OUTER MEMBRANE OF SALMONELLA XIV. REDUCED TRANSMEMBRANE DIFFUSION RATES IN PORIN-DEFICIENT MUTANTS

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SUMMARY: Rates of diffusion of a β-lactam antibiotic, cephaloridine, across the outer membrane of Salmonella typhimurium cells were measured by determining the rates of its hydrolysis by β-lactamases located in the periplasmic space. It was shown that the permeability coefficient of the outer membrane toward cephaloridine decreased to about one-tenth of that in the wild type, in mutant strains deficient in two "porin" proteins, previously shown to produce transmembrane pores in in vitro reconstitution experiments. In contrast, the loss of the 33,000 dalton outer membrane protein did not have any noticeable effect on the permeability coefficient.

The outer membrane, the outermost layer of the cell envelope of Gram-negative bacteria such as Salmonella and Escherichia coli, allows a rapid transmembrane diffusion of hydrophilic molecules with molecular weights less than 600 (1,2). Outer membrane of S. typhimurium contains four "major" proteins, called 36K, 35K, 34K, and 33K on the basis of their apparent molecular weights (3). Nakae in our laboratory has shown that the 36K, 35K, and 34K proteins can produce transmembrane channels or "pores" when reconstituted with phospholipids and lipopolysaccharides, and proposed the name "porins" for these proteins (4,5).

It was obviously desirable to obtain mutants deficient in porins, and to ascertain that the rates of diffusion of small molecules across the outer membrane were indeed reduced in these mutants. Such porin-deficient mutants have been isolated (6). Furthermore, measurement of diffusion rates across the outer membrane has recently become possible through the work of Zimmermann and Rosselet (7), who showed that the rates of hydrolysis of β-lactam antibiotics

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by intact cells of an E. coli strain, containing β-lactamase in the periplasmic space, could be explained by assuming the diffusion across the outer membrane (determined by Fick's law) was balanced by the hydrolysis of β-lactam molecules in the periplasmic space (determined by the Michaelis-Menten relationship). We used this method by introducing R plasmids R1 and R471a, which code for periplasmic β-lactamases (8), into porin-deficient mutants of S. typhimurium, and then by determining the rates of hydrolysis of a β-lactam antiobiotic, cephaloridine, by intact cells. The results showed that the outer membrane of porin-deficient mutants have drastically reduced permeability toward cephaloridine.

MATERIALS AND METHODS

Bacterial Strains and Their Cultivation. S. typhimurium mutants producing reduced amounts of 34K, 36K, or both of these proteins have been described (6); they are derivatives of SH5014, which carries multiple auxotrophic markers (ilv thr trp his met). A mutant (SL1917) resistant to bacteriocin 4-59, recently isolated by Dr. B.A.D. Stocker, was found to lack the 33K protein; this mutant was derived from the parent of SH5014, SL1027. R plasmids R1 and R471a (9) were introduced into these S. typhimurium strains by conjugational transfer from E. coli K12 strains containing the plasmids. The donor strains were the kind gift of Dr. M. Yoshikawa. The transconjugants were selected in supplemented mininal media containing citrate as the sole carbon source and ampicillin (100 μg/ml).

For β-lactamase assay, the bacteria were grown with shaking at 37 C in LM broth (1% tryptone, 1% yeast extract, 0.5% NaCl, and 5 mM $MgSO_4$) until the culture density reached 0.5 mg (dry weight) /ml. Then the cells were centrifuged at 3,000 x g for 5 min, washed once in 10 mM sodium phosphate-5 mM $MgCl_2$ (pH 7.0), and were resuspended in the same buffer. After the harvest, the cells were always kept and handled at room temperature. Portions of the suspensions were sonicated in ice-water bath with a microtip of a Bronwill Biosonik IV sonicator.

