JOURNAL OF BACTERIOLOGY, Feb. 1999, p. 791–798 Vol. 181, No. 3
0021-9193/99/$04.00+0

Excretion of Endogenous Cadaverine Leads to a Decrease in Porin-Mediated Outer Membrane Permeability

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Received 25 August 1998/Accepted 19 November 1998

The permeability of the outer membrane of *Escherichia coli* to hydrophilic compounds is controlled by porin channels. Electrophysiological experiments showed that polyamines inhibit ionic flux through cationic porins when applied to either side of the membrane. Externally added polyamines, such as cadaverine, decrease porin-mediated fluxes of β-lactam antibiotics in live cells. Here we tested the effects of endogenously expressed cadaverine on the rate of permeation of cephaloridine through porins, by manipulating in a pH-independent way the expression of the *cadBA* operon, which encodes proteins involved in the decarboxylation of lysine to cadaverine and in cadaverine excretion. We report that increased levels of excreted cadaverine correlate with a decreased outer membrane permeability to cephaloridine, without any change in porin expression. Cadaverine appears to promote a sustained inhibition of porins, since the effect remains even after removal of the exogenously added or excreted polyamine. The cadaverine-induced inhibition is sufficient to provide cells with some resistance to ampicillin but not to hydrophobic antibiotics. Finally, the mere expression of *cadC*, in the absence of cadaverine production, leads to a reduction in the amounts of OmpF and OmpC proteins, which suggests a novel mechanism for the environmental control of porin expression. The results presented here support the notion that polyamines can act as endogenous modulators of outer membrane permeability, possibly as part of an adaptive response to acidic conditions.

The outer membrane of gram-negative bacteria forms a natural barrier that protects the cell from harmful agents such as proteases, some antibiotics, bile salts, and toxins. Its permeability depends largely on porins, abundant trimeric proteins that form nonspecific, mostly open channels. These proteins have been characterized extensively at the biochemical and molecular levels (33). β-Lactam antibiotics have been shown in intact cells to permeate the outer membrane through porins at high rates, strengthening the belief that porins are permanently open pores (33). Many other biochemical and electrophysiological studies of reconstituted purified porins have confirmed that porins are mostly open pores (2, 9, 33). This property along with their role as molecular filters that discriminate against solutes of high molecular weight makes porins the major pathway for fast nutrient flux in a highly protective outer membrane.

Recent patch-clamp studies on reconstituted porins, however, have revealed that closures of porins are induced in the presence of polyamines or membrane-derived oligosaccharides applied to the periplasmic side (7, 9, 20). In addition, externally applied polyamines inhibit the flux of β-lactam antibiotics through porins and thus decrease the permeability of the outer membrane (8).

Polyamines are a class of naturally occurring polycationic molecules that have been implicated in a wide range of biological phenomena, including modulation of ion channels of heart, muscles, and neurons (11, 22, 27). They are associated with the outer membrane of *Escherichia coli* and are likely to accumulate in the periplasmic space during their synthesis and transport (4, 23, 25, 30). Cadaverine, one of the smallest polyamines, is the end product of a pH-induced lysine decarboxylation. The *E. coli cadBA* operon encodes a lysine decarboxylase (*cadA*) and a lysine-cadaverine antiporter (*cadB*) and is coinduced by external low pH, anaerobiosis, and lysine (36, 40). A positive regulator of *cadBA* expression has been identified as the membrane-bound protein CadC, whose gene lies upstream to the *cadBA* operon (10, 31, 32). The periplasmic domain of CadC senses both external pH and lysine as positive regulators and possibly cadaverine as a negative regulator of *cadBA* (10, 32). Upon induction, CadC binds the *cadBA* promoter and activates the operon. It is proposed that the acid-induced synthesis of cadaverine from lysine by CadA and its subsequent excretion through the lysine-cadaverine antiporter CadB lead to some neutralization of the external pH, thus protecting the cell from the acidic conditions. Under this mechanism, the levels of the endogenously expressed and excreted cadaverine are increased during *cadBA*-inducing conditions (28).

The study reported here was undertaken with the goal of understanding the physiological relevance of porin inhibition by polyamines. Based on our observations that polyamines inhibit porins from both membrane sides (20) and that external polyamines decrease porin-mediated flux of antibiotics (8), we hypothesized that endogenous cadaverine should also affect the porin-mediated outer membrane permeability as it transits the periplasm and/or becomes excreted. To test this hypothesis, we induced the synthesis of cadaverine in three ways: (i) by low-pH induction, (ii) by placing both *cadA* and *cadB* under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, and (iii) by constitutive *cadBA* expression from a pH-independent *cadC* mutant. The results suggest that multiple pathways are used for the decrease in outer membrane permeability induced at acidic pH, including a reduction in porin expression and a cadaverine-dependent inhibition of porin-mediated fluxes.

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MATERIALS AND METHODS

Strains and plasmids. *E. coli* K-12 strains were used, and their relevant characteristics are shown in Table 1. Strains HS200, EP243, EP247, and EP314

TABLE 1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristics	Reference
E. coli strains		
W3110	F^- IN(*rrnD-rrnE*)	31
HS200	W3110 (F′ Tn*10 proAB lacI*^{q} ZΔM15)	This work
XL1-blue	*lac* (F′ Tn*10 proAB lacI*^{q} ZΔM15)	5
EP243	W3110Δ(*lacIOPZYA*) *exa*-1::Mu dI*1734*(Km *lac*)[a]	31
EP247	W3110 *cadC1*::Tn*10*	31
EP314	EP243 *cadC1*::Tn*10*	31
AW737	Wild type for porins, *hisG4 thr-1*(Am) *tonA31 tsx-78*	19
HS111	AW737 (*ompF* Δ*ompC zei*::Tn*10*)	This work
Plasmids		
pBR322	Cloning vector, Tet^r Ap^r	3
pCD470	pGB2-based plasmid expressing a mutant *cadC*, Sp^r Sm^r	10
pCADA	pBGS18-based plasmid expressing *cad*A under the control of *lacUV5* promoter, Km^r	28
pCADB	pBR322-derived plasmid expressing *cadB* under the control of the *tac* promoter, Tet^r Ap^r	28
R_{471a}	R factor, Ap^r	15

