BILE ACID BIOTRANSFORMATION RATES OF SELECTED GRAM-POSITIVE AND GRAM-NEGATIVE INTESTINAL ANAEROBIC BACTERIA

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SUMMARY: Treatment of whole cell suspensions of Eubacterium aerofaciens and Bacteroides fragilis with lysozyme resulted in a marked increase (>100-fold) in the rates of biotransformation of cholate to 7-ketodeoxycholate (7-KD) in the former but only a 2-fold increase in the latter bacterium. In B. fragilis the total activity of both NAD-dependent 7-α-hydroxysteroid dehydrogenase (7-α-OHSDH) and bile salt hydrolase (BSH) increase markedly during the stationary growth phase. Both enzymes were found in the spheroplast lysate and the Triton-soluble washed membrane fractions but only BSH was found in the spheroplast medium.

INTRODUCTION: Greater than 99% of the intestinal bacterial flora of man are obligate anaerobes of which B. fragilis and E. aerofaciens strains account, respectively, for about 20-25% and 8% (1,2).

The normal intestinal bacteria of man are able to transform conjugated and free bile acids into a variety of metabolites. The major reactions include the hydrolysis of bile salt conjugates, dehydroxylation of the hydroxyl group at carbon 7 and dehydrogenases that oxidize the hydroxyl groups at either carbons 3,7 or 12 as well as reduce the 3-keto group to both α and β hydroxyls (3-6). B. fragilis strains are reported to oxidize only the 7-α-hydroxyl group (7,8).

There is a paucity of published information on the cellular location and regulation of the bile acid enzymes in the intestinal anaerobic bacteria nor is it clear which physiological features in these bacterial cells are important in regulating the rates of bile acid biotransformation.

In this communication, we report the rates of selected bile acid transformations by whole and lysozyme treated cells of a Gram-positive (G^+) (E. aerofaciens) and Gram-negative (G^-) (B. fragilis) intestinal anaerobic bacterium. In B. fragilis the stage of growth was shown to effect the level of two such enzymes

MATERIALS AND METHODS: Strains of B. fragilis ssp. thetaiotaomicron NCTC 10852, B. fragilis ssp. fragilis ATCC 25285 and E. aerofaciens VPI J4-26 were kindly donated by J. L. Johnson and W. E. C. Moore of the Virginia Polytechnic Institute (VPI) and State University, Anaerobe Laboratory. Stock cultures of bacteria were maintained in chopped-meat medium under CO_2 as described by Holdeman and Moore (9). E. aerofaciens was cultured in Peptone-Yeast Extract-Glucose (PYG) medium containing 3 µM hemin and 0.5 mM sodium cholate. B. fragilis was cultured in either chemically defined growth medium as described previously (10) or PYG medium. Growth of bacteria was determined by measuring the culture absorbancy with a Bausch and Lomb Spectronic 20 at 660 nm.

Preparation of lysozyme treated cell suspensions: Cells harvested by centrifugation at 13,700 g for 15 min (4 C) from a 3 liter culture during the early stationary growth phase were suspended in 20 ml of freshly prepared 50 mM Tris-HCl buffer (pH 7.6) containing 0.5 M sucrose, 10 mM 2-mercaptoethanol (2-ME) and 1 mM sodium EDTA. The cell suspensions were divided into two and one portion treated with lysozyme. With B. fragilis ssp. thetaiotaomicron 50 µg/ml lysozyme yielded maximal spheroplast in 30 min at 25 C using procedures described by Bell et al. (11). The E. aerofaciens cells were incubated with 100 µg/ml lysozyme for 3 h at 37 C. Samples of the suspensions were solubolized in 1 N NaOH and protein measured according to Lowry et al. (12).

Approximately 3.5 X 10^6 dpm of cholic-carboxyl-C^{14} acid (40 Ci/M)(New England Nuclear, Boston, Mass.) or deoxycholic-carboxyl-C^{14} acid (52 Ci/M) (Amersham-Searle, Arlington Heights, Ill.) was added individually to whole cell suspensions and lysozyme treated cell suspensions to a final concentration of 0.5 mM (total volume of reaction mixture was 5 ml). Samples (1.0 ml) were removed at selected time intervals up to 6 h, immediately acidified to pH 1.0 with concentrated HCl and extracted with 1.0 ml redistilled ethylacetate. The extracted radiolabeled products were spotted onto Silica gel thin-layer chromatography plates (20 by 20) and separated in solvent systems described by Eneroth (13). Radiolabeled products were located by using a Packard radio-

chromatogram scanner. Individual spots corresponding to bile acids and keto-bile acids were scrapped into scintillation vials and counted by liquid scintillation spectrometry. The percent conversion of each bile acid to keto-bile acid was determined for each time point.