Assay of β-lactamase. A two-step modification (7) of Novick's microiodometric procedure (10) was used. The reaction mixture contained in 1.00 ml, 0.01 M sodium phosphate buffer, pH 7.0, 0.8 mM cephaloridine (a kind gift of Dr. L.F. Ellis, Eli Lilly Co.), and the "enzyme" (cell suspension, sonicate, or supernatant obtained after the centrifugation of cell suspension). After 10 min at 25 C, the reaction was stopped by adding 1.00 ml of a solution containing 1 M acetic acid, 0.2 M sodium tungstate, 0.2% soluble starch, 0.12 mM I_2 and 4.8 mM KI. The reduction of I_2 by the hydrolyzed β-lactams was determined by reading the optical density of the mixture at 623 nm, after the mixture had been kept further for exactly 20 min at 25 C.

RESULTS

Conditions of Assay. Incubation of crude sonicates with various concentrations of cephaloridine showed that the enzymes coded for by R1 as well as

Table 1. Transmembrane Diffusion of Cephaloridine as Measured by the "Intact Cell" Activity of β-Lactamase

Strain	"Major proteins" of the outer membrane[a]				β-Lactamase			Permeability Coefficient(P)[c] (cm/sec)
	Porins				Activity[b] (nmoles/min/mg cells)		Ratio (Cells/ Sonicate)	
	36K	35K	34K	33K	Intact Cells	Sonicate		
							%	x 10^{-6}
SH5014(R1)	+++	+	+++	+++	44	108	41	9.3
SH5551(R1)	+++	+	±	+++	26	97	27	5.0
SH6017(R1)	±	+	+++	+++	18	105	17	3.1
SH6260(R1)	±	+	±	+++	3.4	121	2.8	0.57
SH6261(R1)	±	+	±	+++	8.0	91	8.8	1.34
SH6263(R1)	±	+	±	+++	4.2	99	4.2	0.69
SH6264(R1)	±	+	±	+++	4.2	92	4.6	0.69
SL1917(R1)	+++	+	+++	-	35	64	55	9.0
SH5014(R471a)	+++	+	+++	+++	95	1850	5.1	15.4
SH6260(R471a)	±	+	±	+++	<10	1840	<0.5	<1.3

[a]Protein patterns of strains without the R plasmids were determined previously (6). Most of the R-plasmid-containing strains were reexamined and were found to have major protein patterns indistinguishable from the non-plasmid-containing parents.

[b]Measured as described in Methods, with 0.8 mM cephaloridine.

[c]Calculated as described in text.

R471a had a K_M of 0.8 mM for this substrate. With the strains of SH5014 series, the "leakage" of the periplasmic β-lactamase into the suspending medium could be minimized by the addition of Mg^{++} to the growth medium and the buffer used for washing and resuspension, and by avoiding the exposure of the cells to low temperature. The "osmotic shock" (11) released more than 85% of β-lactamase into the medium both in SH5014 (R1) and in SH6260 (R1).

β-Lactamase Activity in Intact Cells. Table I shows the results of β-lactamase assay. At the time the cell suspension was used for assay, another portion of the suspension was centrifuged and the supernatant was later assayed in order to determine the degree of "leakage" of the β-lactamase into the medium. With the precautions mentioned above, only 1-2% of the total activity was found in the medium. The "intact cell" activities in Table I have been corrected for the activities due to the leaked-out enzymes; with R1-containing wild type and mutant strains this correction amounted to less than 4%, and less than 30%, of the "intact cell" activities, respectively. A similar fraction (about 1%) of the total β-lactamase leaked out in R471a-containing cells. In this case, however, the activity of the leaked out enzyme contributed heavily toward the total "intact cell" activity, and the accurate determination of intact cell activity in the mutant (SH6260) was therefore impossible.

Permeability Coefficients. Zimmermann and Rosselet (7) calculated the β-lactam concentration in the periplasm (C_i) from the V_{max} and K_M (obtained from assays with sonicates) as well as the "intact cell" rate (v), by utilizing the classical Michaelis-Menten relationship, $v = V_{max} \cdot K_M/(K_M + C_i)$. Then the constant, k, related to the permeability of the outer membrane could be calculated, since under steady state conditions v must equal the diffusion rate across the outer membrane, $k\ (C_o - C_i)$, where C_o is the β-lactam concentration in the medium. We followed this procedure, and furthermore calculated the permeability coefficient P, from $k = P \times A$, by using the area A of *S. typhimurium* cell surface previously determined (12). Permeability coefficients obtained in the presence of widely different amounts of β-lactamases in the periplasmic space [compare SH5014 (R1) with SH5014 (R471a) in Table I] were very similar. Furthermore, assays with three different external concentrations of cephaloridine gave permeability coefficients of an almost identical value for SH5014 (R1) (results not shown). These results suggest that the assumptions made above are likely to be correct.