[a] The *exa*-1::Mu dI*1734* allele is an insertion of Mu dI*1734* in *cad*A, resulting in a CadA$^-$ phenotype.

are all derived from wild-type strain W3110 and are therefore isogenic except for the genes being manipulated or a deletion of the *lac* operon. For most of the antibiotic permeation assays, the β-lactamase-encoding R_{471a} factor (15) was introduced into the strains by conjugation as previously described (6). Strain HN37 (kindly provided by H. Nikaido) was used as the donor for the R factor, and the conjugants were selected on plates containing mitomycin C (1 μg/ml) and ampicillin (100 μg/ml). For antibiotic permeation assays on strains carrying plasmid pCADA (Table 1), the β-lactamase was produced from either the R_{471a} factor or a pBR322 plasmid. Other plasmids are described in Table 1. To acquire a better control of *lac* promoter-dependent expression of *cadA* and *cadB*, a *lacI*^{q} gene on an F factor was introduced in strains harboring plasmids pCADA and/or pCADB by mating with *E. coli* XL1 (5). Successful conjugants (HS200) were selected on minimal medium plates with lactose as the sole carbon source and in the presence of tetracycline (15 μg/ml).

HS111, a strain deficient in OmpC and OmpF, was constructed as follows. Strain AW739 (19) was transformed with the replication temperature-sensitive plasmid pMAK705 (12) on which *ompF* had been cloned. P1 transduction was then used to move an *ompC* deletion from strain AW738 (Δ*ompC zei*::Tn*10*) (19) into the chromosome. The resulting strain expressed *ompF* only when grown at a temperature permissive for maintenance of plasmid pMAK705 (30°C). To obtain an *ompC ompF* mutant strain, the cells were grown at the nonpermissive temperature (42°C) for 6 h in liquid medium and then plated on modified Luria-Bertani (MLB) plates in the absence of antibiotics. After overnight incubation at 30°C, 50 colonies were tested for sensitivity to chloramphenicol (the antibiotic marker carried by pMAK705). Clones that did not grow in the presence of the antibiotic were identified and designated HS111.

Growth conditions. *E. coli* was normally grown in MLB containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl, with the pH adjusted to 7.0 with NaOH. For experiments where the effects of growth at pH 7.6 and 5.8 were compared, cells were first grown for 1 h in MLB at pH 7.6 and harvested by centrifugation in two centrifuge tubes. The pelleted cells were then resuspended in the same volume of fresh MLB either at pH 7.6 or at pH 5.8 and allowed to grow for 30 more min at 32°C before being harvested again for the various assays. Buffering of MLB was done at pH 7.6 with 100 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] or at pH 5.8 with 100 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (10). For the experiments with strains carrying plasmids with *lac*-controlled genes, cells were grown in MLB at pH 7.6 to an optical density at 650 nm (OD_{650}) of 0.3 before the addition of IPTG at a final concentration of 1 mM. After growing further to an OD_{650} of 0.6 (~1 h), the cells were harvested for assay. Strains that express *cadA* from plasmid pCADA grow as fast as the wild-type strain, but strains that overexpress *cadA* and *cadB* grow slowly.

Plasmid-containing cells were grown in the presence of the appropriate antibiotic at the following concentration: ampicillin sodium salt, 100 μg/ml; kanamycin sulfate or spectinomycin, 50 μg/ml; or tetracycline, 15 μg/ml. All cultures, solid and liquid, were maintained at 32°C. This was necessary for maintenance of the Mu dI*1734* prophage in strains EP243 and EP314 and thus was used for growth of all strains. Antibiotics and organic chemicals were purchased from Sigma Chemical Co., inorganic chemicals were purchased from Fisher Scientific, and medium components were purchased from Difco Laboratories.

Preparation of porin extracts and SDS-urea-PAGE. Cells from overnight cultures were washed in 30 mM Tris (pH 8.1) and broken by Tris-EDTA-lysozyme treatment followed by sonication (three 20-s bursts). After removal of the unbroken cells, the supernatant was centrifuged again at 65,000 rpm for 15 min, to collect the cell envelopes. The final pellet was resuspended in 50 mM KCl–5 mM HEPES–1 mM K-EGTA (pH 7.2); 30 μg of protein was analyzed by sodium dodecyl sulfate-urea (6 M)-polyacrylamide gel electrophoresis (SDS-urea-PAGE). The level of porin expression was quantified with an EagleEye densitometer and calculated relative to OmpA (standard control).

Antibiotic permeation assays. The rates of permeation of the β-lactam antibiotic cephaloridine were determined as described elsewhere (35). Fifteen-milliliter cultures were inoculated from overnight cultures and grown at 32°C in MLB supplemented with 5 mM $MgCl_2$ to an OD_{650} of 0.6. The cells were harvested, washed twice in permeability buffer (10 mM NaH_2PO_4, 5 mM $MgCl_2$ [pH 6.0]), and resuspended in 6 ml of the same buffer. One hundred microliters of cells was mixed with 300 μl of permeability buffer and 100 μl of a 5 mM cephaloridine stock solution; 400 μl of the mixture was transferred immediately 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 Unikon 810 double-beam spectrophotometer (Kontron Instruments). Continuous readings were taken for 4 min following addition of the antibiotic. Such measurements were performed with both intact cells and cell-free culture supernatants as a control for any possible enzyme leakage from the periplasm. Typically, supernatant values were less than 5% of the rate measured with intact cells, and the measured rates were not corrected for leakage.

Cadaverine excretion assay. Fresh cultures inoculated from overnight cultures were grown at 32°C with shaking to an OD_{650} of 0.6. The cells were then pelleted, and the supernatant was tested for cadaverine content. All reagents and procedures were as described elsewhere (37), with the following modifications. One milliliter of culture supernatant was mixed with 1 ml of K_2CO_3 and 1 ml of 10.2 mM of 2′,4′,6′-trinitrobenzylsulfonic acid, and the mixture was incubated for 5 min at 42°C. The colored product, *N*,*N*′-bistrinitrophenylcadaverine, was extracted with 2 ml of toluene after vortexing for 20 s and centrifugation at 2,500 rpm for 5 min. The A_{340} of the extract was read. Appropriate medium blanks were used to verify that trace amounts of other amines and amino acids present in the supernatant did not interfere with the cadaverine measurement. Standard curves show that the assay is linear between absorbance values of 0.1 and 1.0, the latter corresponding to 250 μM cadaverine.

