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Polyamines Decrease *Escherichia coli* Outer Membrane Permeability

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The permeability of the outer membranes of gram-negative bacteria to hydrophilic compounds is mostly due to the presence of porin channels. We tested the effects of four polyamines (putrescine, cadaverine, spermidine, and spermine) on two processes known to depend on intact porin function: fluxes of β-lactam antibiotics in live cells and chemotaxis. In both cases, inhibition was observed. Measurements of the rate of permeation of cephaloridine and of chemotaxis in swarm plates and capillary assays were used to determine the concentration dependence of this modulation. The effective concentration ranges depended on the nature of the polyamine and varied from submillimolar for spermine to tens of millimolar for cadaverine. Both OmpC and OmpF porins were inhibited, although the effects on OmpC appeared to be milder. These results are in agreement with our observations that polyamines inhibit porin-mediated ion fluxes in electrophysiological experiments, and they suggest that a low-affinity polyamine binding site might exist in these porins. These results reveal the potential use of porins as targets for blocking agents and suggest that polyamines may act as endogenous modulators of outer membrane permeability.

The permeability of the outer membranes of gram-negative bacteria is largely dependent on porins, which are abundant proteins that form nonspecific channels (26). High rates of flux of β-lactam antibiotics, which permeate the outer membrane through porins, have been measured in intact cells, substantiating the belief that porins are permanently open pores (24). The combined properties of favored open state and discrimination against solutes of high molecular weight make porins the major pathway for fast nutrient fluxes in an otherwise highly protective membrane. Electrophysiological and biochemical studies of reconstituted purified porins have indeed confirmed that these 16-stranded β-barrels are mostly open (3, 23, 34).

Recent patch-clamp investigations of porins in reconstituted membrane fractions, however, have revealed that closed states become favored when membrane-derived oligosaccharides or cadaverine is presented to the periplasmic side of the porins (7, 8). In both cases, the compounds reduce the probability of porins being in the open state in a concentration-dependent fashion. In addition, it was clearly demonstrated that the modulation of porin activity by cadaverine introduced a voltage-dependent component to porin behavior (7). These channel modulators are found in the vicinity of porins in vivo (16, 28) and might therefore play some roles as natural regulators of porin activity.

Cadaverine and other polyamines, such a putrescine, spermidine, and spermine, are linear molecules that terminate at both ends with an amine functional group. Spermidine and spermine also carry additional amine groups at other internal positions. These functional groups confer to the molecules multiple positive charges at physiological pH. Spermine is not endogenous to *Escherichia coli*, but putrescine, cadaverine, and spermidine are produced through the action of basic amino acid decarboxylases (33) and are found associated with the outer membrane (16). It is of interest that these polyamines, in particular cadaverine, are lost during fractionation (16), which might explain the discrepancy observed between the probabilities of finding permanently open porins in electrophysiological experiments done in reconstituted systems (3, 9) and in experiments with giant cells or spheroplasts (5).

In order to assess the physiological impact of the polyamine modulation of porins, we have tested the effects of the four polyamines (putrescine, cadaverine, spermidine, and spermine) on cellular processes that depend on intact porin function, namely, antibiotic fluxes and chemotaxis. β-Lactam antibiotics need to cross the outer membrane barrier in order to exert their bactericidal actions of interfering with cell wall synthesis in the periplasm. Resistant strains have been shown to synthesize a plasmid-encoded β-lactamase, which resides in the periplasm and degrades the antibiotics (32). It has been shown that the permeation of these antibiotics through the outer membrane is mediated by porins (10). In fact, porin permeability has been measured in intact cells because the degradation of the antibiotic by the periplasmic β-lactamase is rate limited by the flux of the compounds through the porins (24). This β-lactamase activity is greatly reduced in strains deficient in the major porins OmpF and OmpC (2).

The cellular mechanisms by which motile bacteria perform chemotaxis and thus migrate towards high concentrations of nutrients and away from noxious chemicals have been elucidated in molecular terms (18, 30). The first step in this signaling cascade is the activation of inner membrane receptors by chemoeffectors alone or in complexes with periplasmic binding proteins. For gram-negative bacteria the efficiency of this first step requires that the flux of attractants through the outer membrane not be limiting. Ingham and colleagues (13) have shown that chemotaxis of motile *E. coli* is greatly impaired in strains lacking porins, thus linking the chemotactic cascade to outer membrane permeability. Chemotaxis is performed efficiently only under conditions in which the ability of attractants to permeate through the porins is high enough for periplasmic concentrations to be maintained above threshold levels. Therefore, we can infer that a decrease in the number of open porins, due to either reduced porin synthesis or inhibitory modulation,

