Vol. 83, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 28, 1978 Pages 550-557

DIFFUSION OF TETRACYCLINE ACROSS THE OUTER MEMBRANE OF ESCHERICHIA COLI K-12: INVOLVEMENT OF PROTEIN I_a

I. Chopra and Sarah J. Eccles

Department of Bacteriology,
The Medical School,
Bristol BS8 1TD
United Kingdom

Received May 16,1978

SUMMARY

The possibility that passage of tetracycline across the outer membrane of E. coli K-12 is controlled by one or more of the proteins I_a, I_b and II* (Henning's nomenclature) was investigated. A mutant lacking protein I_a (obtained by selection for resistance to phage TuI_a) was more resistant to tetracycline than wild-type strains or those lacking only proteins I_b or II*. The envelope protein composition of a tetracycline-resistant mutant (cmlB) was altered in several respects, but the major change involved loss of protein I_a. These data support our previous suggestion [12] that tetracycline diffuses across the outer membrane through hydrophilic regions. Furthermore, they imply that only protein I_a plays a significant role in the passage of this antibiotic across the outer membrane.

INTRODUCTION

The outer membrane of wild-type strains of Escherichia coli K-12 contains five major proteins (with respect to content) [1]. Four of these proteins (described according to Henning's nomenclature, see 1) are linked to peptidoglycan. For proteins I_a, I_b and II* the association with peptidoglycan appears to be non-covalent [2-5], whereas approximately one-third of protein IV is covalently linked at its carboxyl terminus to peptidoglycan [1].

Much evidence has accumulated to implicate proteins I_a, I_b and II* in the permeability of the outer membrane to small hydrophilic molecules including nutrients (involving proteins I_a, I_b and II*) [6-8] and β-lactam antibiotics (involving proteins I_a and I_b) [9]. On the other hand, the fourth peptidoglycan associated protein (protein IV) probably has no role in the permeation of nutrients or antibiotics across the outer membrane [10,11].

We showed recently [12] that tetracycline crosses the outer membrane of E. coli K-12 through hydrophilic regions. This raises the question of whether passage of tetracycline across the outer membrane is controlled by one

0006-291X/78/0832-0550$01.00/0

or more of proteins I_a, I_b and II*. The data presented here suggest that only protein I_a plays a significant role in the passage of tetracycline across the outer membrane.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains are listed in Table 1.

Growth media for bacteria

Nutrient broth and nutrient agar were used [15].

Determination of IC_{50}† values for antibiotics

Bacteria (1-2 x 10^8 organisms/ml) growing exponentially at 37°C in nutrient broth were challenged with various concentrations of antibiotics. The growth rates of drug-inhibited and drug-free cultures were determined from semi-logarithmic plots of culture turbidity at 675nm versus time. IC_{50} values were determined from the growth rate data.

Propagation and titration of phages TuI_a, TuI_b and TuII*

Nutrient agar plates containing 4mM calcium chloride but lacking sodium chloride were lawned with PC0479 and overlaid with the individual phages in soft nutrient agar (0.7%). Phages were harvested from confluently lysed plates by scraping the soft agar into nutrient broth containing 4mM calcium chloride. The phages were titred against the strains described in Table 1 by analogous methods.

Preparation of whole cell envelopes

This was performed as previously described [12].

Separation of envelope proteins by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate

This was performed as described [16]. Staining and scanning densitometry of disc gels were performed as described previously [17]. Molecular weights of polypeptides were estimated as described [15].

Isoelectric focusing of envelope proteins in polyacrylamide disc gels

This was performed essentially as described by Ames and Nikaido [18] except that envelopes were denatured by heating at 100°C for 5 minutes. The isoelectric focusing gels produced pH gradients (3.9 - 6.8) that were approximately linear. Proteins were visualized according to a procedure described by Righetti and Drysdale [19] which involves sequential immersion of gels (a) for 4-6 hours in Coomassie brilliant blue (0.05%) and cupric sulphate (0.1%) in acetic acid-ethanol-water (19:25:65), (b) for 4 hours in the same solution but containing only 0.01% of the dye, (c) for 12-15 hours in acetic acid-ethanol-water (10:10:80) (destaining solution).

