

# Permeation rates of penicillins indicate that *Escherichia coli* porins function principally as nonspecific channels

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Small, hydrophilic compounds such as  $\beta$ -lactams diffuse across the outer membrane of Gram-negative bacteria through porin channels, which were originally thought to be nonspecific channels devoid of any specificity. However, since the discovery of an ampicillin-binding site within the OmpF channel in 2002, much attention has been focused on the potential specificity of the channel, where the binding site was assumed either to facilitate or to retard the penetration of  $\beta$ -lactams. Since the earlier studies on porin permeability were done without the knowledge of the contribution of multidrug efflux pumps in the overall flux process across the cell envelope, in this study we have carefully studied both the porin permeability and active efflux of ampicillin and benzylpenicillin. We found that the influx occurs apparently by a spontaneous passive diffusion without any indication of specific binding within the concentration range relevant to the antibiotic action of these drugs, and that the higher permeability for ampicillin is totally as expected from the gross property of this drug as a zwitterionic compound. The active efflux by AcrAB was more effective for benzylpenicillin due to the stronger affinity and high degree of positive cooperativity. Our data now give a complete quantitative picture of the influx, efflux, and periplasmic degradation (catalyzed by AmpC  $\beta$ -lactamase) of these two compounds, and correlate closely with the susceptibility of *Escherichia coli* strains used here, thus validating not only our model but also the parameters obtained in this study.

AcrB | OmpC

The permeability of *Escherichia coli* porins OmpF and OmpC was studied soon after the discovery of their pore-forming functions, by reconstitution of purified proteins and by using  $\beta$ -lactams as probes in intact cells (1–3). The results suggested that the porin channels lack specificity, and that the permeation rates of solutes were determined by their gross physicochemical properties such as size, hydrophobicity, and charges. This conclusion was important at that time because there were indeed claims that porin channels had some specificity (4).

These 30-y-old conclusions, however, currently need reexamination because of two reasons. The first is the discovery of constitutively expressed multidrug efflux pumps that function synergistically with the barrier function of the outer membrane (5). The second was the discovery in 2002 that ampicillin (AMP) at high concentrations can produce transient blockage of OmpF channel in planar bilayer assays, a result suggesting the presence of a binding site for AMP within the channel (6). This result, reported in an article entitled “Designed to penetrate: Time-resolved interaction of single antibiotic molecules with bacterial pores” (6), led many scientists to hypothesize that the porin channels are not entirely nonspecific, and that the binding to this site accelerated the permeation of certain  $\beta$ -lactams (7, 8) or are even necessary for their permeation (8, 9). For example, ref. 8 states, “It has become clear that the transport of  $\beta$ -lactams or fluoroquinolones through OmpF-type porins is not by passive diffusion through an inert tube, but involves specific interactions with porin channels.” On the other hand, some scientists argued that this binding must cause significant retardation in AMP influx, and reported that benzylpenicillin (PEN), which cannot bind to this site, diffuses faster than AMP through OmpF (10).

Unfortunately, the permeability of AMP and PEN was estimated in the era when active efflux of drugs was not known (11). Although permeability determination was possible with AMP using porin-containing liposomes (12), this system could not be used with PEN because it permeates across the lipid bilayers rapidly (13). Our previous efflux study of various acid-stable penicillins, including AMP, determined both the permeability and efflux kinetics; however, PEN could not be included because of its instability in the acidic conditions used for the termination of the reaction (14). Furthermore, an unnatural *E. coli* construct with a much wider mutant porin channel had to be used to facilitate the uptake of some penicillins (14).

In view of this situation, a quantitative study with high precision appeared necessary to clarify the porin-mediated influx of both AMP and PEN in *E. coli*. In this study, we first determined, using intact cells of efflux-deficient mutants, the influx process across the outer membrane of these drugs by coupling it with their subsequent hydrolysis by a  $\beta$ -lactamase in the periplasm (15), by stopping the  $\beta$ -lactamase reaction with a brief heating in a boiling water bath. Our results showed that the very low-affinity binding site within the porin channel does not visibly affect the permeation of AMP, and that porin channels should be treated as nonspecific channels as proposed in 1983, at least for the first approximation. We then used cells expressing the major efflux pump AcrAB, which is mostly responsible for active efflux of  $\beta$ -lactams (16, 17), to determine the efflux kinetics as was previously done for cephalosporins (18) and acid-stable penicillins (14), and evaluated the movement of these drugs across the cell envelope.

## Results

**Setting Up the Assay Conditions.** Previous studies for  $\beta$ -lactam influx and efflux were conducted with strains that were modified extensively to optimize their behavior during the assay, such as the overexpression of plasmid-coded  $\beta$ -lactamase or the mutational alteration of channel size of porin (3, 14, 15, 18). The former may lead to the leakage of significant amounts of the

## Significance

Porin channels in the outer membrane of Gram-negative bacteria were originally thought to be nonspecific channels that discriminate among solutes only by their gross physicochemical properties, such as size, hydrophobicity, and charge. Since the discovery of the ampicillin-binding site inside one porin channel, however, such “specific” interactions are hypothesized to play significant roles especially for the permeation of drugs. We show, by the careful examination of influx and efflux of ampicillin and benzylpenicillin, that the original nonspecific model is still valid and that it is misleading to overemphasize the role of specific binding sites.

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The authors declare no conflict of interest.

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overproduced  $\beta$ -lactamase into the medium, which may affect the accuracy of the assay. The latter directly affects the permeability. It was thus desirable to use a strain with minimal modifications. In this study, we chose *E. coli* LA51 (19), a K-12 derivative with wild-type porins but possessing an “up-mutation” in the promoter region of chromosomal *ampC*  $\beta$ -lactamase gene, producing a 10-fold increase in AmpC activity (11). The increased hydrolysis rates in this strain were sufficient for the assay of permeability and efflux for both AMP and PEN (see below).