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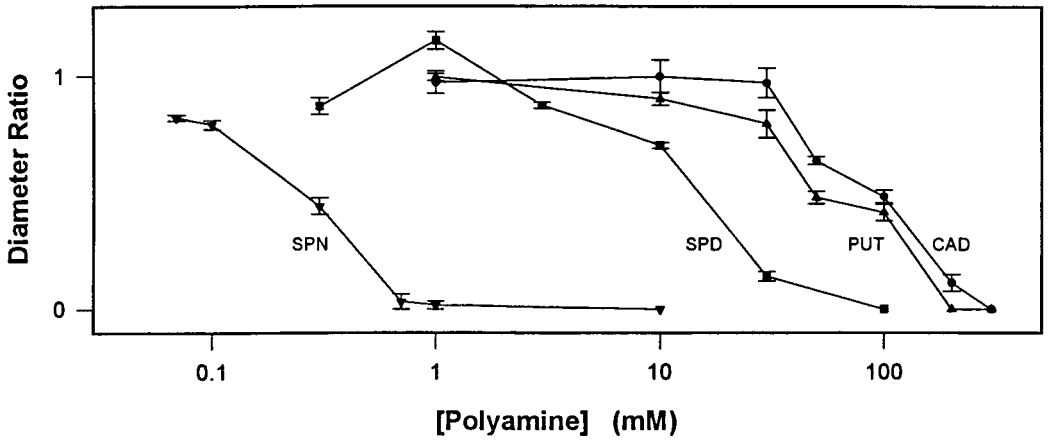

FIG. 1. Polyamines inhibit swarming ability. The diameter of the outermost swarm ring was measured after 8 h of incubation at 35°C in control plates or plates containing putrescine (PUT), cadaverine (CAD), spermidine (SPD), or spermine (SPN). The ratio of diameters in the presence and the absence of polyamines (diameter ratio) is plotted against polyamine concentrations. Points are averages of 5 to 30 data points obtained in a total of one to three experiments. Error bars represent standard errors of the mean and in some cases lie within the symbol.

would greatly affect chemotactic ability. In this study, we have used this concept to demonstrate that polyamines reduce outer membrane permeability in vivo.

MATERIALS AND METHODS

Materials and strains. The *E. coli* K-12 derivatives AW737 (*ompC*$^+$ *ompF*$^+$), AW738 (*ompC ompF*$^+$), and AW739 (*ompC*$^+$ *ompF*) were used (13). For antibiotic permeation assays, the β-lactamase-encoding plasmid R_{471a} (24) was introduced into the strains. *E. coli* was grown in tryptone broth (T-broth) containing 1% tryptone and 0.5% NaCl or in Luria-Bertani medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. The polyamines used in this study were putrescine [NH_2-$(CH_2)_4$-NH_2], cadaverine [NH_2-$(CH_2)_5$-NH_2], spermidine [NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2], and spermine [NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2]. They were purchased from Sigma Chemical Co. as the hydrochloride species, and they did not alter the pH of solutions. Medium components were from Difco Laboratories, all organic chemicals were from Sigma Chemical Co., and all inorganic chemicals were from Fisher Scientific.

Swarm assays. A single colony of motile bacteria was inoculated into soft T-agar (0.3% agar) with or without polyamines, and the plates were incubated at 35°C for 8 h. Five to 10 colonies were tested for each condition, and the diameters of the rings were averaged.

Capillary assays. One-hour capillary assays were performed at 35°C essentially as described by Adler (1). We used 10 mM serine as an attractant. Polyamines were added to both the pond of cells and the capillary. Triplicate measurements were made for each condition.

Antibiotic permeation assays. The ability of cephaloridine to permeate through porins was assayed as described by Nikaido et al. (24). Briefly, 50 ml of cells was grown at 37°C in Luria-Bertani medium with 5 mM $MgCl_2$ to an A_{650} of 0.6 and then harvested, washed twice in 10 mM NaH_2PO_4–5 mM $MgCl_2$ (pH 6), and resuspended in 10 ml of the same buffer. Fifty microliters of cells was incubated for 5 min with 400 μl of buffer either with or without polyamines, and then 50 μl of a cephaloridine stock solution was added to a final concentration of 1 mM. Immediately, 400 μl of this mixture was transferred to a 1-mm-path-length quartz cuvette, and the rate of cephaloridine degradation by the periplasmic β-lactamase was measured as a decrease in A_{260} in a Uvikon 810 dual-beam spectrophotometer (Kontron Instruments). The readings were made during the 4 min immediately following the addition of the antibiotic. These measurements were performed with intact cells and with cell-free supernatants in order to correct for enzyme leakage out of the periplasm. These latter values were typically no more than 10% of the rate measured with intact cells.