Antibiotic sensitivity assay. Cells were grown in MLB (pH 7.6) at 32°C. For cells containing pCADA, IPTG was added 1 h after the growth culture started. When the OD_{650} of the culture reached 0.6, the culture was split into two batches, one of which received the antibiotic to be tested. After a 1-h incubation at 32°C with shaking, each batch was diluted, and a 100-μl aliquot was plated onto LB plates and maintained at 32°C overnight. A viable count was obtained from the number of colonies present the next day, and percent survival in the presence of the antibiotic was calculated.

RESULTS

pH-induced decrease in porin expression and outer membrane permeability. Growth at pH 5.8 induces a change in outer membrane composition seen as a reduction in total porin expression. A 30-min exposure to acidic pH during growth promoted a major (34% ± 18% [mean ± standard deviation {SD}; $n = 3$) decrease in OmpF expression and no significant change in OmpC expression (decreased by 3% ± 5%, $n = 3$), as shown previously (16). These values were obtained by using the constant levels of OmpA as an internal control. Figure 1A shows that the total amount of porins (total porin expression = OmpC + OmpF) is reduced by 14% ± 9% at pH 5.8. The flux of cephaloridine measured in cells grown at this low pH is also lower than that in cells grown at pH 7.6. The 32% decrease in flux likely arose from not only the reduced abundance of porins in the membrane but also from the shift in the relative proportion of OmpC and OmpF in favor of OmpC, which has a narrower pore.

A drop in pH is known to have pleiotropic effects on cells (36, 40). Are there other factors that might contribute to the flux reduction when cells have been grown at acidic pH? A well-documented response to acidic pH is the induction of the *cad* operon, which leads to the excretion of cadaverine (28, 36, 40), a known inhibitor of porin function (7, 20). Upon acid induction in the presence of lysine, the *cadBA* operon activated

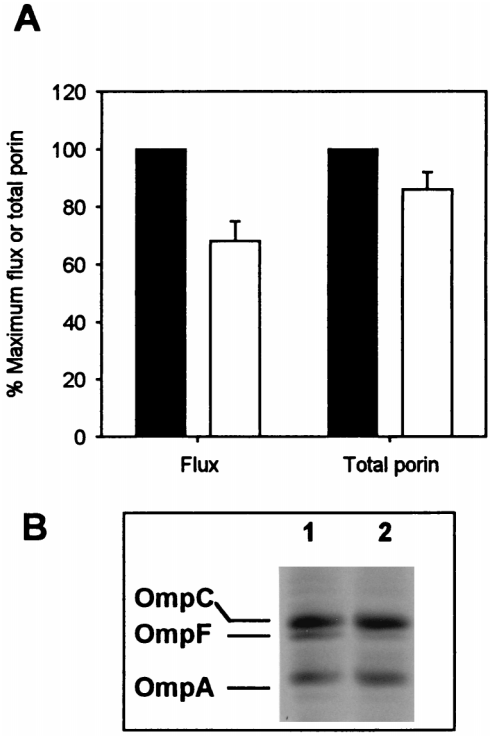

FIG. 1. (A) Effect of acidic pH on outer membrane permeability and porin expression in *E. coli* wild-type strain W3110 containing the R_{471a} factor and grown at pH 7.6 (black) or 5.8 (white). The flux rate (absolute value of $[5.2 \pm 0.3] \times 10^{-5}$ cm/s) and total porin expression of cells grown at pH 7.6 were set to 100%. Bars for flux data represent the averages of four experiments (triplicate measurements per experiment), and the error bars indicate SEM; bars for porin expression data represent the averages of three experiments (±SD), one of which is shown in panel B. (B) Representative SDS-urea-PAGE analysis of outer membrane proteins obtained from W3110 grown at pH 7.6 (lane 1) or 5.8 (lane 2).