We constructed LA51A, the *acrAB* deletion mutant of LA51 by transducing  $\Delta$ *acrAB::kan* marker from AG100A (17), and used this strain for the influx assay, and used LA51 for the efflux assay caused by AcrAB. As is well known, AcrAB is responsible for the major part of efflux of  $\beta$ -lactams in *E. coli* (17, 20). Although AcrD can make a significant contribution to  $\beta$ -lactam efflux when overexpressed, it is expressed at a very low level in uninduced cells (21).

Our assays always involved the determination of the velocity of hydrolysis ( $V_h$ ) of penicillins by the periplasmic AmpC in intact cells. The prerequisite conditions for the assay were (i) no leakage of AmpC enzyme, (ii) the movement of the substrates is in a steady state, so that we can consider that  $V_h = V_{in}$  in LA51A, and  $V_h = V_{in} - V_e$  in LA51, where  $V_{in}$  and  $V_e$  denote the rate of influx and efflux, respectively. If the assay condition satisfies both,  $V_{in}$  or  $V_e$  can be determined from the value of  $V_h$ .

To meet condition (i), 5 mM MgCl<sub>2</sub> was added to growth media and assay mixture, to increase the outer membrane stability and to prevent the undesirable leakage of AmpC. We have examined the possible leakage as well as the time course of hydrolysis (SI Text and Fig. S1), and the results clearly indicated that there was no significant leakage of the enzyme and that we are justified in assuming a steady-state condition during the assay.

**Quantitative Analysis of Influx.** Fig. 1A shows the relationship of  $V_{in}$  and extracellular substrate concentration ( $C_o$ ) of AMP and PEN in LA51A, in which the active efflux was abolished. For both of them,  $V_{in}$  showed linear increase proportional to  $C_o$ , displaying the characteristic of passive diffusion process. This was refined further

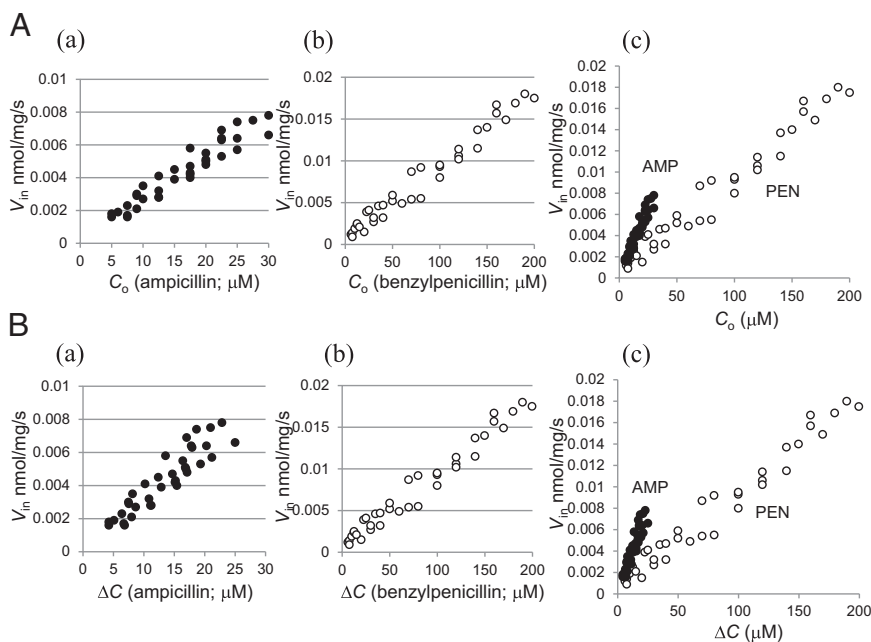
by analyzing the correlation between  $V_{in}$  and the concentration gradient across the outer membrane ( $\Delta C$ ) at each point of  $C_o$  (Fig. 1B). The periplasmic substrate concentration ( $C_p$ ) was calculated by Michaelis–Menten equation of  $C_p = V_h \times K_m / (V_{max} - V_h)$ , using  $V_{max}$  (0.014 and 0.255 nmol·mg<sup>-1</sup>·s<sup>-1</sup> for AMP and PEN, respectively) and  $K_m$  (4.93 and 6.21  $\mu$ M for AMP and PEN) of AmpC determined with sonicated cell extract (Table 1). The results clearly showed a linear correlation between  $V_{in}$  and  $\Delta C$ , with no sign of saturation that might be expected from the specific drug-binding site within the channel. Fick’s first law of diffusion,  $V_{in} = P \times A \times \Delta C$ , where  $P$  is permeability coefficient of the outer membrane, and  $A$  is the surface area of the cell [132 cm<sup>2</sup>/mg [cell dry weight] (22)], is thus obviously applicable to describe the influx process of AMP and PEN.  $P$  values for AMP and PEN were  $0.28 \times 10^{-5}$  cm/s and  $0.07 \times 10^{-5}$  cm/s, respectively. The permeability of AMP is therefore shown to be four times higher than PEN.