Growth curves. Two hundred fifty microliters of an overnight culture was inoculated into 25 ml of T-broth with or without polyamines and incubated at 37°C for 1 h. One-milliliter aliquots were subsequently taken from each culture every half hour, and their A_{590}s were read. The doubling time was obtained as (ln 2/k), where k represents the growth rate calculated from the following equation: $\ln (N_2/N_1) = k \times (t_2 - t_1)$ (N_2 and N_1 are the A_{590}s at times t_2 and t_1, respectively).

Toxicity assays. Two types of toxicity assays were performed. In the first one, toxicity was assessed on plates by first growing cells in T-broth with shaking at 37°C to an A_{590} of 0.5 and then spreading 100 μl on T-agar plates (1.5% agar) containing either no polyamine or a given polyamine at different concentrations. The plates were incubated for 24 h at 37°C, and the colonies were counted. In the second assay, toxicity was measured in liquid cultures by first growing cells in T-broth to A_{590} of ~0.3 and then transferring 500 μl of that culture into 500 μl of fresh medium with or without polyamine. The cultures were incubated for 0.5, 1, or 3 h, at which time 25-μl portions of serial dilutions were plated onto T-plates. The number of colonies counted after 24 h of incubation at 37°C represented the number of surviving cells.

RESULTS

Chemotaxis. The ability of cadaverine to affect chemotaxis has been determined qualitatively in our previous study (7). We were interested in obtaining more-quantitative information on the effects of the four major polyamines over a wide range of concentrations. Our first set of data was obtained with chemotaxis swarm plate assays, which, although they do not separate true chemotaxis effects from those on growth, allow for an easy and reproducible assessment of chemotactic ability. Figure 1 shows a graph of the swarm inhibition caused by polyamines. To normalize the results obtained from 5 to 10 colonies in several experiments (n = 1 to 3), the results are represented as ratios of ring diameters in polyamine-containing plates to those observed in plates without polyamine addition. A concentration-dependent decrease in chemotactic ability is consistently found for the four polyamines studied. The potencies of the inhibitory compounds follow the series spermine > spermidine > putrescine ~ cadaverine. The swarm plate assays take 8 h to complete and are carried out in a nutrient-containing medium. Thus, it is not possible to separate effects of the polyamines on chemotaxis from those on growth or viability. As will become apparent in other figures, it is likely that the inhibition observed at the highest concentrations used is attributable in part to slowed growth and/or lethality.

In order to assess the effect of polyamines on chemotaxis per se, we have measured the number of cells entering an attractant-filled capillary. This type of assay is growth independent, since both cells and attractant are placed in a phosphate buffer, and takes only 1 h. Figure 2 shows that the capillary assays confirmed the results obtained from swarm plate assays. The chemotaxis of strain AW737 to 10 mM serine was measured in the presence of increasing concentrations of polyamines in both the pond and the capillary. Positive control (absence of polyamines only) and negative control (absence of attractant and polyamines) experiments were also performed. A concentration-dependent inhibition of chemotaxis was observed in all