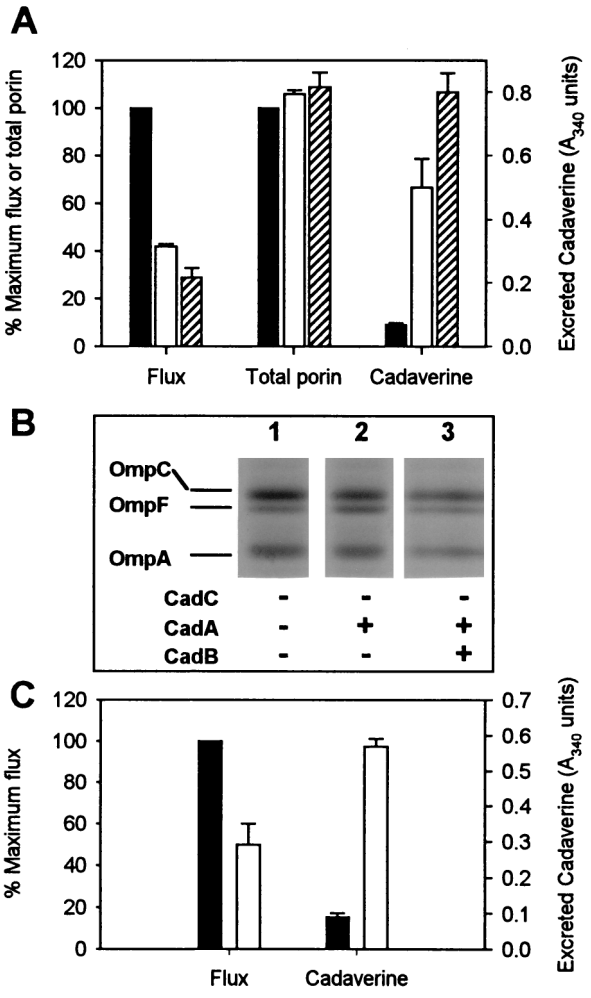

FIG. 2. (A) Outer membrane permeability, total porin expression, and excreted cadaverine in strains HS200/pBR322 (black), HS200/pCADA/pBR322 (white), and HS200/pCADA/pCADB (hatched). The left ordinate represents the ratio of the flux rate and total porin expression in the presence of CadA or CadBA to that in the absence of CadA and CadB. Bars for flux and excreted cadaverine data represent the averages of four experiments (triplicate measurements per experiment), and the error bars indicate SEM. Bars for total porin expression represent the averages of three experiments (error bars indicate SD), one of which is shown in panel B. The rate of permeation in the control strain HS200/pBR322 was $5.2\ 10^{-5}$ cm/s. (B) Representative SDS-urea-PAGE of outer membrane proteins obtained from HS200/pBR322 (lane 1), HS200/pCADA/pBR322 (lane 2), and HS200/pCADA/pCADB (lane 3). The minus sign in the CadC row indicates that CadC was not activated. (C) Outer membrane permeability and excreted cadaverine in HS200/R_{471a} (black) and HS200/R_{471a}/pCADA (white). The left ordinate represents the ratio of the flux rate in the presence of CadA to that in the absence of CadA. Bars represent the averages of three experiments (triplicate measurements per experiment), and the error bars indicate SEM.

by the positive regulator CadC expresses large quantities of lysine decarboxylase (32). The low-pH exposure used in the previous experiments also resulted in the excretion of a large amount of cadaverine into the medium. The average concentration of external cadaverine in the growth medium of the wild-type strain W3110 jumped from 25 to 350 μM ($n = 8$) upon a decrease in pH from 7.6 to 5.8. Although no lysine was added directly, sufficient induction of the *cadBA* can be obtained in LB medium at pH 5.8 (36a).

The endogenous production of cadaverine decreases outer membrane permeability. To determine whether the endogenous production of cadaverine leads to a decrease in outer membrane permeability independently from changes in porin levels, we decided to bypass the induction by pH and test directly the effect of inducing expression of the *cadA* and *cadB* genes.

We introduced into the wild-type strain W3110 an F′ episome containing the *lacI*^{q} gene and used this strain (HS200) as a host for the plasmids pCADA and pCADB, which encode *cadA* and *cadB* under the control of the *lac* and *tac* promoters, respectively (see Materials and Methods and Table 1). All experiments were performed at pH 7.6 to ensure that there was little expression of the chromosomal *cadBA* operon.

Three types of measurements were made: (i) rate of cephaloridine permeation through porins, (ii) total porin expression, and (iii) amounts of cadaverine excreted. Figure 2 shows the results for cells grown in the presence of 1 mM IPTG. Expression of both the *cadA* and *cadB* genes leads to a 10-fold increased amount of excreted cadaverine, with medium concentrations reaching 200 μM. Intermediate values are observed when only the *cadA* gene is present. Presumably, the export of cadaverine is still possible in these conditions because of a low-level expression of chromosomal *cadB* or via transporters other than the lysine-cadaverine exchanger. The expression of OmpC and OmpF (normalized to OmpA levels used as a loading control) is not affected by the presence of external cadaverine, as shown in Fig. 2A (the 9% ± 10% increase in the presence of pCADA and pCADB is not signif-

icant; $P > 0.05$, $n = 3$) and B. The amounts of OmpC and OmpF relative to OmpA were found to be, respectively, 1.20 ± 0.50 and 0.73 ± 0.13 for HS200, 1.15 ± 0.06 and 0.89 ± 0.16 for HS200/pCADA, and 1.25 ± 0.14 and 0.87 ± 0.08 for HS200/pCADA/pCADB. Although a slight increase in OmpF expression is found in the latter two strains, none of the values obtained from strains containing the plasmids are significantly different from those for the control strain HS200 ($P > 0.05$, $n = 3$).

The extent of β-lactam flux through porins is, however, greatly inhibited when the *cadA* and *cadB* genes are expressed (Fig. 2A). The amounts of excreted cadaverine and maximum inhibition are observed under conditions when both the lysine decarboxylase and the lysine-cadaverine antiporter are produced. The strong correlation between the decrease in flux and increase in secreted cadaverine, coupled with the steady levels of porin expression, suggests that flux inhibition is mediated through a cadaverine-dependent effect on porin function. Reduced cadaverine excretion (A_{340} of 0.1 or 0.3 in the presence of plasmid pCADA alone or with pCADB, respectively) and a smaller inhibition of antibiotic flux (25% or 30% in the presence of plasmid pCADA alone or with pCADA and pCADB, respectively) were also obtained in the absence of IPTG, because of the well-known leakiness of these plasmids (1a). Despite the presence of the single copy of the *lacI*^{q} gene, the *lac* operon is intrinsically leaky, especially on high-copy-number plasmids.

Although plasmids pCADA and pCADB have been used together by others (28), there was concern about the validity of the results in Fig. 2A and B because the two plasmids share the same origin of replication and may be incompatible (both are ColE1 derivatives). To address this issue, we grew cells containing the two plasmids in exactly the same conditions as used for the flux assays and plated them in the presence of either ampicillin or kanamycin or both. In all cases, the numbers of CFU were identical. In addition, we picked the colonies grown on kanamycin and tested them for ampicillin sensitivity; all were found to be resistant. These experiments clearly indicate that both plasmids are stably maintained, which is not surprising because they are multicopy plasmids and carry different antibiotic resistance markers.

Since one might still question whether the results are influenced by fluctuations in plasmid copy numbers, we repeated the experiments shown in Fig. 2A and B with cells that express β-lactamase from the R_{471a} factor and do or do not harbor pCADA as well (the two plasmids are compatible). As shown in Fig. 2C, a 50% ± 10% inhibition of cephaloridine flux is observed concomitantly with a 6.3-fold increase in cadaverine production in cells that harbor pCADA. An SDS-urea-PAGE analysis demonstrated that levels of porin expression were identical in cells lacking and cells containing plasmid pCADA (total amounts of OmpC plus OmpF relative to OmpA were 1.25 and 1.34 in the absence and presence of pCADA, respectively). These observations agree with the results in Fig. 2A and B and demonstrate that cadaverine inhibits porin function to the same extent, regardless of the plasmid combination used.