#### Evaluation of the Influence of Diffusion Across the Cytoplasmic Membrane.

The monoanionic, hydrophobic  $\beta$ -lactams were shown to diffuse across the lipid bilayer and the cytoplasmic membrane rather rapidly (13). It was thus necessary to evaluate the potential influence that this may have on the result of influx assay of PEN. When the diffusion rate across the cytoplasmic membrane,  $V_{out}$ , is taken into consideration of the influx assay,  $V_h$  should be described as  $V_h = V_{in} - V_{out}$ . In this equation,  $V_{in}$  can be derived from  $P_{OM} \times A_{OM} \times (C_o - C_p)$ , and  $V_{out}$  from  $P_{CM} \times A_{CM} \times (C_p - C_c)$ , where OM and CM stand for outer membrane and cytoplasmic membrane, respectively, and  $C_c$  denotes the substrate concentration in cytoplasm. Substitution and rearrangement give the following:

$$P_{OM} = \frac{V_h}{A_{OM}(C_o - C_p)} + \frac{A_{CM}(C_p - C_c)}{A_{OM}(C_o - C_p)} \cdot P_{CM}. \quad [1]$$

The degree of influence by diffusion across the cytoplasmic membrane is thus expressed in the second term of right side of



**Fig. 1.** Influx kinetics of AMP and PEN in LA51A. (A) Relationship between  $V_{in}$  and  $C_o$  of AMP (a) and PEN (b). These were combined in graph (c). (B) Relationship between  $V_{in}$  and  $\Delta C$  of AMP (a) and PEN (b). These were combined in graph (c). Abbreviations:  $\Delta C$ , concentration gradient across the outer membrane ( $=C_o - C_p$ );  $V_{in}$ , rate of influx.

**Table 1. Kinetic constants of AmpC  $\beta$ -lactamase**

Strain	AMP		PEN	
	$K_{m_r}$ , $\mu\text{M}$	$V_{max_r}$ , $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{s}^{-1}$	$K_{m_r}$ , $\mu\text{M}$	$V_{max_r}$ , $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{s}^{-1}$
LA51	4.48	0.019	5.28	0.330
LA51A	4.93	0.014	6.21	0.255

Eq. 1. Within this term,  $A_{OM}/A_{CM}$  should be close to 1 because there should not be any significant difference between  $A_{OM}$  and  $A_{CM}$ , and in our influx assay of PEN, the ratio of  $C_p/C_o$  was always less than 1/200.  $P_{CM}$  can be estimated from the half-equilibration time of PEN across the cytoplasmic membrane, which was previously determined to be around 3 min (13). In *E. coli* cell, the half-equilibration time of 3 min corresponds to  $\sim 1 \times 10^{-7}$  cm/s of permeability coefficient (23). Hence, this term is estimated to be as follows:

$$\frac{A_{CM}(C_p - C_c)}{A_{OM}(C_o - C_p)} \cdot P_{CM} \leq \frac{1}{200} \cdot P_{CM} \approx 0.5 \times 10^{-9} \text{ cm/s.}$$

This corresponds to less than 0.1% of first term in the right side of Eq. 1. Thus, the influence of the diffusion across the cytoplasmic membrane seems insignificant and will not be considered in our study.

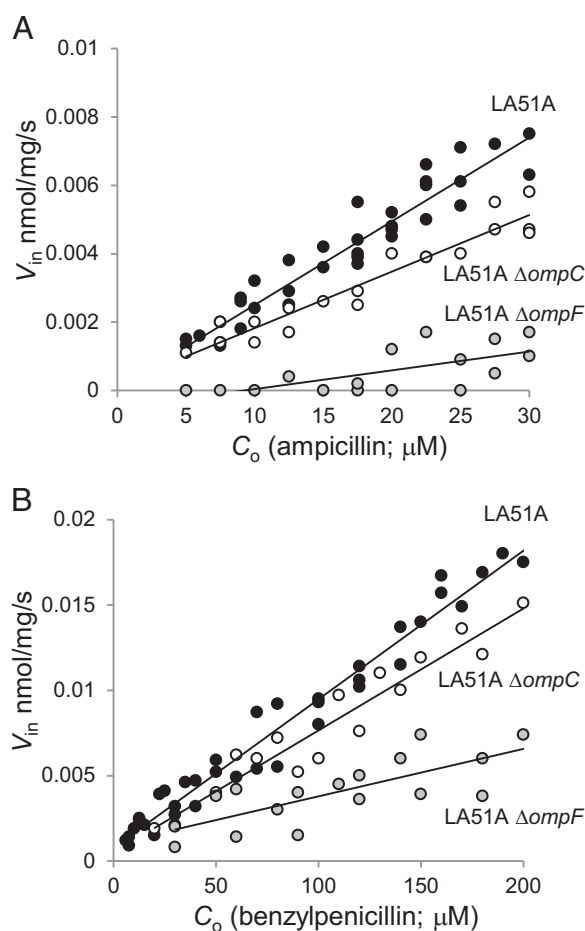
**Determination of Influx Route.** For the understanding of the influx process, determining the influx route is essential. The  $\beta$ -lactams have been shown to diffuse mainly through the OmpF channel (2, 12, 24). We confirmed this by analyzing  $V_{in}$  in the LA51A with *ompF* or *ompC* deletion (Fig. 2). Deletion of *ompF* gene caused drastic decrease in  $V_{in}$  for both of AMP and PEN. Deletion of *ompC*, however, caused only slight decreases of  $V_{in}$ . The majority of AMP and PEN were thus shown to diffuse through OmpF channel, consistent with previous research.

**Quantitative Analysis of Efflux.** With  $P$  values obtained as above, we were now able to analyze the efflux activity using LA51, which expresses *acrAB* at its wild-type, constitutive level, and the equation of  $V_e = V_{in} - V_h$ , where  $V_{in}$  can be calculated from Fick's first law of diffusion,  $V_{in} = P \times A \times \Delta C$ , using the value of  $P$  obtained in the assay with LA51A (14, 18). Fig. 3 shows the efflux kinetics of AcrAB for AMP and PEN. There was significant cooperativity, and the best fit was obtained by assuming a Hill coefficient of 1.9 and 4.0 for AMP and PEN, respectively. The  $K_{0.5}$  value, where the efflux attains a half-maximal velocity, was 2.16  $\mu\text{M}$  for AMP but was exceptionally low for PEN, 0.30  $\mu\text{M}$ .  $V_{max}$  was 0.069 and 0.085  $\text{nmol}\cdot\text{mg cells}^{-1}\cdot\text{s}^{-1}$  for AMP and PEN. The efflux kinetics for AMP showed good agreement with the previous result (14), except  $V_{max}$ , which was about sixfold lower. This difference, however, seems reasonable because the previous study was conducted in a strain with deletion of *acrR* and the cells from stationary phase were used, conditions that enhance the expression of *acrAB* by about sixfold (25).