---

† IC_{50}, concentration of antibiotic causing a 50% reduction in growth rate

Table 1 *Escherichia coli* K-12 strains

Strain	Parental strain	Strain description†	Reference
PC0479		polyauxotroph	13
CE1122	PC0479	lacks protein I_a	7,9
CE1110	PC0479	rich in protein I_a, lacks protein I_b	7,9
CE1131	PC0479	lacks protein II*	7,9
DU1004	J5-3	*pro-1*, *met-2*, *rif*	14
DU2503	DU1004	*cmlB*	14

†proteins referred to are the major outer membrane proteins in the nomenclature of Henning [1]

RESULTS

Strains CE1122 and DU2503 were approximately 3-fold more resistant to tetracycline and 2-fold more resistant to chloramphenicol than their parental strains (Table 2). CE1122 was slightly more resistant to minocycline than PC0479 (Table 2). Strains PC0479, CE1110 and CE1131 were essentially equally sensitive to tetracycline (Table 2).

These data (a) suggest that the increased levels of resistance to tetracycline and chloramphenicol in CE1122 result from loss of protein I_a, and (b) imply that a similar change is responsible for resistance in DU2503. To confirm the latter, envelope proteins from DU2503 and DU1004 were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis [16]. DU1004 contained two major proteins (Fig.1a) which were identified as proteins I and II* on the basis of their apparent molecular weights (38,000 and 31,000 respectively) and by the fact that the protein of molecular weight 31,000 was virtually absent in envelopes from CE1131 (data not shown). DU2503 (*cmlB*) contained less protein I than DU1004, but was enriched for proteins with apparent molecular weights of 25,000 and 50,000 (Fig.1). The significance of the latter changes is at present unknown, and they were not noted when

Table 2 Sensitivity of strains of E. coli K-12 to various antibiotics

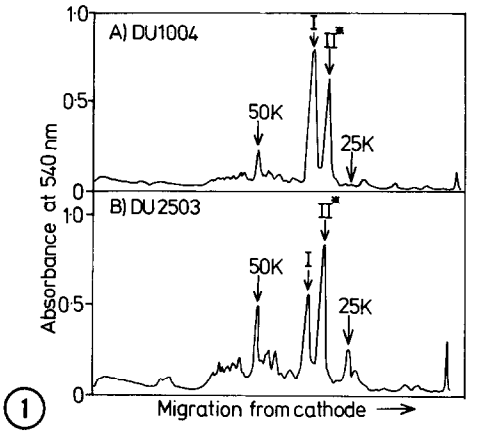
Strain	Outer membrane proteins			IC_{50}† (μg/ml) Tetracycline	Minocycline	Chloramphenicol
PC0479	I_a	I_b	II*	0.26 ± 0.10	0.65 ± 0.06	1.34 ± 0.20
CE1122		I_b	II*	0.70 ± 0.12	0.95 ± 0.15	2.13 ± 0.14
CE1110	I_a		II*	0.21 ± 0.10	ND	ND
CE1131	I_a	I_b		0.25 ± 0.06	ND	ND
DU1004	I_a	I_b	II*	0.26 ± 0.12	ND	1.12 ± 0.13
DU2503		I_b	II*	0.82 ± 0.16	ND	2.25 ± 0.15

†IC_{50} values determined as described in Methods. Values are the means of at least two separate determinations for each strain and are quoted ± standard error of the mean.

ND, not determined.

CE1122 was compared with PC0479 (data not shown).

Although the electrophoresis system used here can separate proteins I_a and I_b from each other [16], we have been unable to resolve these proteins as separate species (Fig.1 and reference 17). However, proteins I_a and I_b were adequately resolved by isoelectric focusing (Fig.2) and this method demonstrated that DU2503 (cmlB) contained less protein I_a than DU1004 (Fig.2, gels 5 and 6). Isoelectric focusing of envelope proteins from strains PC0479, CE1122, CE 1110 and CE1131 (Fig.2) confirmed the reported outer membrane protein compositions of these bacteria (Table 1). The approximate isoelectric points of proteins I_a, I_b and II* were, respectively, 5.0, 4.9 and 6.4. These values agree with those reported in the literature [20,21]. Protein II* from strains DU1004 and DU2503 had a lower isoelectric point than the protein from the other strains (Fig.2). The reason for this is unclear. The sensitivities of the strains described in Table 1 to phages TuI_a, TuI_b and TuII* (data not shown) were consistent with the outer membrane protein compositions of these bacteria.