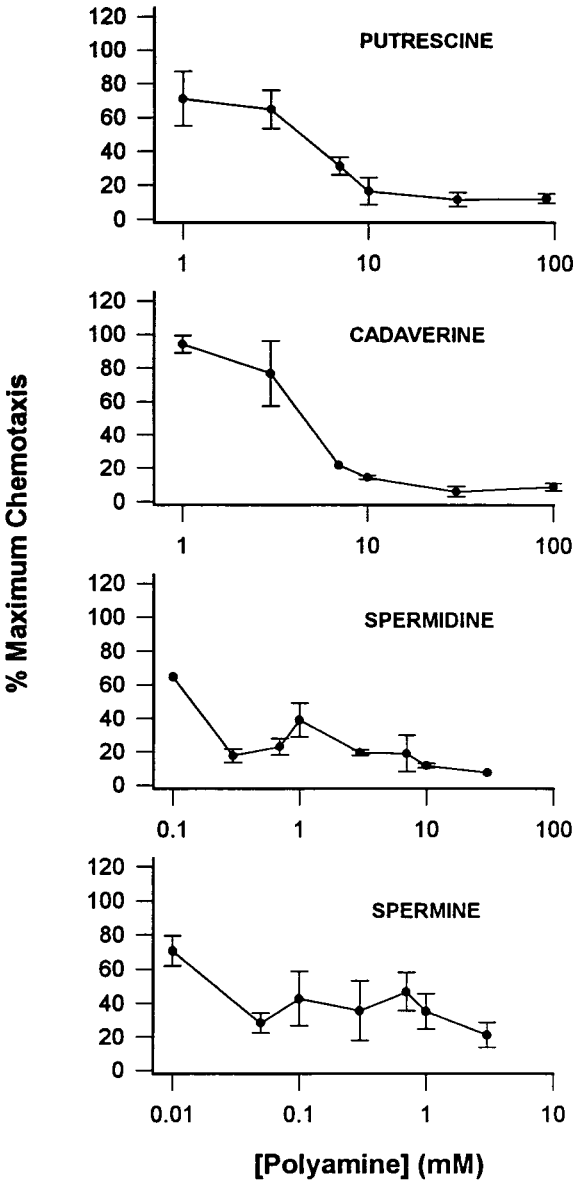

FIG. 2. Polyamines inhibit chemotaxis to 10 mM serine in capillary assays. Polyamines were present in the pond and the capillary. Triplicate data points were collected for each experiment. One to six experiments were performed for each polyamine concentration tested. Error bars represent standard errors of the mean. Maximum chemotaxis is defined as the chemotaxis to 10 mM serine in the absence of polyamine and averaged ~60,000 cells per ml accumulated in the capillary.

cases, although the concentration ranges were typically shifted towards concentrations lower than those in swarm plate assays. We ascribe this shift to the fact that swarm plate assays are performed in a medium of higher ionic strength than capillary assays, which would tend to shield the ionic interactions involved in the modulation of porins by polyamines (7). Although there is some scatter of the data, as a result of the nature of the assay itself, the inhibitory trend is consistently observed for the four polyamines tested, with potencies that follow a series identical to that obtained with swarm plate assays. The inhibition is clearly established at concentrations that are lower than those shown to have toxic effects after a 1-h incubation (see Fig. 6). A combination of capillary assays, plug assays, and temporal assays (data not shown) revealed that polyamines neither are repellents nor perturb bacterial motility. Thus, we conclude that the observed impairment in chemotactic ability is due to reduced porin-mediated permeation of the attractant through the outer membrane, similar to what has been reported for porin-deficient mutants (13).

Antibiotic permeation. The inhibition of porin-mediated fluxes by polyamines can be directly tested in intact cells by antibiotic permeation assays. The hydrolysis of cephaloridine by the periplasmic β-lactamase is rate limited by the ability of the antibiotic to permeate through the outer membrane. Previous studies have shown that porins are the main pathway for entry of these antibiotics into the periplasm (10). Thus, the rate of hydrolysis of cephaloridine in intact cells can be used as a measure of porin-mediated outer membrane permeability. Our rationale was that if porins close in response to the presence of polyamines, the rate of permeation of the β-lactam antibiotic should be reduced. Cephaloridine was chosen because of its high rate of penetration (24) and neutrality. We wanted to avoid complications that might arise with the use of charged antibiotics in an assay that already tests the effect of a charged compound (the polyamine) on the channel.

Initial experiments with AW737, which expresses both porin types, were somewhat unreliable because of the greater leakage of the β-lactamase in this strain. Pilot experiments yielded 77 and 65% inhibition of cephaloridine flux with 0.1 and 0.3 mM spermine, respectively, but the leakiness rate was 30% of the control value. It is not clear why this strain was different from those expressing only OmpC or OmpF. However, high leakage rates are usually indicative of a somewhat damaged outer membrane (21) and thus may lead to misinterpretation of the data. In addition, we wanted to avoid extensive correction of the data by leakage rate subtraction. Since we also had some interest in knowing whether OmpC and OmpF have different sensitivities to polyamines, we decided to focus our analysis on the rate of permeation of cephaloridine in strains expressing a single porin type.

Figure 3 shows that polyamines reduce the flux of cephaloridine through OmpF in a concentration- and polyamine-dependent manner. The potencies of the polyamines were in the order spermine > spermidine > cadaverine ~ putrescine. Identical concentration ranges were tested with a strain expressing *ompC* only (Fig. 4). Although effects are clearly observed, it appears that OmpC is less sensitive to all polyamines, most notably to spermine and spermidine. Our electrophysiological experiments demonstrated that the binding of the polyamine is sensitive to the transmembrane electric field (7). This suggests that the polyamine does not bind to proteinic loops at the membrane surface but rather needs to enter the channel to exert its effect. This model predicts that the smaller OmpC channel would be less sensitive to the polyamines, especially the larger spermine and spermidine, because steric considerations would prevent these molecules from reaching their binding sites. This is supported by the antibiotic permeation experiments reported above.