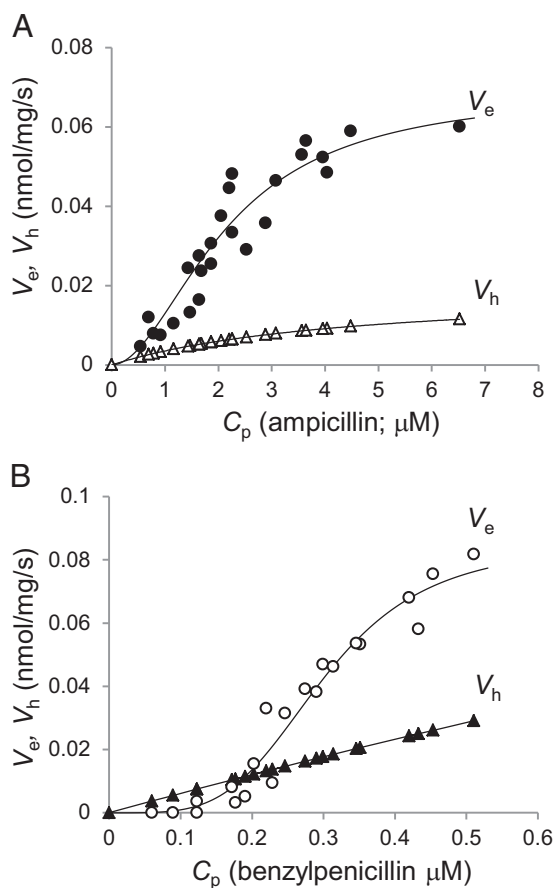
**Periplasmic Drug Concentration Required for Growth Inhibition.** The data obtained in the experiments of Figs. 1 and 3 were replotted to show  $C_p$  values at various values of  $C_o$  (Fig. 4). In this manner, we can assess the periplasmic drug concentration needed for growth inhibition. When the external concentration equals minimum inhibitory concentration (MIC), we assume that the drug reaches a critical concentration ( $C_{inh}$ ) needed to inhibit growth (11). Determination of MIC of AMP and PEN was done first by the microdilution method, and produced values of 32 and 4  $\mu\text{g/mL}$  AMP and 512 and 64  $\mu\text{g/mL}$  PEN for LA51 and LA51A,

respectively. The gradient plate method also produced similar values of MIC, i.e., 35 and 5  $\mu\text{g/mL}$  AMP and 300 and 60  $\mu\text{g/mL}$  PEN (Fig. S2). Because the twofold microdilution method cannot give precise values, we assumed that the MICs determined by gradient plate method represent true MICs. The values of  $C_{inh}$  can be estimated from Fig. 4. It is important that two strains, LA51 and LA51A, produced similar values of  $C_{inh}$ , serving as a further confirmation of both our approach and our data. The estimated values of  $C_{inh}$  were 1.7  $\mu\text{M}$  for AMP and 0.37  $\mu\text{M}$  for PEN. The  $C_{inh}$  values determined here are similar to those estimated on the basis of affinities for penicillin-binding proteins (11) in the case of AMP, but much lower than that for PEN.

**The Parameters Obtained Are Consistent with the MIC Values.** One possible way to assess the validity of parameters obtained in this study is to calculate the movement of AMP and PEN when  $C_o = \text{MIC}$ . Here,  $C_p$  reaches  $C_{inh}$ , and  $V_e$  and  $V_h$  in LA51 can be derived using the parameters obtained earlier (Tables 1 and 2). Calculated values are shown in Table 3. The influx of the drug at this condition, however, should provide the sufficient rate to overcome the sum of the activities of efflux and hydrolysis.  $V_{in}$  at  $C_o = \text{MIC}$  is described as  $V_{in} = P \times A \times (C_o - C_{inh})$ . The calculated  $V_{in}$  of AMP and PEN in LA51 are 0.034 and 0.075  $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{s}^{-1}$ , respectively. For both of AMP and PEN,  $V_{in}$  is close to the sum of  $V_e$  and  $V_h$ , confirming the validity of our approach as well as the parameters obtained.



**Fig. 2.** Influx route of AMP and PEN.  $V_{in}$  of AMP (A) and PEN (B) was measured in LA51A, LA51A  $\Delta ompF$ , and LA51A  $\Delta ompC$ . Abbreviations:  $C_o$ , extracellular substrate concentration;  $V_{in}$ , rate of influx.



**Fig. 3.** Efflux kinetics of AcrB for AMP (A) and PEN (B). Abbreviations:  $C_p$ , periplasmic substrate concentration;  $V_e$ , rate of efflux;  $V_h$ , rate of hydrolysis.

