of 1 M NaCl extractions in SDS-PAGE. Supernatants contained about 2% membrane proteins and about 4% LPS (data not shown). Thus, significant OM damage was not observed. In addition, the leakage of periplasmic β-lactamase to the supernatant of 1 M NaCl extraction was less than 3% of total activity (65 U/mg of cells [wet weight]), studied using *S. typhimurium* SH9178 (data not shown).

One location of polyamines in the cell surface could be the membrane-derived oligosaccharide, the acidic saccharide compound in periplasmic space. To elucidate the significance of membrane-derived oligosaccharide as a source of NaCl-releasable polyamines, the *E. coli mdo* mutants were employed. Total amounts of polyamines released in 1 M NaCl extractions from wild-type *E. coli* and its *mdo* mutant strains were essentially similar. Accordingly, the results in Table 1 show that polyamines released from intact cells were apparently bound to membrane component of the cell envelope.

Effect of sodium azide on 1 M NaCl extractions of intact bacteria. In 1972, Munro et al. (22) reported that an increase in osmolarity causes rapid excretion of putrescine from *E. coli* B and that this excretion can be blocked by 6 mM sodium azide. To test whether sodium azide has any effect on the amounts of polyamine released during our extraction procedure, cells were preincubated with 6 mM sodium azide for 5 min and then extracted with 1 M NaCl plus 6 mM NaN_3 (see details in Materials and Methods). As shown in Table 2, sodium azide had no effect on the amounts of putrescine or

TABLE 1. Polyamine contents of 1 M NaCl extractions from intact cells

Strain	Treatment[a]	Amt[b] (μmol/g of cells [wet weight]) of polyamine			G6PDH activity[c] (%)
		Putrescine	Cadaverine	Spermidine	
S. typhimurium SH5014	1 M NaCl	1.8 (± 0.3)	0.5 (± 0.1)	0.2 (± 0.1)	<2
	1 M sucrose	0.4	0.1	<0.05	ND[d]
	10 mM HEPES	0.2	0.1	<0.05	ND
	10% TCA	20.3 (± 3.4)	5.4 (± 1.5)	3.8 (± 1.2)	
E. coli					
MA1008	1 M NaCl	3.6	1.7	0.3	ND
	10% TCA	31.8	5.9	6.0	
NFB200 (*mdoA*)	1 M NaCl	5.3	1.9	0.8	ND
	10% TCA	29.8	4.3	8.2	
NFB80 (*mdoA1*)	1 M NaCl	2.9	1.0	0.5	ND
	10% TCA	25.7	4.0	7.7	

[a] Total polyamines were extracted from the cells with 10% TCA. Other polyamine extractions were performed at 37°C for 10 min.
[b] Each value is the average (± standard deviation) of at least duplicate determinations.
[c] Percentage from total G6PDH activity (6.5 U/mg of cells [wet weight]), determined from cells forced through a French press.
[d] ND, not determined.

other polyamines released by 1 M NaCl. The experiment was also performed with a higher sodium azide concentration (20 mM). It gave results identical to those obtained with 6 mM NaN_3 (data not shown).

Polyamine contents of the isolated OM and CM. In an isopycnic sucrose gradient, the OM and CM were isolated from *S. typhimurium* SH5014 that had been grown to the logarithmic growth phase and forced through a French press. On the basis of the LPS content determined (see Materials and Methods), we can regard the isolated OM as a typical OM. However, the isolated CM was contaminated with OM material (approximately 15% contamination; see Materials and Methods).

NaCl (1 M) was only slightly less effective than TCA in extracting the membrane-bound polyamines. Putrescine and spermidine were found in both membranes (Table 3). Compared with whole cells (Table 1), both OM and CM contained much spermidine and were devoid of cadaverine. Importantly, there was four times more polyamine in the OM fraction (total polyamines, 69 nmol/mg of protein) than in the CM fraction (total polyamines, 17 nmol/mg of protein). Furthermore, most (approximately 10 to 11 nmol/mg of protein) of the polyamines found in the CM may be derived from cross contamination with the OM. Thus, the amounts of polyamines bound to the typical CM are apparently lower than those determined in Table 3.