To substantiate the above data, we controlled the expression of *cadA* differently but still in a pH-independent way. We transformed EP247 (CadA$^+$) and EP314 (CadA$^-$), two strains derived from the wild-type strain W3110, with plasmid pCD470, which expresses a constitutively active *cadC* mutant allele (*cadC*^{c}) (Table 1). Both strains have a transposon insertion in the chromosomal *cadC* gene and thus produce CadC only from the plasmid-borne gene *cadC*^{c}. This mutant CadC protein is insensitive to pH and confers permanent activation of the *cadBA* operon (10). Figure 3A shows measurements of β-lactam flux, total porin expression levels, and amounts of excreted cadaverine. Since these two strains have the same genetic background, we set to 100% the flux rate and total porin expression obtained in the CadA$^-$ strain (EP314/*cadC*^{c}) and plotted the relative values for the CadA$^+$ strain (EP247/*cadC*^{c}). Any observed difference between the two strains can be attributed solely to the presence of CadA and the resulting cadaverine excretion.

Figure 3A shows that the pH-independent expression of *cadA* leads to greatly enhanced excretion of cadaverine (external concentration of 200 μM) but no change in the level of porin expression. The amounts of OmpC and OmpF relative to OmpA were found to be, respectively, 0.88 ± 0.07 and 0.67 ± 0.11 for the CadA$^-$ strain (EP314/*cadC*^{c}) and 0.89 ± 0.04 and 0.67 ± 0.18 for the CadA$^+$ strain (EP247/*cadC*^{c}) ($n = 3$ for all). The excretion of cadaverine is accompanied by a significant decrease in porin-mediated cephaloridine flux (Student *t* test, $P < 0.05$). These results agree with those shown in Fig. 2 and support the hypothesis that the inhibition of porin-mediated flux is due to cadaverine, not to a reduction in porin amounts. They also document that external cadaverine does not regulate expression of the *omp*C and *omp*F genes.

CadC alone influences porin expression and outer membrane permeability. As a control for the experiments described above, we compared the antibiotic flux rates and porin levels of EP314 in the presence and the absence of the constitutively expressed *cadC*^{c} allele. Figures 3C and D show that, surprisingly, the mere expression of *cadC* leads to a reduction of porin level and the resulting decrease in cephaloridine flux. It is important to point out that EP314 has a transposon insertion in *cadA*, and the resulting CadA$^-$ phenotype leads to no cadaverine excretion, even in the presence of constitutive CadC (Fig. 3C).

Interestingly, the profile of porin expression obtained through a CadC-dependent means of regulation is slightly different from that induced by a drop in pH. The constitutive expression of *cadC* leads to a decrease in amounts of both OmpF (by 15% ± 5%, $n = 3$) and OmpC (by 25% ± 5%, $n = 3$), not only OmpF as in Fig. 1B. The control exerted by CadC over the expression of porin genes may originate from cross-talk with the OmpR regulatory system (see Discussion). It represents a novel mechanism by which acidic pH can control outer membrane permeability.

Sustained inhibition of porins by cadaverine. Since the cells are washed of the externally released cadaverine before the permeability assay, how can cadaverine still exerts an inhibitory effect on porin during the measurement of antibiotic permeation?

One possibility is that there is a continuous excretion of cadaverine during the time required to set up and perform the flux assay (~10 min). Figure 4A shows that this is not the case. Immediately after the cadaverine-excreting cells were washed twice and resuspended in permeability buffer (0 min), the amount of external cadaverine drops to the background level observed in control cells that do not make cadaverine. This low level of cadaverine extrusion is maintained for at least 60 min. Thus, the inhibition of cephaloridine flux observed after cadaverine-excreting cells had been resuspended in permeability buffer for ~10 min (Fig. 2 and 3) cannot be attributed to the presence of external cadaverine during the flux assay.

The lysine decarboxylase and lysine-cadaverine antiporter are still present in the cell though, because a boost in cadaverine excretion can be obtained when 10 mM lysine is provided. For the experiment represented in Fig. 4B, cells remained in permeability buffer for 10 minutes and then were spun down and resuspended in permeability buffer containing