## Discussion

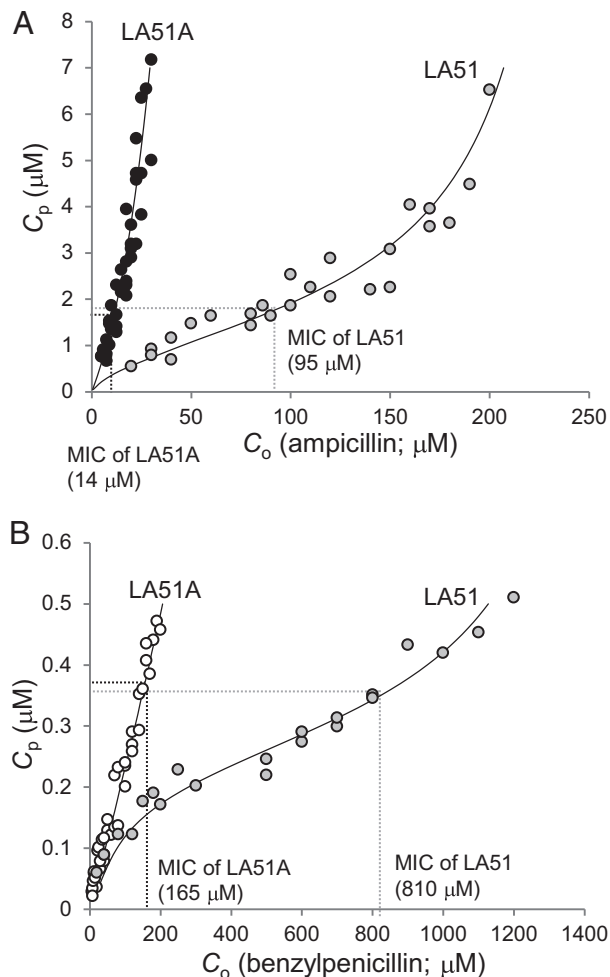
**Porin Channels Are Essentially Nonspecific.** A major purpose of this study was to examine the outer membrane permeation of two penicillins, AMP and PEN, in view of the presence of an AMP-binding site within the OmpF channel. We performed the study with an *acrAB* deletion mutant so that the results would not be affected by the active efflux process.

We found that both AMP and PEN diffused across the outer membrane apparently by a spontaneous passive diffusion process, without any hint of saturation kinetics (Fig. 1). This is perhaps not surprising, because the affinity of AMP to the binding site is extremely weak, with the dissociation constant reported as 1.9 M (by planar bilayer assay) (6) and 1 M (by molecular dynamics simulation) (10). Thus, in the range of concentrations we used (less than 30  $\mu\text{M}$  for AMP), the drug was at least four orders of magnitude below its  $K_D$ .

We can examine first if our permeation data fit with the model of the porin channels as nonspecific channels. We found that AMP permeated about 2.2 times faster than PEN through the OmpF channel (Fig. 2). This is precisely what is expected from the effect the gross physicochemical properties of the solute exert on its penetration rate through this nonspecific channel: monoanionic compounds diffuse at one-half of the rates of uncharged analogs (for example compare lactobionic acid with lactose in ref. 2), and zwitterionic compounds appeared to diffuse somewhat faster than uncharged compounds of a similar molecular weight [for example, L-Ser-L-Ala,  $M_r$  of 176, diffused 50% faster than D-glucose,  $M_r$  of 180 (2)]. [It is theoretically possible that the interior-negative Donnan potential could retard the permeation of anionic PEN. However, under our assay

conditions (50 mM KPi buffer, 5 mM  $\text{MgSO}_4$ ), the Donnan potential would be quenched to about 10 mV (26) and is unlikely to affect the permeation rates strongly.] These results thus fit the notion that diffusion through porins may be treated, at the level of first approximation, as a process through a large, open channel, which is affected by the gross physicochemical properties of the solute, i.e., size, charge, and lipophilicity (2, 3).

More recently, however, models have been published that assumes a major role for the “specific” binding of AMP and other drugs within the porin for drug penetration, and we can examine whether our result fits with such models. There are two classes for these models. Some workers assume that permeation of a drug (such as AMP) is retarded by the specific binding (10). Such a model predicts that drugs that do not bind to the binding site (such as PEN) will permeate more rapidly than those that bind (such as AMP). Our data (Figs. 1 and 2) show that PEN permeates more slowly than AMP and clearly contradict the model. Although PEN has been claimed to penetrate faster than AMP (10) in liposome-swelling assays, this was apparently caused by the improper application of the technique, without the knowledge that PEN diffuses through the phospholipid bilayers quite rapidly (2, 13). Another class of the specific binding models



**Fig. 4.** Quantitative monitoring of periplasmic concentration ( $C_p$ ) of AMP (A) and PEN (B), in LA51 and LA51A. Combined with MIC values, which are shown in graphs,  $C_p$  that is lethal for the cells can be obtained. Theoretical relationship between  $C_o$  and  $C_p$ , which satisfies the equation of  $V_{in} = V_h - V_e$ , is shown as curves in each graph. Abbreviation:  $C_o$ , extracellular substrate concentration.

**Table 2. Parameters for the drug flux in the *E. coli* cell envelope**

Drug	Influx P, cm/s	Efflux		
		$V_{max}$ , nmol·mg <sup>-1</sup> ·s <sup>-1</sup>	$K_{0.5}$ , μM	h
AMP	$0.28 \times 10^{-5}$	0.069	2.16	1.9
PEN	$0.07 \times 10^{-5}$	0.085	0.30	4.0

assumes that the binding site facilitates drug diffusion (7, 8). In extreme cases, it has been claimed that drug permeability can be calculated simply from its on-constant to the binding site (8). Our results clearly do not fit with such a model, because PEN, which has never been shown to bind to the AMP-binding site, nevertheless permeates at a significant rate that corresponds to 25–45% of the diffusion rate of AMP (Figs. 1 and 2). This model also does not fit with the observation that many dozens of solutes, such as simple sugars, that are most unlikely to bind to the AMP-binding site, nevertheless diffuse through the porin channels quite rapidly (1). The proponents of this model made a mistake when they applied the model for LamB (27), the phage lambda receptor, which has a narrow channel and requires the tight binding of ligands to slide them through it, to general-purpose, wide-open porins that allow a rapid, nonspecific diffusion of any small solutes. In conclusion, the specific binding site may produce subtle influence on the diffusion rates of drugs, but this effect is minor, and the rates are predominantly determined by the gross physicochemical properties of the drug. Overemphasizing the role of the specific binding site [for example, in an article entitled “Toward screening for antibiotics with enhanced permeation properties through bacterial porins” (10)] is misleading and does not contribute to the progress in the field.