Bile acid enzyme activities in spheroplast fractions of B. fragilis ssp. fragilis: Cells were harvested, as indicated in methods, from one 6 liter culture sample taken at different growth stages and washed once at 25 C in 200 ml of 20 mM potassium phosphate (PP) buffer (pH 7.0). Spheroplasts were prepared by suspending 8 g wet weight of cells in 20 ml of 20 mM PP buffer which was 0.5 M in sucrose, 20 mM in 2-ME and 1 mM in sodium EDTA. One liter of cell culture at 1.6 absorbance yielded about 2.2 g wet weight of cells. Cell suspensions were treated with lysozyme (50 µg/ml) for approximately 1 h at 30 C, centrifuged at 10,000 g for 20 min at 20 C and the supernatant fluid designated as "spheroplast medium". The sedimented spheroplasts were diluted into 20 ml of 20 mM PP buffer containing 1 mM sodium EDTA and 20 mM 2-ME and passed once through an automatic French Pressure Cell (4 C) at 8,000 lb/in^2. The lysed spheroplasts were centrifuged at 105,000 g for 30 min at 4 C and the supernatant fluid designated as "spheroplast lysate". The sedimented membranes were washed in 20 ml PP buffer by homogenization in a tissue homogenizer. The homogenate was again centrifuged at 105,000 g for 30 min and the sediment treated for 1 h with Triton X-100 (2% vol/vol) at 4 C. The Triton-treated membrane fraction was again centrifuged at 105,000 g for 60 min and the supernatant fraction designated "Triton-soluble washed membrane fraction". Each spheroplast fraction was assayed for 7-α-OHSDH activity as described previously (7). BSH activity was assayed according to the method of Nair et al. (14) and glutamate dehydrogenase (GDH) activity was assayed according to the method of Phibbs and Bernlohr (15). Using this procedure, we were able to recover approximately 60% and 70% of the initial amount of 7-α-OHSDH and BSH activity in the lysed spheroplast sediment, respectively. Enzyme activities are reported as total International Units (I.U.) of enzyme activity per spheroplast fraction.

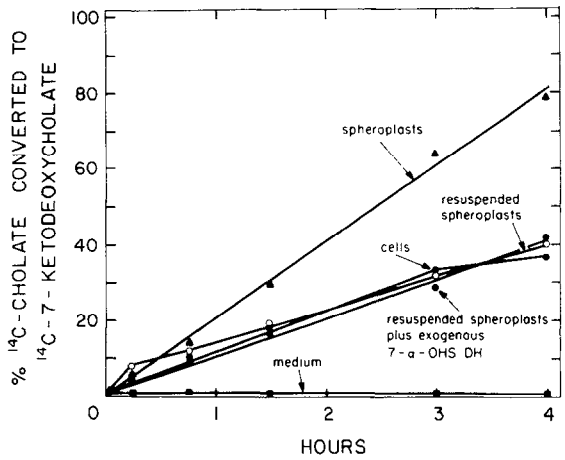

Fig. 1. Rates of conversion of cholate to 7-ketodeoxycholate in whole cell suspensions (●), spheroplasts (▲), resuspended spheroplast (○), resuspended spheroplasts plus 0.65 I.U. of exogenously added NAD-dependent 7-α-hydroxysteroid dehydrogenase (●) and "spheroplast medium" (■) of B. fragilis ssp. thetaiotaomicron NCTC 10852. The initial cell suspension contained 3.28 mg of whole cell protein per ml.

RESULTS: Growing cultures of B. fragilis (G^-) converted 70-80% cholate to 7-KD in 24-36 h, whereas E. aerofaciens (G^+) cultures converted only about 6-8% in 72 h. The data presented in Figure 1 show the rates of transformation of cholate to 7-KD in B. fragilis cells and spheroplasts. The lysozyme treated cells showed a 2-fold increase in the rate of conversion of cholate to 7-KD.