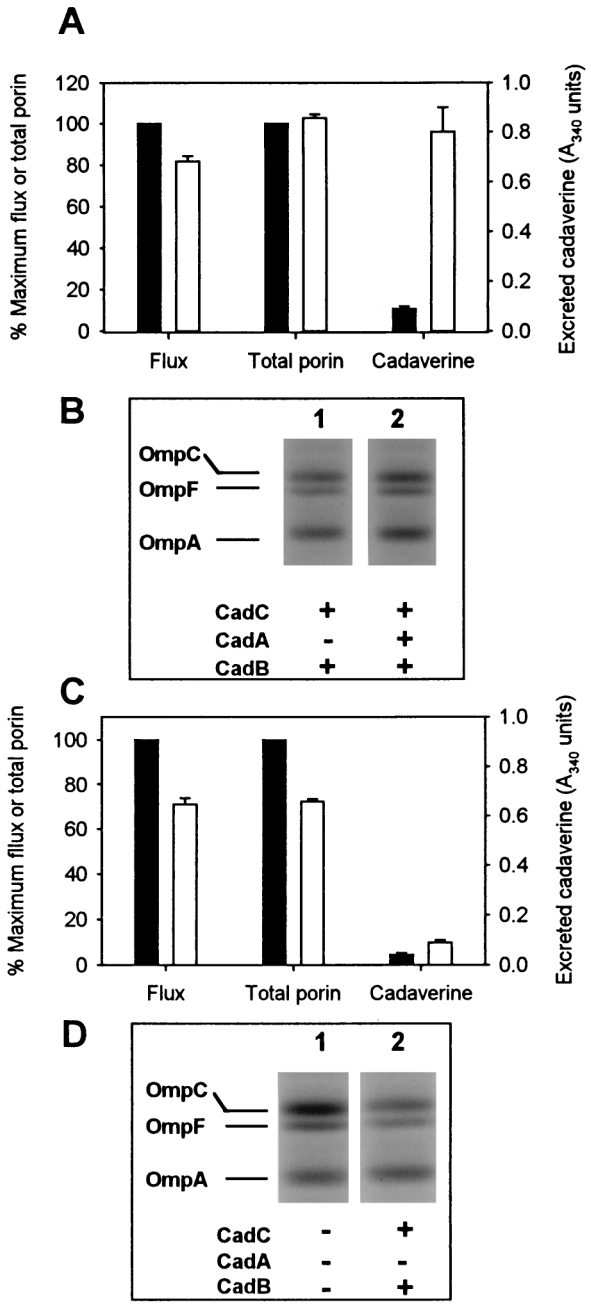

FIG. 3. (A) Outer membrane permeability, total porin expression, and excreted cadaverine in strains EP314/*cadC*^{c} (black) and EP247/*cadC*^{c} (white), containing the β-lactamase-encoding plasmid R_{471a}. The *cadC*^{c} allele is constitutive and allows cadaverine production in EP247 ($CadA^+$). The flux rate (absolute value of 4.2 10^{-5} cm/s) and total porin expression of EP314/*cadC*^{c} are set to 100%. Bars for flux and excreted cadaverine data represent the averages of four experiments (triplicate measurements per experiment), and error bars indicate SEM; the bars for total protein expression represent the averages of three experiments (±SD), one of which is shown in panel B. (B) Representative SDS-urea-PAGE analysis of outer membrane proteins obtained from EP314/*cadC*^{c} (lane 1) and EP247/*cadC*^{c} (lane 2). (C) R_{471a} factor-containing strains EP314 ($CadA^-$ $CadC^-$; black bars) and EP314/pCD470 ($CadA^-$ $CadC^+$ constitutively; white bars) were grown at pH 7.6. The cephaloridine flux rate (absolute value of $[5.1 \pm 0.1] \times 10^{-5}$ cm/s) and total porin expression in the absence of constitutively expressed *cadC* are set to 100%. Bars for flux and cadaverine data represent the averages of four and three experiments, respectively (triplicate measurements per experiment), and the error bars indicate SEM; the bars for total protein expression represent the averages of three experiments (±SD), one of which is shown in panel D. (D) SDS-urea-PAGE analysis of outer membrane proteins obtained from EP314 (lane 1) and EP314/*cadC*^{c} (lane 2).

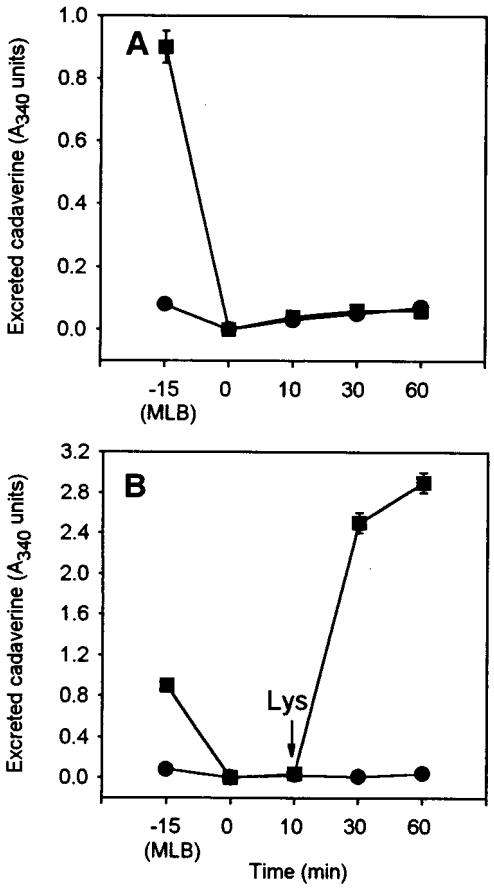

FIG. 4. Measurements of external cadaverine (i) in permeability buffer at various times after strain HS200/pBR322 (circles) and HS200/pCADA/pCADB (squares) cells had been washed and resuspended in this buffer and (ii) in medium at the end of growth (MLB; i.e., 15 min before resuspension in permeability buffer). Symbols represent the averages of four experiments (error bars indicate SEM and sometimes lie within the thickness of the symbol). For panel B, cells were spun at the 10-min time point (arrow) and resuspended in permeability buffer containing 10 mM lysine (Lys). The presence of external lysine does not interfere with the cadaverine assay (37).

10 mM lysine (pH adjusted to 6.0). Within 20 min after lysine addition, the amount of excreted cadaverine had increased more than 20-fold. Interestingly, the extent of antibiotic flux inhibition remained unchanged after the lysine-induced boost in cadaverine excretion (data not shown).

An alternative explanation for the antibiotic flux reduction is that the excretion of cadaverine during growth has produced an effect on porin function that is retained even after removal of the external polyamine. To test this hypothesis, we performed the following experiment. We grew the wild-type strain W3110 at pH 7.6, a condition in which it does not excrete cadaverine. After being washed with permeability buffer, the cells were resuspended in permeability buffer with or without 100 mM external cadaverine, and incubated for 5 or 30 min. At the end of the incubation period, an aliquot of cells was assayed for cephaloridine flux rate in the presence of the external cadaverine initially added. The remaining cells were washed twice of the external cadaverine, resuspended in permeability buffer for 5 min, and then tested for antibiotic flux rate in the absence of external cadaverine. Figure 5 shows that the presence of 100 mM external cadaverine during the flux assay has reduced the cephaloridine rate by ~40%, as shown previously

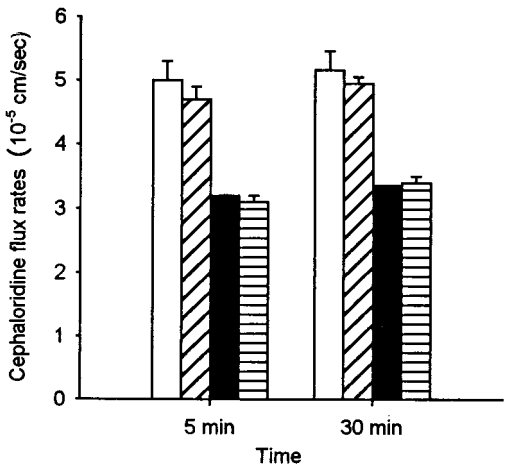

FIG. 5. Cadaverine induces a sustained inhibition of porins. Cephaloridine flux rates were obtained from wild-type strain W3110 containing the R_{471a} factor. The following treatments were carried out in parallel for each batch of cells: (i) cells were incubated in permeability buffer in the presence (black) or absence (white) of 100 mM cadaverine for the times indicated and assayed immediately thereafter in the same solution; and (ii) cells were incubated in permeability buffer in the presence (horizontal stripes) or the absence (hatched) of 100 mM external cadaverine for the times indicated, spun down, resuspended in permeability buffer only, and assayed in permeability buffer only. Values are the averages of four experiments (triplicate measurements per experiment), and error bars represent SEM.