The binding of drugs within the channel, however, can produce subtle effects on the permeation rates. A recent study by Ziervogel and Roux (28) is instructive. They found by cocrySTALLIZATION that AMP binds to the OmpF channel at a site just proximal to its narrowest point.

Site-directed mutagenesis of several residues inside OmpF channel was shown to produce alterations of MIC of various antibiotics (29–31). These data, although interesting, cannot be interpreted immediately as the consequence of the changes in the binding process, because no structural data are available for the interaction of the drugs with the mutant channel. Additionally, Ziervogel and Roux (28) also showed that conversion of several crucial residues (D121, S125, R167, R168) to others decreased AMP MIC from 2 to 1 μg/mL, presumably by a slight increase in its permeation rate. However, they pointed out that some of the mutations could cause significant changes in the size of the channel. Indeed, mutations of often targeted residues, such as D113 or R132, were reported to produce increased permeability for sugars, which are not known to bind to the AMP-binding site (32, 33).

**Active Efflux of AMP and PEN by AcrAB-TolC System.** In the cells with functional AcrAB pump, the drug molecules that penetrated into the periplasm are either pumped out by the AcrB pump or hydrolyzed by the periplasmic AmpC β-lactamase. Determination of efflux kinetics for AMP and PEN showed that efflux plays a predominant role, in comparison with hydrolysis, for the removal

**Table 3. Calculated values (in nmol·mg<sup>-1</sup>·s<sup>-1</sup>) of  $V_{in}$ ,  $V_e$ , and  $V_h$  in LA51 at  $C_0 = \text{MIC}$** 

Rate	AMP	PEN
$V_{in}$	0.034	0.075
$V_e$	0.028	0.057
$V_h$	0.005	0.022

of drugs from the periplasm (Fig. 3). The efflux of PEN was especially effective due to the low  $K_{0.5}$  (0.3 μM) and a high degree of cooperativity (Hill coefficient of 4.0). This value of  $K_{0.5}$  is exceptionally low among β-lactams that have so far been studied. Thus, the  $K_{0.5}$  value ranged from about 5 (nitrocefin) to 288 μM (cephaloridine) among cephalosporins (18), and averaged at 0.98 μM among acid-stable penicillins (14). The Hill coefficient of 4.0 is similar to the average value, 3.8, for the acid-stable penicillins previously studied (except AMP) (14), and are higher than those found for cephalosporins, which ranged from 1.0 to 3.2 (18).

In Fig. 3, we can see quantitatively the relative importance of efflux over enzymatic hydrolysis, when the drugs are present at MIC concentrations (producing  $C_p$  of 1.7 and 0.36 μM for AMP and PEN, respectively, from Fig. 4). We note that this situation is found even in LA51, where the level of AmpC β-lactamase has been increased about 10-fold over the wild-type level. Thus, we can safely assume that the poorly expressed wild-type levels of AmpC contribute very little to the AMP and PEN susceptibility in the strains with the wild-type level expression of AcrAB.

Finally, we can compare the present results with the analysis, performed in 1987, without the knowledge of active efflux (11). First, in the earlier study, the kinetic parameters of AmpC β-lactamase were not correct. The  $K_m$  values obtained in this study (4.9 and 4.5 μM for AMP and PEN, respectively) are close to those reported by Galleni and Frère (3.5 and 4.4 μM) (34) and appear to be more reliable than those used in 1987, 0.9 and 1.9 μM. Presumably because efflux was not considered, the permeability coefficients published in 1987 ( $9.8 \times 10^{-5}$  and  $0.2 \times 10^{-5}$  cm/s for AMP and PEN) are very far from our values of  $0.28 \times 10^{-5}$  and  $0.07 \times 10^{-5}$  cm/s. The values of  $C_{inh}$ , which in this study were estimated from the influx and efflux parameters, and validated by the concurrence of values from two strains, were 1.7 and 0.36 μM for AMP and PEN, whereas in the 1987 study they had to be estimated from the penicillin-binding protein binding data and were assumed to be 1.9 and 2.2 μM, and the large difference between these two compounds was not detected. With all these problems, it is not surprising that, for AMP and PEN, the MIC values predicted from the influx rate and the hydrolysis rate did not fit with the observed values in our 1987 study. However, the fit was somewhat better for cephalosporins, where efflux may not play such a major role especially in concentrations at or below MIC values (18).

## Materials and Methods

**Bacterial Strains.** LA51 is an *E. coli* K-12 derivative, in which the expression level of chromosomal AmpC is substantially increased due to the mutated promoter of *ampC* (19). LA51A, a  $\Delta\text{acrAB}$  derivative of LA51, was constructed by transducing the *acrAB::Km<sup>r</sup>* gene from strain AG100A (17), selecting transductants with 35 μg/mL kanamycin. LA51A  $\Delta\text{ompC}$  was constructed by introducing the *ompC* null genotype by cotransduction with *zei-298::Tn10* that is located nearby, from strain CS1253 (35). Tn10 was then removed as described by Bochner et al. (36). To construct LA51A  $\Delta\text{ompF}$ , we first transduced  $\Delta\text{acrAB::Km<sup>r</sup>$  gene from RAM1337 (37), and kanamycin resistance gene was removed by using FLP recombinase from pCP20 (38). Then the *ompF* gene was disrupted by transducing *ompF::Tn5* from strain MH450 (39). Transduction was done using P1cml, *clr100* phage according to the standard protocols (40).