Control experiments showed that the enzymatic rate determined with the supernatant of sonicated AW738 or AW739 cells (containing the released periplasmic β-lactamase) was not affected by the polyamines at the lowest concentrations used in the assays whose results are presented above. A 20 to 25% inhibition was observed with 250 mM putrescine, 300 mM cadaverine, and 30 mM spermidine, but no inhibition was observed with 3 mM spermine. At these concentrations (the highest used in the assays), the reduction in enzymatic rate for intact cells ranged from ~60 to ~80% for the $OmpF^+$ strain and was about 40% for the $OmpC^+$ strain. Thus, it is clear that

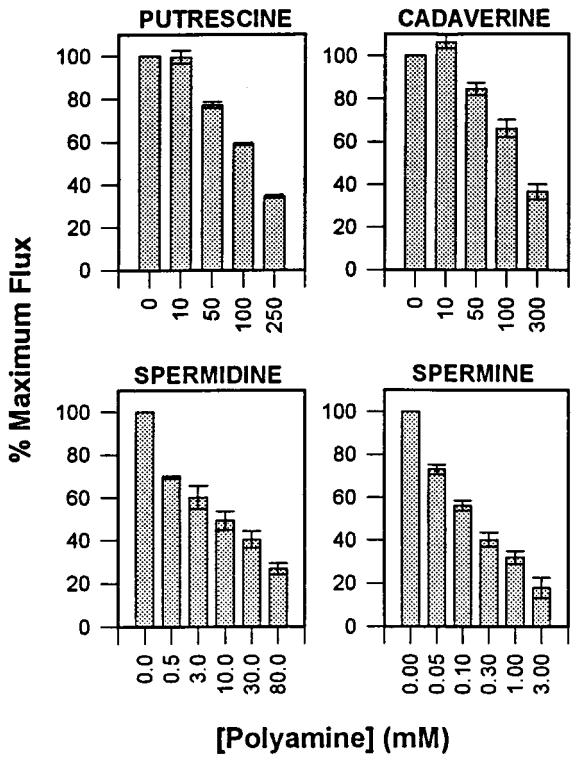

FIG. 3. Polyamines inhibit cephaloridine permeation through OmpF porin. Strain AW738 ($OmpC^-$ $OmpF^+$) containing the β-lactamase-encoding plasmid R_{471a} was used to measure outer membrane permeability in the presence of polyamines. The *y* axis of each graph represents the ratio of the permeation rate in the presence of the polyamine to that in the absence of the polyamine. Each bar represents the average of six data points collected in two independent experiments. The average permeability coefficient under control conditions was 2.5×10^{-5} cm/s.

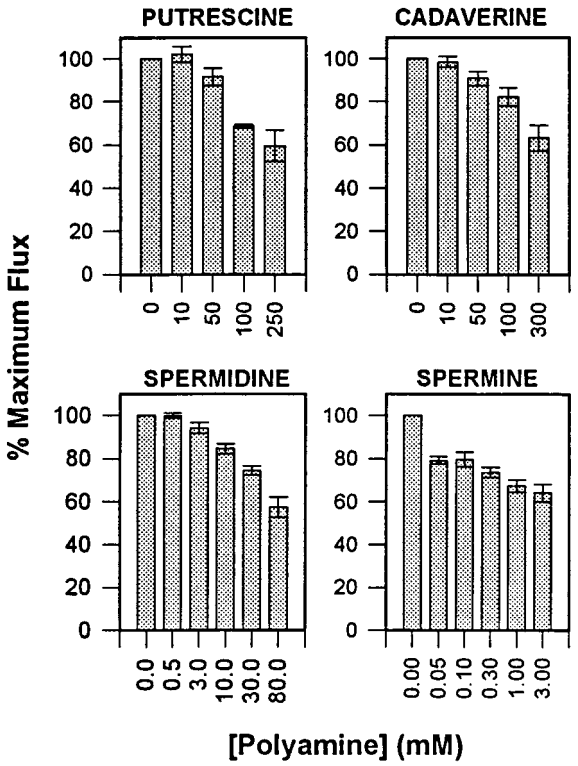

FIG. 4. Polyamines inhibit cephaloridine permeation through OmpC porin. Strain AW739 ($OmpC^+$ $OmpF^-$) containing the β-lactamase-encoding plasmid R_{471a} was used to measure outer membrane permeability in the presence of polyamines. The *y* axis of each graph represents the ratio of the permeation rate in the presence of the polyamine to that in the absence of the polyamine. Each bar represents the average of six data points collected in two independent experiments. The average permeability coefficient in control conditions was 1.9×10^{-5} cm/s.

the observed rate of inhibition in live cells is mostly due to a decrease in the rate of permeation of the antibiotic through the porins rather than to an inhibition of the enzyme activity per se.