Polyamine contents of the isolated rough LPS. The polyamines are found in isolated LPS (5) and are known to bind to purified LPS (28). Furthermore, the results obtained above by using isolated membranes strongly suggested that the anionic LPS molecules may be the major binding site of polyamines in the membranes of gram-negative enteric bacteria. Therefore, we also measured the polyamine content of isolated LPS preparations (Table 4). NaCl (1 M) proved to be as efficient as 10% TCA in the extraction of polyamines from this material also. The rough LPSs (LPS chemotype Rb_2) isolated from strains SH6482 and SH9178, both grown into logarithmic growth phase, contained 65 to 102 nmol of putrescine, 36 to 42 nmol of cadaverine, and 23 to 37 nmol of spermidine per mg of LPS (Table 4). The rough LPS (LPS chemotype Rb_2) from SH5014 (grown into stationary growth phase on L agar) contained somewhat less.

TABLE 2. Effect of 6 mM sodium azide on polyamine release by 1 M NaCl from *S. typhimurium* SH5014[a]

Preincubation treatment	Extraction treatment	Amt[b] (μmol/g of cells [wet weight]) of polyamine		
		Putrescine	Cadaverine	Spermidine
6 mM NaCl	1 M NaCl	1.7	0.8	0.3
6 mM NaN_3	1 M NaCl + 6 mM NaN_3	1.6	0.7	0.2
6 mM NaN_3	6 mM NaN_3	0.2	<0.05	<0.05

[a] Bacteria were suspended in 6 mM NaN_3 and preincubated at 37°C for 5 min. Extractions were performed as described in footnote *a* of Table 1.
[b] Values are averages of duplicate determinations.

DISCUSSION

In this paper, we have shown that 1 M sodium chloride extraction releases a significant proportion of all major polyamines (putrescine, cadaverine, and spermidine) from intact cells of *S. typhimurium* and *E. coli*. Released polyamines represent approximately 8% of total polyamines in *S. typhimurium* and approximately 13% in *E. coli* (Table 1). The relative proportion of different polyamines found in the NaCl extracts is quite similar to that of total cellular polyamines. However, during the extraction process, no major cell lysis or membrane damage was detected. SDS-PAGE analysis revealed that only approximately 2% of the mem-

TABLE 3. Polyamine contents of the isolated OM and CM of *S. typhimurium* SH5014

Preparation	Extraction treatment	Amt[a] (nmol/mg of protein) of polyamine		
		Putrescine	Cadaverine	Spermidine
OM	10% TCA	39	<1	30
	1 M NaCl	37	<1	28
CM	10% TCA	5	<1	12
	1 M NaCl	5	<1	10

[a] Values are averages of duplicate determinations.

TABLE 4. Polyamine contents of isolated LPS of three *S. typhimurium* strains

Strain	Extractment treatment	Amt[a] (nmol/mg of LPS) of polyamine		
		Putrescine	Cadaverine	Spermidine
SH6482[b]	10% TCA	102	42	37
	1 M NaCl	89	36	32
SH9178[b]	10% TCA	65	41	26
	1 M NaCl	65	41	23
SH5014[c]	10% TCA	42	21	28
	1 M NaCl	38	19	25

[a] Values are averages of duplicate determinations.
[b] Cells grown up to logarithmic growth phase in L broth.
[c] Cells grown overnight on L agar.

brane proteins and 4% of LPS were released. The typical pattern of lysed cells was not found with SDS-PAGE. The leakage of the periplasmic β-lactamase was less than 3% and that of the cytoplasmic marker enzyme (G6PDH) was less than 2%. Accordingly, these results suggest that 1 M NaCl releases those polyamines from a peripheral cellular compartment, i.e., the cell envelope. However, the acidic membrane-derived oligosaccharide molecules in periplasm are not the major source of released polyamines (Table 1). In the present paper, we also measured the polyamine content of isolated OM and LPS and showed that 1 M NaCl is able to quantitatively release the polyamines bound to these materials.