**Influx Assay.** Cells were precultured overnight in LB broth containing 5 mM MgCl<sub>2</sub> at 37 °C with shaking. This culture was diluted 50-fold in a fresh medium, and cultivated further at 37 °C with shaking until it reached an optical density at 600 nm (OD<sub>600</sub>) of 1.0–1.2. Cells were harvested by centrifugation at 3,000 × g for 10 min at room temperature (RT) and washed once with KP buffer [50 mM potassium phosphate buffer (pH 7.0) containing 5 mM MgCl<sub>2</sub>]. Cells were resuspended in KP buffer, and OD<sub>600</sub> of the cell suspension was adjusted to 4.0 (corresponding to 1.5 mg [dry weight]/mL). For the assay, 0.4 mL of the cell suspension was mixed with 0.6 mL of KP buffer containing desired concentration of AMP or PEN, and incubated at RT. The reaction was done in a glass test tube (18 mm × 150 mm). After the incubation period of 0, 5, 10, and 15 min, the reaction was stopped by

heating the test tube in a boiling water bath for 50 s with constant shaking. The test tube was then cooled by plunging it into a water bath at RT.

The hydrolysis product was quantified by microiodometry (41) as follows. After the reaction as above, 1 mL of KP buffer and 1 mL of starch-iodine solution were added to the reaction mixture, and OD<sub>592</sub> was determined after 30 min at RT. Complete hydrolysis of 1 μM substrate decreases OD<sub>592</sub> by 0.128 in a cuvette with a 10-mm light path (41). Using this value, the amount of hydrolysis product ([P]) was calculated. The rate of hydrolysis was obtained by linear fitting of [P] vs. reaction period (s) data.

To obtain the kinetic parameter of the AmpC β-lactamase, the assay was done using sonicated cell extracts (30-s rest and 30-s pulse, eight times). The activity was measured as described above, but with the following modifications: (i) reaction was done in 2 mL of total volume, (ii) the volume of cell extract used in the assay was adjusted according to the level of hydrolytic activity that differs significantly depending on the substrates, and (iii) incubation period was fixed to 5 min.  $V_{max}$  and  $K_m$  values were derived by curve fitting by CurveExpert (version 1.4), using Michaelis–Menten equation.

**Efflux Assay.** The hydrolysis rates by the intact cell of LA51, which expresses *acrAB*, were determined under various extracellular substrate concentrations,  $C_o$ , according to the same protocol for influx assay as described above. The rate of efflux,  $V_e$ , was obtained using the equation of  $V_e = V_{in} - V_h$ , where  $V_{in}$  denotes the rate of influx.  $V_{in}$  was calculated by Fick's first law of diffusion,  $V_{in} = P \times A \times (C_o - C_p)$ , where  $P$ ,  $A$ , and  $C_p$  denote permeability coefficient, surface area of the cell [=132 cm<sup>2</sup>/mg [dry cell weight] (22)], and

periplasmic substrate concentration.  $C_p$  was derived from Michaelis–Menten equation using the observed value of  $V_h$  and the kinetic parameters of AmpC, and  $P$  was determined in the influx assay using LA51A. Because the efflux activity showed sigmoidal curves when plotted against  $C_p$ , we used the Michaelis–Menten equation modified for cooperativity,  $V_e = V_{max} \cdot (C_p)^h / [(C_p)^h + (K_{0.5})^h]$ , where  $h$  is the Hill coefficient, and  $K_{0.5}$  is the substrate concentration that gives the half-maximum velocity. These constants were derived by curve fitting using CurveExpert (version 1.4).

**MIC Determination.** Linear gradient plates containing AMP or PEN in the bottom layer were made in square petri dishes, using LB agar supplemented with 5 mM MgCl<sub>2</sub> (42). Cells were cultured in LB broth containing 5 mM MgCl<sub>2</sub> at 37 °C with shaking, and at a late exponential phase (OD<sub>600</sub> = 1.0) the culture was diluted to OD<sub>600</sub> of 0.1 and used as inoculum. One microliter of inoculum was streaked as a line across the plate, in parallel to the gradient. The plates were incubated for 18 h at 37 °C.

Microdilution method was done using a series of 200 μL of LB broth supplemented with 5 mM MgCl<sub>2</sub> and containing twofold serial deletion of AMP or PEN, which were prepared in the 96-well microtiter plates. Approximately 10<sup>4</sup> cells from the inoculum as described above were inoculated. The plates were incubated for 18 h for 37 °C.