(8), and that the inhibition is maintained even after the external cadaverine has been removed by washing.

This experiment shows that a prolonged effect on outer membrane permeability is elicited by the presence of external cadaverine and retained after removal of the polyamine. This inhibition is rapid, being completed within 5 min, with no additional effect after 30 min of incubation. Such apparently irreversible inhibition is in agreement with results of our electrophysiology experiments. When cadaverine is applied to the periplasmic side of porin-containing membrane patches, a rapid decrease in porin-mediated current is observed due to the permanent closure or inactivation of a number of pores. Such an effect remains even after the polyamine has been washed away from the patch (7, 20). Mechanisms for this intriguing form of inhibition are suggested in Discussion.

Physiological impact. To assess the extent of outer membrane permeability in the exact environmental conditions that are experienced by the cells, we measured the antibiotic flux directly in the growth medium from which they were harvested. Although the medium is rich in compounds absorbing at 260 nm (the wavelength used for the antibiotic flux assay), we were able to blank the sample successfully, most likely because of the use of a 1 mm-path-length cuvette. In growth medium, the flux rate was $(2.8 \pm 0.2) \times 10^{-5}$ cm/s for control cells that do not produce cadaverine (HS200/pBR322; the plasmid encodes the β-lactamase) and $(1.1 \pm 0.2) \times 10^{-5}$ cm/s for cells that excrete cadaverine (HS200/pCADA/pCADB; average cadaverine concentration, 200 ± 15 μM [$n = 3$]). The reduced cephaloridine flux observed with cells that excrete cadaverine is comparable whether cells are maintained in their own growth medium or washed in permeability buffer ($[1.8 \pm 0.2] \times 10^{-5}$ cm/s). This result is another example of the prolonged nature of the inhibition, as discussed above. Even in the absence of cadaverine, the flux rate measured in growth medium is much lower than that of the same cells washed in permeability buffer ($[5.3 \pm 0.8] \times 10^{-5}$ cm/s). The rate may be lower in growth medium because many porin channels are occupied by nutrients fluxing into the periplasm or because of the presence of yet unidentified inhibitors. It is worth noting that the combined presence of the medium and the released cadaverine results in 80% reduction in outer membrane permeability compared to the values traditionally measured in permeability buffer.

Since β-lactam antibiotics use porins as an uptake pathway, the cadaverine-induced porin inhibition is expected to restrict the entry of these types of antibiotics and possibly to provide the cells with some resistance. We tested this hypothesis by measuring the survival of cells in the presence of one of the following antibiotics at a concentration close to its MIC: ampicillin (5 μg/ml), which uses porins to gain access to its periplasmic target, or polymyxin B (2.5 μg/ml) or erythromycin (50 μg/ml), which both enter the cell through a lipid-mediated pathway (13). Percent survival was calculated as the ratio of viable counts from cells grown for 1 h in the presence of the antibiotic to those of cells grown in the absence of the antibiotic. We found that only $66\% \pm 8\%$ of wild-type (HS200) cells survived in a medium containing 5 μg of ampicillin per ml, while wild-type cells containing plasmid pCADA were not affected by the presence of the antibiotic at this concentration ($109\% \pm 5\%$ survival). Cells containing plasmid pCADA grow as fast as wild-type cells, and so the resistance of the cadaverine-producing cells to ampicillin is not due to changes in growth rate. The presence of plasmid pCADA resulted in the excretion of 125 ± 12 μM cadaverine (as opposed to 17 ± 12 μM for cells lacking the plasmid). Higher concentrations of ampicillin (>50 μg/ml) were, however, equally effective at killing cells regardless of their ability to produce cadaverine. It is noteworthy that cadaverine-excreting cells displayed no change in sensitivity to 2.5 μg of polymyxin B per ml (survival of $7\% \pm 1\%$ in HS200 and $8\% \pm 1\%$ in HS200/pCADA) or 50 μg of erythromycin per ml (survival of $47\% \pm 10\%$ in HS200 and $48\% \pm 12\%$ in HS200/pCADA), even though similar amounts of cadaverine had been released. In conclusion, the production of cadaverine confers some resistance specifically to antibiotics requiring porins for uptake.

DISCUSSION

The outer membrane of *E. coli* behaves as an effective molecular filter whose permeability depends mostly on porins. We previously documented that externally applied polyamines, including cadaverine, effectively decrease outer membrane permeability by promoting porin closure (7, 20). This observation, in conjunction with the well-known environmental control of the bacterial polyamine pools (30, 36, 40), raises some important issues. Can endogenous polyamines modulate porin activity? Can this modulation be part of a mechanism to respond to environmental changes? What is the physiological significance of porin inhibition by polyamines? The present study is an initial attempt to answer such questions.

We assessed outer membrane permeability from the rate of β-lactam antibiotic permeation, a process that requires the presence of functional porins for maximum efficiency (14, 34). We chose to manipulate the endogenous levels of cadaverine because this polyamine is the end product of a pH-dependent degradative pathway that has been well characterized (36, 40). Because of the pleiotropic effect of acidic pH on cells and outer membrane function in particular (16, 26, 36, 39, 40), we designed strategies that bypassed the drop in pH to isolate effects on porins that are due solely to the production of cadaverine. Our results clearly demonstrate that increased synthesis and excretion of cadaverine in these conditions correlate

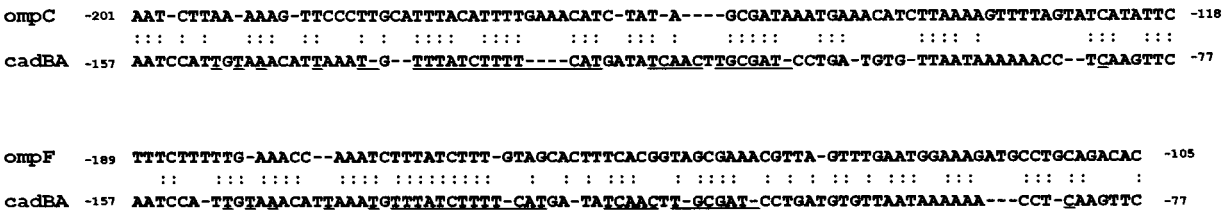

FIG. 6. Nucleotide sequence alignment of promoter regions of *ompC*, *ompF*, and the *cadBA* operon. The sequences were aligned by performing pairwise comparisons with the ALIGN program (University of Wisconsin Genetics Computer Group software), with some minor adjustments made by visual inspection. Dots mark positions of identical nucleotides; underlined nucleotides represent the motif essential for acid induction of the *cadBA* operon (29).

with a decreased porin-mediated outer membrane permeability, independently of the method used to induce the production of endogenous cadaverine. The cadaverine-mediated inhibition of outer membrane permeability resulted solely from a modulation of porin function, not from a cadaverine-dependent modification in porin expression.