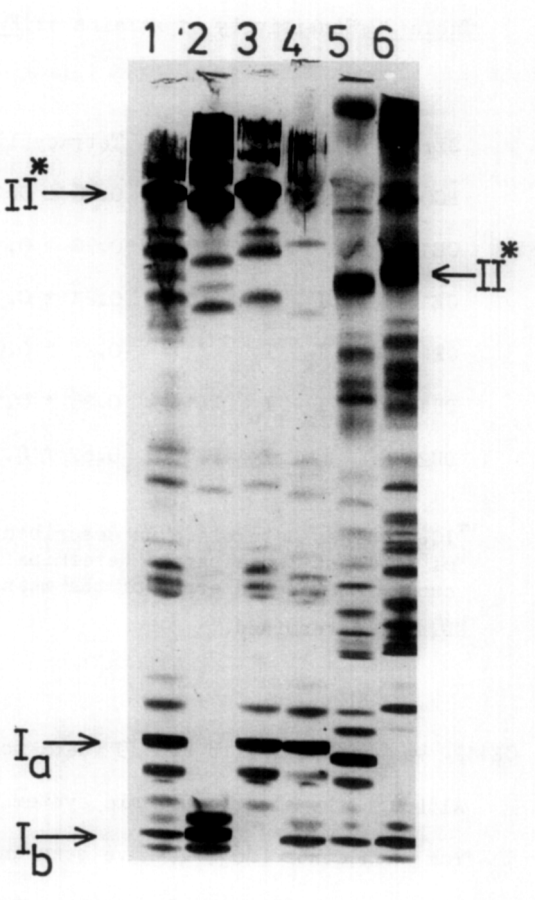


Figure 1

Scanning densitometer tracings of sodium dodecyl sulphate polyacrylamide disc gels containing separated proteins from envelopes of DU1004 (A) and DU2503 (B). Envelopes were prepared and analyzed as described in Methods. Proteins I and II* and polypeptides with apparent molecular weights of 25,000 and 50,000 are indicated.

Figure 2

Isoelectric focusing of proteins from the envelopes of bacteria listed in Table 1. Envelopes were prepared and analyzed as described in Methods. Bands corresponding to proteins I_a, I_b and II* are indicated. Identity of gels: 1 = PC0479; 2 = CE1122; 3 = CE1110; 4 = CE1131; 5 = DU1004; 6 = DU2503. Protein II* from DU1004 and DU2503 (gels 5 and 6) has a lower isoelectric point than the protein from the other strains. Some minor proteins present in gels 1-4 are not apparent in this photograph.

DISCUSSION

There have been many elegant studies on the nature and organization of the major outer membrane proteins of *E. coli* (for a review see 1). Several authors have concluded from this work that proteins I_a, I_b and II* aggregate

in the outer membrane to produce transmembrane pores through which small hydrophilic molecules diffuse (the pore model). Although we have been sceptical about the pore model [17], we now admit that there is an overwhelming body of evidence to favour it. However, there are still some observations that are apparently inconsistent with the pore model:

a) extensive peptidoglycan degradation can lead to artifactual formation of protein I aggregates in the outer membrane [17]

b) Manning et al [8] show that protein II* controls permeability in vivo, yet Nakae's work [22] with liposomes implies that protein II* does not form pores

c) Proteins I_a, I_b and II* together comprise about 2-3 x 10^5 molecules per outer membrane [23]. The area available to accommodate protein in the outer half of the outer membrane bilayer [24] seems insufficient for this number of protein molecules.

Data presented in this paper show that both a phage TuI_a resistant mutant and a cmlB mutant are deficient in protein I_a and are more resistant to tetracycline than their wild-type parental strains. Loss of protein I_b or II* was not associated with changes in the level of resistance to tetracycline. These results imply that tetracycline preferentially diffuses through pores formed by protein I_a. Furthermore, our results support the contention [25] that the cmlB locus is either identical or closely related to tolF. The tolF locus probably determines a post-translational system that specifically processes protein I_a [20]. Loss of the tolF function alone (as in a cmlB mutant) is therefore expected to produce $I_a^- I_b^+$ strains, a prediction supported by the data presented here. We reported [12] that minocycline (a tetracycline derivative) is unlikely to diffuse primarily through outer membrane pores. A corollary of the previous work [12] (which did not utilize mutants specifically lacking outer membrane proteins) is that mutants deficient in protein I_a should not be markedly more resistant to minocycline than wild-type organisms. Although CE1122 (I_a^-) was more resistant to minocycline than PC0479 (wild-type) (Table 2), the ratio of the IC_{50} values was only 1.5, whereas for tetracycline

the ratio was 2.7.