No detectable cholate transformation occurred in cell suspensions of E. aerofaciens over a time course of 6 h (Fig. 2). In contrast, deoxycholate was converted rapidly to 12-ketolithocholate (12-KL), whereas lysozyme treatment markedly increased (>100-fold) the rate of conversion of cholate to 7-KD. However, the rate of conversion of deoxycholate to 12-KL increased only 2-fold in the same lysozyme treated cell suspensions (Fig. 2).

Figures 3 and 4 shows the total units of enzyme activity in spheroplast fractions derived from cells taken at different growth stages. The total units of BSH (Fig. 3) and 7-α-OHSDH (Fig. 4) activity increased in the "Triton-soluble washed membrane" and "spheroplast lysate" fractions derived from culture samples taken at time points indicated by numbered arrows during the stationary peroid

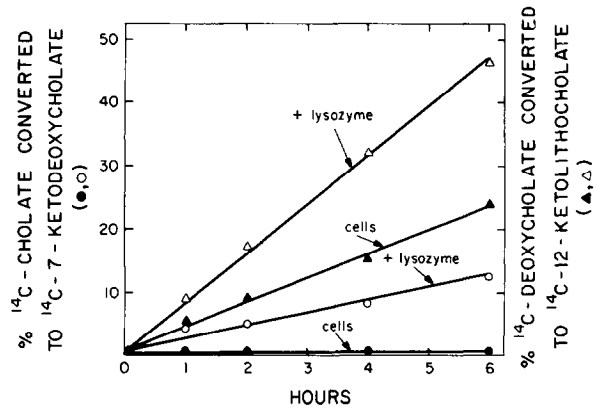

Fig. 2. Rates of conversion of selected bile acid to keto-bile acid in whole cell suspensions (●,▲) and lysozyme treated cell suspensions (○,△) of E. aerofaciens VPI J4-26. The initial cell suspension contained 4.5 mg whole cell protein per ml.

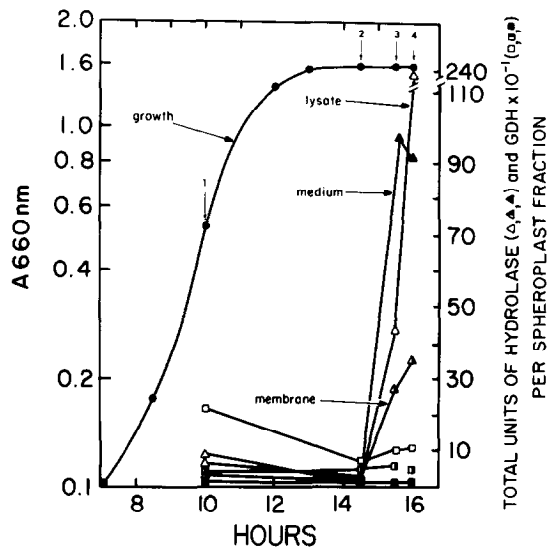

Fig. 3. Regulation and cellular location of bile salt hydrolase (BSH) (triangles) and NADP-dependent glutamate dehydrogenase (GDH) (squares) in B. fragilis ssp. fragilis ATCC 25285 cultured in PYG medium. Symbols indicate total international units (I.U.) of enzyme activity in "spheroplast lysate" (△,□), "Triton-soluble washed membrane" (▲,◨) and "spheroplast medium" (▲,■). Cell growth was recorded as culture absorbancy at 660 nm (●).

of growth. In contrast, the total units of GDH remained relatively constant (Figs. 3 and 4). Moreover, BSH activity was detected in the "spheroplast medium' (Fig. 3) in these same spheroplast preparations in contrast to both 7-α-OHSDH (Fig.4) and GDH activities.