In other experiments, we first incubated AW738 cells for 5 min with the polyamines at the highest concentrations used for Fig. 3. We then measured the β-lactamase activity in the supernatant of such cells to test whether the polyamines had caused any leakage of the periplasmic enzyme. The hydrolysis rates were comparable to those of the supernatants of cells not exposed to polyamines (data not shown). This indicates that the polyamines by themselves did not cause any disruption of the outer membrane at the concentrations used, in agreement with previous observations (36).

Growth. In order to estimate the contribution of growth inhibition to the decreased chemotaxis monitored in swarm plates, we decided to measure growth rates of cells in liquid cultures containing polyamines. This assay would also provide an indication of whether the inhibition of porin by polyamines was sufficient to effectively reduce the permeation of nutrients across the outer membrane and to slow growth. Bacteria were grown in T-broth with or without polyamines, and the turbidity of the culture was measured every half hour for 13 h. The results in Fig. 5 represent the averages from two separate experiments. For this assay, we chose polyamine concentrations that produced almost complete inhibition of ring formation in swarm plates.

Estimation of the doubling time was performed by fitting the datum points obtained between 2.5 and 5 h (for no addition, putrescine, cadaverine, and spermidine) or between 7 and 11 h (for spermine) to an equation describing logarithmic growth (see Materials and Methods). Spermine (0.5 mM) clearly

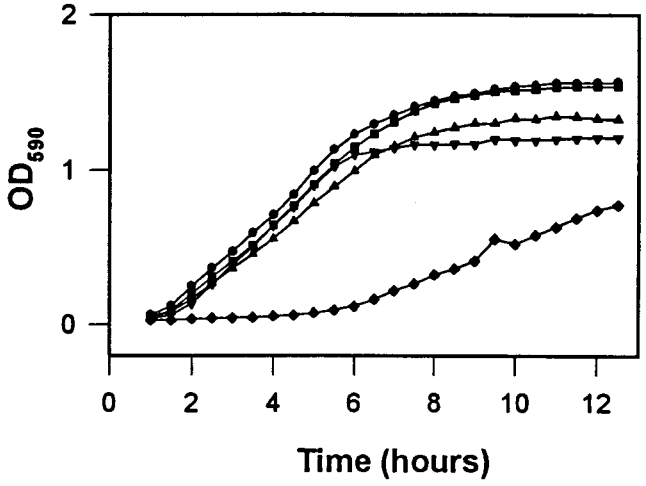

FIG. 5. The OD_{590} of T-broth cultures with or without polyamine was measured every half hour for 13 h. The graph represents the average for two experiments. Symbols: ●, no addition; ■, 100 mM putrescine; ▲, 100 mM cadaverine; ▼, 20 mM spermidine; ◆, 0.5 mM spermine.

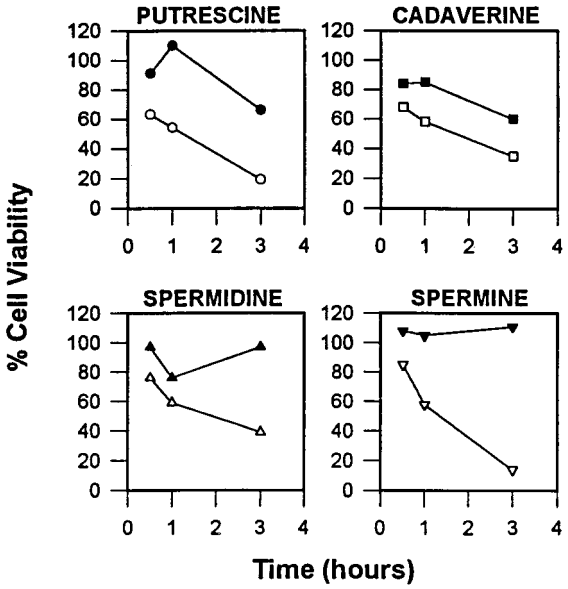

FIG. 6. Toxicities of polyamines. The percent cell viability is plotted against the culture incubation time. Polyamine concentrations were 80 mM (●) and 250 mM (○) putrescine, 100 mM (■) and 300 mM (□) cadaverine, 10 mM (▲) and 100 mM (△) spermidine, and 0.2 mM (▼) and 1.5 mM (▽) spermine.

slowed the growth by a factor of ~2 (doubling time with spermine, 2.66 h; doubling time with no addition, 1.78 h). The other polyamines caused a slight increase in the growth rate (doubling times were 1.61 h in 100 mM putrescine, 1.65 h in 100 mM cadaverine, and 1.44 h in 20 mM spermidine). Except with putrescine, a more substantial effect was seen during stationary phase. The maximal absorbance values obtained in stationary phase were decreased when cadaverine, spermidine, or spermine was present. The maximal absorbance was reduced to 85 and 77% of the control value by 100 mM cadaverine and 20 mM spermidine, respectively, and to 49% of the control value by 0.5 mM spermidine. The cadaverine- and spermidine-induced decreases in outer membrane permeability occur mostly during stationary phase. Spermine, however, exerts an inhibitory action on growth directly after its addition to the culture.