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- Nikaido H, Rosenberg EY (1981) Effect of solute size of diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. *J Gen Physiol* 77(2): 121–135.
- Nikaido H, Rosenberg EY (1983) Porin channels in *Escherichia coli*: Studies with liposomes reconstituted from purified proteins. *J Bacteriol* 153(1):241–252.
- Nikaido H, Rosenberg EY, Foulds J (1983) Porin channels in *Escherichia coli*: Studies with beta-lactams in intact cells. *J Bacteriol* 153(1):232–240.
- Nikaido H, Luckey M, Rosenberg EY (1980) Nonspecific and specific diffusion channels in the outer membrane of *Escherichia coli*. *J Supramol Struct* 13(3):305–313.
- Nikaido H (1996) Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 178(20):5853–5859.
- Nestorovich EM, Danelon C, Winterhalter M, Bezrukov SM (2002) Designed to penetrate: Time-resolved interaction of single antibiotic molecules with bacterial pores. *Proc Natl Acad Sci USA* 99(15):9789–9794.
- Danelon C, Nestorovich EM, Winterhalter M, Ceccarelli M, Bezrukov SM (2006) Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. *Biophys J* 90(5):1617–1627.
- James CE, et al. (2009) How beta-lactam antibiotics enter bacteria: A dialogue with the porins. *PLoS One* 4(5):e5453.
- Page JM, James CE, Winterhalter M (2008) The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. *Nat Rev Microbiol* 6(12): 893–903.
- Hajjar E, et al. (2010) Toward screening for antibiotics with enhanced permeation properties through bacterial porins. *Biochemistry* 49(32):6928–6935.
- Nikaido H, Normark S (1987) Sensitivity of *Escherichia coli* to various beta-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic beta-lactamases: A quantitative predictive treatment. *Mol Microbiol* 1(1):29–36.
- Yoshimura F, Nikaido H (1985) Diffusion of β-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrob Agents Chemother* 27(1):84–92.
- Li XZ, Ma D, Livermore DM, Nikaido H (1994) Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: Active efflux as a contributing factor to β-lactam resistance. *Antimicrob Agents Chemother* 38(8):1742–1752.
- Lim SP, Nikaido H (2010) Kinetic parameters of efflux of penicillins by the multidrug efflux transporter AcrAB-TolC of *Escherichia coli*. *Antimicrob Agents Chemother* 54(5):1800–1806.
- Zimmermann W, Rosset A (1977) Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. *Antimicrob Agents Chemother* 12 (3):368–372.
- Ma D, et al. (1993) Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J Bacteriol* 175(19):6299–6313.
- Okusu H, Ma D, Nikaido H (1996) AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 178(1):306–308.
- Nagano K, Nikaido H (2009) Kinetic behavior of the major multidrug efflux pump AcrB of *Escherichia coli*. *Proc Natl Acad Sci USA* 106(14):5854–5858.
- Jaurin B, Grundström T, Edlund T, Normark S (1981) The *E. coli* β-lactamase attenuator mediates growth rate-dependent regulation. *Nature* 290(5803):221–225.
- Sulavik MC, et al. (2001) Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob Agents Chemother* 45(4):1126–1136.
- Hirakawa H, Nishino K, Yamada J, Hirata T, Yamaguchi A (2003) Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. *J Antimicrob Chemother* 52(4):576–582.
- Smit J, Kamio Y, Nikaido H (1975) Outer membrane of *Salmonella typhimurium*: Chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. *J Bacteriol* 124(2):942–958.
- Nikaido H (1989) Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob Agents Chemother* 33(11):1831–1836.
- Harder KJ, Nikaido H, Matsushashi M (1981) Mutants of *Escherichia coli* that are resistant to certain β-lactam compounds lack the ompF porin. *Antimicrob Agents Chemother* 20(4):549–552.
- Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE (1996) The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Mol Microbiol* 19(1):101–112.
- Sen K, Hellman J, Nikaido H (1988) Porin channels in intact cells of *Escherichia coli* are not affected by Donnan potentials across the outer membrane. *J Biol Chem* 263(3): 1182–1187.
- Benz R, Schmid A, Vos-Scheperkeuter GH (1987) Mechanism of sugar transport through the sugar-specific LamB channel of *Escherichia coli* outer membrane. *J Membr Biol* 100(1):21–29.
- Zierovogel BK, Roux B (2013) The binding of antibiotics in OmpF porin. *Structure* 21(1): 76–87.
- Simonet V, Malléa M, Page JM (2000) Substitutions in the eyelet region disrupt cefepime diffusion through the *Escherichia coli* OmpF channel. *Antimicrob Agents Chemother* 44(2):311–315.
- Bredin J, et al. (2002) Alteration of pore properties of *Escherichia coli* OmpF induced by mutation of key residues in anti-loop 3 region. *Biochem J* 363(Pt 3):521–528.
- Vidal S, Bredin J, Page JM, Barbe J (2005) Beta-lactam screening by specific residues of the OmpF eyelet. *J Med Chem* 48(5):1395–1400.
- Saint N, et al. (1996) Structural and functional characterization of OmpF porin mutants selected for larger pore size. II. Functional characterization. *J Biol Chem* 271(34):20676–20680.
- Phale PS, et al. (2001) Role of charged residues at the OmpF porin channel constriction probed by mutagenesis and simulation. *Biochemistry* 40(21):6319–6325.
- Galleni M, Frère JM (1988) A survey of the kinetic parameters of class C beta-lactamases. Penicillins. *Biochem J* 255(1):119–122.
- Schnaitman CA, McDonald GA (1984) Regulation of outer membrane protein synthesis in *Escherichia coli* K-12: Deletion of *ompC* affects expression of the OmpF protein. *J Bacteriol* 159(2):555–563.
- Bochner BR, Huang HC, Schieven GL, Ames BN (1980) Positive selection for loss of tetracycline resistance. *J Bacteriol* 143(2):926–933.
- Kim HS, Nagore D, Nikaido H (2010) Multidrug efflux pump MdtBC of *Escherichia coli* is active only as a B<sub>2</sub>C heterotrimer. *J Bacteriol* 192(5):1377–1386.
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
- Hall MN, Silhavy TJ (1981) The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K12. *J Mol Biol* 146(1):23–43.
- Millar JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 201–205.
- Novick RP (1962) Micro-iodometric assay for penicillinase. *Biochem J* 83:236–240.
- Takatsuka Y, Nikaido H (2006) Threonine-978 in the transmembrane segment of the multidrug efflux pump AcrB of *Escherichia coli* is crucial for drug transport as a probable component of the proton relay network. *J Bacteriol* 188(20):7284–7289.