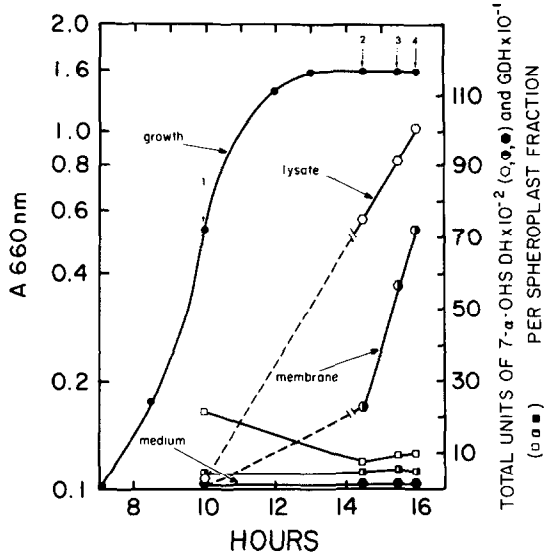

Fig. 4. Regulation and cellular location of NAD-dependent 7-α-hydroxysteroid dehydrogenase (7-α-OHSDH) (hexagons) and NADP-dependent glutamate dehydrogenase (GDH) (squares) in B. fragilis ssp. fragilis ATCC 25285 cultured in PYG medium. Symbols indicate total international units (I.U.) of enzyme activity in "spheroplast lysate" (○,□), "Triton-soluble washed membrane" (◑,◨) and "spheroplast medium" (●,■). Cell growth was recorded as culture absorbancy at 660 nm (●).

DISCUSSION: The selected data on the rates of bile acid transformation by intestinal anaerobic bacteria reported in this communication show that the lysozyme-sensitive peptidoglycan layer of the cell wall act as a significant permeability block to cholate as shown by the greater rates of transformation in lysozyme treated cells. Burman et al. (16) reported that the peptidoglycan layer in E. coli act as a permeability block to cholate. This observation is supported by the fact that G^+ bacteria transform cholate at a markedly reduced rate as compared to G^- bacteria. These data also suggests that G^- intestinal bacteria may play a more important role than G^+ bacteria in cholate transformation in the gastrointestinal tract of man.

We also show that in B. fragilis the activities of both 7-α-OHSDH and BSH increased rapidly during the stationary growth phase. The mechanism of enzyme regulation is unclear. Large amounts of 7-α-OHSDH and BSH activities are shown to be tightly associated with the membrane fraction and in addition, BSH activity can be detected in the "spheroplast medium" suggesting a "periplasmic" location

of this enzyme. The intracellular, GDH was used as a marker enzyme to assure that any spheroplast lysis would be detected. Although, the physiological significance of these different locations of bile acid transforming enzymes is not yet clear, we suggest that the transformation reactions may occur in or on the bacterial cytoplasmic membrane.

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REFERENCES:

1. Darsar, B. S., Shiner, M. and McLoed, G. M. Gastroenterology 56:71-79 (1969).
2. Moore, W. E. C. and Holdeman, L. Appl. Microbiol. 27:961-979 (1974).
3. Aries, V. and Hill, M. J. Biochim. Biophys. Acta 202:526-534 (1970).
4. Aries, V. and Hill, M. J. Biochim. Biophys. Acta 202:534-543 (1970).
5. Midtvedt, T. and Norman, A. Acta Pathol. Microbiol. Scand. 71:629-638 (1967).
6. Lewis, R. and Gorbach, S. Arch. Intern. Med. 130:545-549 (1972).
7. Hylemon, P. B. and Sherrod, J. A. J. Bacteriol. 112:418-424 (1975).
8. MacDonald, I. A., Williams, C. N., Mahony, D. E. and Christie, W. M. Biochim. Biophys. Acta 384:14-24 (1975).
9. Holdeman, L. V. and W. E. C. Moore (ed.) 1973 Anaerobe laboratory Manual, 2nd ed. Virginia Polytechnic Institute and State University, Blacksburg.
10. Hylemon, P. B. and Phibbs, P. V. Biochem. Biophys. Res. Commun. 60:88-95 (1974).
11. Bell, G. R., LeGall, J. and Peck, H. D. J. Bacteriol. 120:994-997 (1974).
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. J. Biol. Chem. 193:265-275 (1951).
13. Eneroth, P. J. Lipid Res. 4:11-16 (1963).
14. Nair, P. P., Gordon, N. and Rebach, J. J. Biol. Chem. 242:7-11 (1967).
15. Phibbs, P. V. and Bernlohr, R. N. J. Bacteriol. 106:375-385 (1971).
16. Burman, L. G., Nordstrom, K. and Bloom, G. D. J. Bacteriol. 112:1364-1374 (1972).