When bacteria are spread on agar plates containing polyamines at concentrations similar to those used for growth curves, the number of colonies appearing overnight is unchanged with respect to that under control conditions but the colony sizes are much smaller (data not shown). This observation supports the notion that polyamine effects on growth take place under conditions of nutrient limitations. Although it is possible that the effects of the polyamines on growth are pleiotropic, these findings are consistent with the previous results that polyamines reduce outer membrane permeability.

Toxicity. In order to ensure that the observed effects are not due to toxicity of the polyamines in the assays performed, the concentration thresholds at which the individual polyamines tested would cause lethality of the cells were determined. Figure 6 represents the time-dependent toxic effects of two different concentrations of each of the four polyamines, studied in liquid cultures (see Materials and Methods). Toxicity is either absent or minimal at the lowest concentrations used (80 mM putrescine, 100 mM cadaverine, 10 mM spermidine, and 0.2 mM spermine), which are similar to those tested for the growth curves. However, it is readily apparent at higher concentrations (250 mM putrescine, 300 mM cadaverine, 100 spermidine, and 1.5 mM spermine). These lethal concentrations are 3 to 10 times higher than those used in the growth assays of Fig. 5. Taken together with the observations on growth in liquid or solid medium, these results indicate that at low concentrations polyamines slow growth, primarily at stationary phase or in colonies, rather than produce acute lethal effects.

In most of the experiments reported above, inhibitory effects were observed in concentration ranges in which toxicity was minimal. In two cases, however, high concentrations were used: in cephaloridine permeation assays and swarm plate assays. In the former case, cells were in the presence of the polyamines for no more than 10 min, and the results of Fig. 6 indicate that the majority of the cells remained alive even after 30-min incubations. Thus, we are confident that toxicity of the polyamines was not a concern during the permeation assays. In swarm plate assays, however, it is possible that lethality might be partly responsible for the lack of ring formation, since swarming rates are dependent on both growth and chemotactic ability.

DISCUSSION

The outer membrane of *E. coli* is an effective cellular barrier whose permeability is determined mostly by the properties of porins. In this work, we have studied two processes that require high porin-mediated fluxes of hydrophilic solutes for maximum efficiency: chemotaxis and β-lactam antibiotic permeation. Chemotaxis is known to be affected by a reduced outer membrane permeability, since attractants must first penetrate into the periplasmic space in order to gain access and bind to receptor sites located in the inner membrane (13). The results from capillary assays clearly indicate that chemotaxis is inhibited by polyamines. The inhibitory trend is obvious even at concentrations that are lower than the lowest concentrations used in the toxicity assay of Fig. 6. At some of the highest concentrations, lethality may play a part, but the observed reduction of chemotaxis is always of a magnitude greater than expected from purely toxic effects. Although not providing direct conclusive evidence because of the growth-dependent component of the assays, the results from swarm plates are also supportive of the notion that chemotaxis is inhibited by polyamines.

The permeation of β-lactam antibiotics, such as cephaloridine, is also highly dependent on the presence of functional porins, since rates are decreased in porin mutants (12, 25). Our results document that polyamines, compounds which have been shown to modulate porin activity electrophysiologically (6, 7), effectively decrease the extents of these processes, as they decrease outer membrane permeability. Some shifts in the concentration dependence are observed among the chemotaxis and flux assays used. Some of these shifts can be ascribed to differences in the natures of the assays themselves. For example, a stronger apparent inhibition can be expected in the swarm plate assays because of growth-dependent effects, which are more readily apparent in this assay, which spans many hours. However, results of the three assays are in agreement in regard to the effective concentration ranges of the four polyamines: submillimolar for spermine, 1 to 10 mM for spermidine, and 10 to 100 mM for putrescine and cadaverine. These concentration ranges are similar to those obtained from electrophysiological studies of polyamine-induced inhibition of porins (6, 7, 14).