Our results suggest that cadaverine triggers a form of sustained inactivation of porins. This phenomenon is seen in electrophysiological experiments (7, 20) as well as in antibiotic flux assays with cadaverine added exogenously or produced endogenously. The retention of the inactivation is prolonged and does not require the continued excretion of the polyamine, since we have measured inhibited flux rates even 60 min after the cadaverine has been washed away. The molecular nature of this puzzling form of inhibition is unclear, and we can only offer some speculative explanations at this point.

Patch-clamp experiments show a lack of effectiveness of cadaverine when applied to the extracellular side of the patch and suggest that the polyamine may exert its effect by binding to a periplasmic site (20). Thus, we propose that in the experiments described here, the transit of cadaverine through the porins and/or the binding of cadaverine to a periplasmic site is responsible for the inhibition. It is difficult to imagine that the irreversibility of the effect stems from cadaverine being trapped in the periplasm because the molecular mass of cadaverine (102 Da) is below the 600-Da cutoff for permeation through porin. In addition, a porin-deficient strain (HS111 [Table 1]) is still capable of excreting substantial amount of cadaverine (up to 450 μM) in low-pH conditions through an OmpF/C-independent pathway. Therefore, the sustained inhibition of porin may be due either to an extremely tight binding of cadaverine to the protein or to an irreversible conformational change that was triggered by the interaction between porin and the polyamine. Alternatively, despite their low molecular weight, cadaverine molecules might still become stuck during their passage through porins as their interactions with the pore lead to conformational changes that trap them. It is noteworthy that some drugs can become trapped inside some types of eukaryotic ion channels (17, 18).

From the results presented here, we propose that multiple pathways exist for the reduction of outer membrane permeability in response to acidic conditions. Some forms of modulation of outer membrane permeability, such as the *envZ*-dependent shutdown of porin expression (16) and the functional closure of open porin pores (26, 39) at acidic pH, have been already documented. Here we propose that two additional mechanisms play a part in the overall response to acidic conditions: porin inhibition by cadaverine, and a *cadC*-dependent reduction in porin expression. Both processes affect only a fraction of the porin population, leaving enough open pores for nutrient import.

The inhibition of OmpC and OmpF expression triggered by the presence of the constitutive $cadC^c$ allele in a *cadA* mutant background prompted us to search for sequence identities in the promoter regions of *ompC*, *ompF*, and the *cadBA* operon. Figure 6 shows an alignment of the −77 to −157 region of the *cadBA* operon with upstream regions of *ompC* and *ompF*. The underlined nucleotides of the *cadBA* promoter region represent the essential residues required for acid induction of the operon (29), which presumably interact with the CadC protein. The sequence alignment yields 58 or 63% identity between the −77 to −157 region of *cadBA* and the −118 to −201 region of *ompC* or the −105 to −189 region of *ompF*, respectively. One of the underlined sequences of *cadBA* (TTTATCTTTT) is almost completely conserved in the upstream sequences of *ompC* and *ompF*. This *cadBA* motif was shown to be the one most essential for regulation of *cadBA* expression (29). Thus, it seems likely that CadC interacts with these *ompC* and *ompF* sequences in a fashion that leads to regulation of porin expression. It is noteworthy that these sequences lie further upstream than important regions for the transcriptional control exerted by OmpR on the *ompF* and *ompC* genes (38). In addition, some level of homology is found between the *cadBA* motif and the Fa and Fd boxes of *ompF* and *ompC*, respectively. These F boxes form part of the binding site for OmpR on the porin gene (38). Thus, in addition to a specific interaction of CadC with the porin promoters, cross-talk with the OmpR-dependent pathway may also occur. Alternatively, stimuli that feed onto OmpR may also influence CadC-dependent promoters. Interestingly, amino acid sequence homologies have been found between the DNA-binding regions of CadC, OmpR, and ToxR, suggesting that CadC may belong to a family of two-component regulators (41).

It is generally supposed that polyamines might help survival at low pH because their excretion would lead to a neutralization of the external milieu (36, 40). This hypothesis is difficult to reconcile with our finding that the pH of the medium is slightly decreased even when large amounts of cadaverine are excreted, probably because the CO_2 released by the pH-induced decarboxylation of amino acids leads to further acidification. Thus, the role for polyamines in the pH response might be other than acid neutralization. Polyamine-mediated response to low pH appears to involve a combination of accumulation of cadaverine and other polyamines in the periplasm, porin inhibition, export through the remaining open porins and other porin-independent pathways, and accumulation in the external milieu. The results presented here support the proposition that cadaverine can act as an endogenous modulator of porin-mediated outer membrane permeability. Porin inhibition by periplasmic cadaverine or by cadaverine in transit is likely to take place before large amounts of the polyamine have diffused outside. The presence of the polyamine molecules in the medium might be an epiphenomenon of their accumulation in the periplasm, without a direct physiological role; al-

ternatively, their association with the external lipopolysaccharides (25) may help stabilize and tighten the outer membrane. This sequence of events may represent one of the strategies in the defense and adaptation mechanisms to acidic stress. The assessment of the contribution of porin inhibition to the overall adaptive response will emerge from future studies with polyamine-resistant porin mutants.

ACKNOWLEDGMENTS

We gratefully acknowledge the gift of strains and plasmids from Eric Olson, Herbert Tabor, George Bennett, and Hiroshi Nikaido. We thank Michael Benedik for useful discussions and reading the manuscript, and we thank Bill Widger for the use of the spectrophotometer.

This work was supported by NIH grant AI34905.

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