The OM and CM differ significantly in their composition and function. It was reported that these membranes also differ in their content of inorganic divalent cations. There are three times more Mg^{2+} and Ca^{2+} in the isolated OM than in the isolated CM (3). Our data revealed that the isolated OM contains at least four times more polyamines than the isolated CM (Table 3). Furthermore, taking into account the estimated 15% contamination of the isolated CM with OM (see Materials and Methods), it can be calculated that more than half of the polyamines found in the isolated CM was of OM origin.

The higher polyamine content in the OM is apparently due to the presence of polyanionic LPS, a unique constituent of the OM. The total amounts of polyamines in LPS (132 to 181 nmol/mg of LPS; Table 4) were quite similar to those found in the isolated OM. Approximately 70 nmol of polyamines per mg of OM protein was found (Table 3). Because the OM contains approximately 0.35 mg of LPS per mg of OM protein, this polyamine content is equivalent to approximately 195 nmol/mg of LPS. However, the proportion of different polyamines varied significantly in the isolated OM and LPS. For instance, LPS preparations contained 60 to 120 times more cadaverine than the isolated OM. On the other hand, the proportions of different polyamines in isolated LPS were fairly similar to those in the NaCl extracts of intact cells (Tables 1 and 4). Apparently most of the putrescine and cadaverine is released from the OM during its isolation, while spermidine remains bound. Related qualitative changes have been reported to take place during the isolation of ribosomes (35).

Accordingly, data from the OM and LPS indicate that polyamines released from intact cells by 1 M NaCl are predominantly bound to the LPS constituent of the OM. On the basis of this assumption and Table 1, about 250 nmol of polyamines is bound to 1 mg of native LPS in the intact OM (molar ratio of 1.5:1; the content of LPS in cells [wet weight] estimated according to reference 30). Consequently, the relative proportions of polyamines (180 nmol of putrescine, 50 nmol of cadaverine, and 20 nmol of spermidine per mg of LPS) could represent the real distribution of polyamines in the OM.

Munro et al. (22) have reported that exposure of *E. coli* to osmotic shock (1 M sucrose or 0.6 M NaCl) causes rapid excretion of putrescine from an intracellular pool (over 50% of total putrescine released) and that this can be blocked by sodium azide or sodium arsenate. There are some discrepancies between their results and our results. (i) In our extraction condition, 1 M NaCl buffer did not cause massive release of putrescine. (ii) All major polyamines were detected in supernatants of 1 M NaCl extractions, whereas Munro et al. detected only the release of putrescine. (iii) In our conditions, 1 M sucrose released only trace amounts (less than 2%) of polyamines. (iv) Sodium azide (6 or 20 mM) did not block the release of polyamines. Furthermore, Munro et al. showed that K^+ ions stimulate putrescine release. However, when we used 10 mM K^+ with 1 M NaCl in our extraction procedure, we did not detect any increase in the release of polyamines (12). Some of the discrepancies could be due to differences in the experimental conditions. We carried out our extractions after washes with 10 mM HEPES (pH 7.4) buffer. This hypotonic treatment released more than 50% of cell-bound rubidium-86 ions (apparently reflecting cell adaption to low osmolarity) but did not cause any release of polyamines (12).

In conclusion, we showed that polyamines are apparently present in the native OM of gram-negative bacteria. The general assumption that the OM contains polyamines is not, to the best of our knowledge, based on any earlier direct experimental data. Another question then is, are those polyamines essential? Polyamine-deficient strains would be practical tools to study the importance of polyamines in the OM.

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