The relationship between outer membrane permeability and bacterial growth is more complex. Bavoil and coworkers (2) made the observation that growth in rich media was not significantly impaired in porin-deficient mutants, even though

antibiotic permeation rates were only 10% of wild-type values. This led to the hypothesis that a large number of porins might be needed under natural conditions in which nutrients are scarce but that flux through only 0.01% of the normal amount of porins would be sufficient to support growth when nutrient concentrations are saturating. Our results, which show that putrescine, cadaverine, and spermidine did not significantly alter the growth rate of bacteria in T-broth, support the notion that the number of uninhibited porins is largely sufficient to support growth in exponentially growing cultures in rich medium. However, in stationary phase, the effect of the polyamines becomes evident. A possible explanation is that at this stage of higher cell density, any decrease in the number of open porins will be felt more drastically, because the cells are competing for nutrients more aggressively. The effect of spermine was distinct from those of the other three polyamines in that the doubling time was increased. In our studies, spermine has consistently been the most potent polyamine, and it therefore could have led to a much stronger inhibition of porins, resulting in a reduced growth rate.

Our finding that polyamines can drastically affect porin-mediated fluxes is important because it shows that (i) porins are functionally regulated channels and (ii) polyamines and related compounds could potentially serve as therapeutic agents specifically targeted at the outer membrane. Although the outer membrane is an effective barrier against hydrophilic and many hydrophobic compounds, some types of antibiotics, such as polymyxin B and aminoglycosides, have been shown to enter the cells by increasing outer membrane permeability (10, 11, 35). In their studies of polycation-induced sensitization of bacteria to antibiotics, Vaara and Vaara (36) reported that, as opposed to cations bearing a large number of positive charges (5 to 50), cadaverine, spermine, and spermidine were neither bactericidal nor active as outer membrane permeability-increasing agents. However, the submillimolar concentrations used in their study were 1 to 2 orders of magnitude lower than those used in the present work.

In contrast to the large number of studies on disruption of outer membrane integrity (10), there have been fewer reports of reduced permeability of this membrane as a consequence of a chemical treatment. It is clear that the control of outer membrane permeability plays an essential role in cell survival. Porins may be ideal targets for inhibitory drugs that would decrease the permeability of the membrane to essential solutes and thus compromise cell survival. At this point, it is not clear whether polyamines influence porin activity directly by binding to the channels or indirectly by affecting the surrounding lipids. For example, divalent cations such as Mg^{2+} and Ca^{2+} appear to stabilize the outer membrane by binding to the highly negatively charged lipopolysaccharides (25, 26, 29). Our electrophysiological results support a direct mechanism of porin modulation, because the voltage dependence of the inhibition suggests that the polyamines bind to a site that is buried within the thickness of the membrane, for example, the channel interior (6, 7). Even at the highest concentrations used in this work, none of the four polyamines significantly altered the MICs of erythromycin or polymyxin B (data not shown), in agreement with previous work (36). This lack of effect on antibiotics known to interact with the outer membrane substantiates the idea that the polyamines specifically associate with porins. The binding of spermidine to OmpF has actually been demonstrated biochemically (15). Our continued biophysical studies will provide information on the precise location of the binding site and the selective design of polycationic molecules with higher affinities for porins than the polyamines tested in this study.

An important issue raised by the present study is the possible involvement of polyamines as natural regulators of porin activity. The location of the endogenous polyamines has been difficult to ascertain, but it appears that the compounds can be in the vicinity of porin channels. Buch and Boyle (4) reported that the arginine decarboxylase responsible for putrescine synthesis is a periplasmic enzyme, and Koski and Vaara (16) documented that polyamines are constituents of the outer membrane. Periplasmic concentrations of polyamines might fluctuate in response to environmental changes. For example, an efflux of putrescine when cells are grown in high-osmolarity media was demonstrated (20), and increased cadaverine concentrations are secreted in the medium when cells are grown at low pH (19). It is possible that if the concentrations of polyamines increased to high levels under detrimental conditions (for example, low pH), a decreased outer membrane permeability through porin inhibition would ensue as a possible defense mechanism. In that respect, it would be of interest to assess the extent of the outer membrane permeability under those conditions in which modulation by endogenous polyamines might occur.

The combination of our previous electrophysiological studies (6–8) and the work presented here strongly supports the newly emerging concept of regulated porin channels. The view of porins as permanently open pores needs to be refined to take into account our observations of the fluctuations of porins between closed and open states (gating) even in the absence of transmembrane voltages (9) and the modulation of this gating activity by ligand binding (7, 8). In reconstituted systems, porins display an open configuration most of the time, but they appear to be mostly closed when electrophysiological experiments are performed on intact cells or spheroplasts (5). It is possible that OmpC and OmpF, which are known to be sensitive to reconstitution protocols (17, 27), change their physiological states during membrane or protein purification. However, the concept of closed porins in cells is not entirely new. Closed-channel forms were identified in *E. coli* OmpA (31) and have been postulated for the *Pseudomonas aeruginosa* OprF porin (22, 37). It is clear that future investigations are required to determine the role of porin modulation in the context of physiologically relevant situations.

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