The values of the permeability coefficients determined (Table I) lead us to several important conclusions.

(a) The value of P for cephaloridine in the wild type strain (SH5014) is quite high. Permeability coefficients for small, uncharged molecules can be predicted for biological membranes of the usual type (13). This leads to the expected P value of the order of 10^{-8} cm/sec for molecules of the size of cephaloridine. Moreover, ionized groups on the antibiotic molecule are expected to reduce the P value further by several orders of magnitude (13). Thus cephaloridine diffuses through the outer membrane much faster than expected, an observation consistent with the presence of diffusion pores.

(b) Among outer membrane proteins, the porins, i.e. the 36K, 35K, and 34K proteins, were found to produce pores in the in vitro reconstitution experiments (4,5). In this study, we found that mutants lacking the 34K or 36K protein singly gave somewhat reduced rates of transmembrane diffusion, and that in mutants lacking both 34K and 36K proteins the diffusion rates of cephaloridine became drastically reduced; this observation clearly indicates that these proteins are indeed needed for the formation of pores in living cells. [The level of 35K protein is quite low in all strains derived from SH5014 (6).]

Although R plasmids may conceivably alter the architecture of the outer membrane, we found similar differences between the wild type and $36K^-$ $34K^-$ mutants by using the two R plasmids belonging to different compatibility groups, and the presence of these plasmids produced no marked alterations in outer membrane protein pattern (P. Bavoil and H. Nikaido, unpublished observation). These results suggest that the differences found are not artefacts caused by the introduction of R plasmids.

c) A mutant lacking the 33K protein (SL1917) was tested and was found to have normal permeability. This is not surprising, as the 33K protein behaves quite differently from porins during sodium dodesyl sulfate extraction of the outer membrane, and cannot produce pores in the reconstitution system (4).

DISCUSSION

The determination of β-lactamase activity in intact cells thus indicates that the permeability of the outer membrane toward cephaloridine, a hydrophilic molecule of 415 daltons, is greatly decreased in porin-deficient mutants. Is the permeability toward other substances, for example nutrients, also decreased? At first sight, the observation that the porin-deficient mutants of Salmonella (6) and E. coli (14) can grow rapidly in conventional media seems to suggest otherwise. However, all "porin-deficient" mutants so far isolated produce residual levels (of the order of 5-20%) of porins, and since the diffusion rates of nutrients are proportional to the concentration difference across the outer membrane, at high nutrient concentrations found in ordinary media it is quite possible that the residual pores are sufficient for growth.

The diffusion through pores, however, can become limiting in porin-deficient mutants if the outside concentration of nutrients is quite low. In fact, von Meyenburg (15) has described an E. coli mutant with a defect of unknown nature, which makes the mutant grow more slowly in very dilute concentrations of all carbon sources, and raises the apparent K_M of the transport of all amino acids. By using his plate culture technique we could show that the double porin mutants (SH6260, SH6264) grew more slowly in very low concentrations of the required amino acids, such as isoleucine or histidine. Furthermore, the apparent K_M values for the transport of leucine and histidine, measured by the "starved cell" method of Ames (16), were increased in SH6260 in comparison with SH5014. Finally, after the decisive results in this work have been obtained, a paper by Beacham et al. (17) has appeared. These workers isolated E. coli mutants that produced normal amounts of a periplasmic enzyme, 5'-nucleotidase, yet showed reduced "intact cell activity" for this enzyme, and found that they were deficient in porins. All these results then indicate strongly that porins are involved in the production of non-specific pores, which form the major pathway of diffusion for a variety of substances including sugars, amino acids, nucleotides, and antiobiotics. It should, however, be added that E. coli and

Salmonella also produce specific diffusion machinery for substances that are too large for pores, or have to be scavenged from the environment at very low external concentrations (e.g., see ref. 18).

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