Those features of the tetracycline molecule that consign it preferentially to diffuse through protein I_a pores are unknown. Although other hydrophilic molecules do show specificity towards these pores the compounds do not have closely related structures. Thus, protein I_a pores are used preferentially by tetracycline and probably chloramphenicol (this paper) and nucleotides [7]. However, diffusion of molecules through pores is unlikely to involve specific interaction between permeant and protein [7].

Resistance to tetracycline mediated by transposon 10 is associated with the synthesis of an outer membrane protein, present at about 2000 copies per cell [26]. That this protein may interact with protein I_a pores to hinder diffusion of tetracycline across the outer membrane is currently under investigation.

ACKNOWLEDGEMENTS

This work was supported by a project grant (G975/1004/C) to I.C. from the Medical Research Council. We thank B. Lugtenberg and T.J. Foster for the provision of bacteria, and U. Henning for the provision of phages.

REFERENCES

1. Braun, V. (1978) In Relations between structure and function in the prokaryotic cell, Symposium 28 of the Society for General Microbiology pp.111-138, Cambridge University Press.

2. Rosenbusch, J.P. (1974) J. Biol. Chem, 249, 8019-8029.

3. Lugtenberg, B., Bronstein, H., van Selm, N., and Peters, R. (1977) Biochim. Biophys. Acta, 465, 571-578.

4. Yu, F., and Mizushima, S. (1977) Biochem. Biophys. Res. Commun, 74, 1397-1402.

5. Endermann, R., Kramer, C., and Henning, U. (1978) FEBS Lett, 86, 21-24.

6. Bavoil, P., Nikaido, H., and von Meyenburg, K. (1977) Molec. gen. Genet, 158, 23-33.

7. van Alphen, W., van Selm, N., and Lugtenberg, B. (1978) Molec. gen. Genet, 159, 75-83.

8. Manning, P.A., Pugsley, A.P., and Reeves, P. (1977) J. Mol. Biol, 116, 285-300.

9. van Alphen, W., van Boxtel, R., van Selm, N., and Lugtenberg, B. (1978) FEMS Micro. Lett, 3, 103-106.

10. Hirota, Y., Suzuki, H., Nishimura, Y., and Yasuda, S. (1977) Proc. Natl. Acad. Sci. USA, 74, 1417-1420.

11. Nikaido, H., Bavoil, P., and Hirota, Y. (1977) J. Bacteriol, 132, 1045-1047.

12. Ball, P.R., Chopra, I., and Eccles, S.J. (1977) Biochem. Biophys. Res. Commun, 77, 1500-1507.

13. Verhoef, C., de Graaff, P.J., and Lugtenberg, E.J.J. (1977) Molec. gen. Genet, 150, 103-105.

14. Foster, T.J. (1975) J. gen. Microbiol, 90, 303-310.

15. Chopra, I., Lacey, R.W., and Connolly, J. (1974) Antimicrob. Agents Chemother, 6, 397-404.

16. Lugtenberg, B., Peters, R., Bernheimer, H., and Betendsen, W. (1975) FEBS Lett, 58, 254-258.

17. Chopra, I., Howe, T.G.B., and Ball, P.R. (1977) J. Bacteriol, 132, 411-418

18. Ames, G.F-L., and Nikaido, K. (1976) Biochemistry, 15, 616-623.

19. Righetti, P.G., and Drysdale, J.W. (1974) J. Chromatog, 98, 271-321.

20. Henning, U., Schmidmayr, W., and Hindennach, I. (1977) Molec. gen. Genet, 154, 293-298.

21. Sato, T., Ito, K., and Yura, T. (1977) Eur. J. Biochem, 78, 557-567.

22. Nakae, T. (1976) Biochem. Biophys. Res. Commun, 71, 877-884.

23. Hindennach, I., and Henning, U. (1975) Eur. J. Biochem, 59, 207-213.

24. Smit, J., Kamio, Y., and Nikaido, H. (1975) J. Bacteriol, 124, 942-958.

25. Foulds, J. (1976) J. Bacteriol, 128, 604-608.

26. Chopra, I. (1978) Submitted to Molec. gen. Genet.