Alcohol & Alcoholism Vol. 33, No. 3, pp. 273–280, 1998

CHARACTERISTICS OF ALDEHYDE DEHYDROGENASES OF CERTAIN AEROBIC BACTERIA REPRESENTING HUMAN COLONIC FLORA

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(*Received* 12 *August* 1997; *in revised form* 26 *November* 1997; *accepted* 1 *December* 1997)

Abstract — We have proposed the existence of a bacteriocolonic pathway for ethanol oxidation resulting in high intracolonic levels of toxic and carcinogenic acetaldehyde. This study was aimed at determining the ability of the aldehyde dehydrogenases (ALDH) of aerobic bacteria representing human colonic flora to metabolize intracolonically derived acetaldehyde. The apparent Michaelis constant (K_m) values for acetaldehyde were determined in crude extracts of five aerobic bacterial strains, alcohol dehydrogenase (ADH) and ALDH activities of these bacteria at conditions prevailing in the human large intestine after moderate drinking were then compared. The effect of cyanamide, a potent inhibitor of mammalian ALDH, on bacterial ALDH activity was also studied. The apparent K_m for acetaldehyde varied from 6.8 ($NADP^+$-linked ALDH of *Escherichia coli* IH 13369) to 205 μM (NAD^+-linked ALDH of *Pseudomonas aeruginosa* IH 35342), and maximal velocity varied from 6 nmol/min/mg (NAD^+-linked ALDH of *Klebsiella pneumoniae* IH 35385) to 39 nmol/min/mg (NAD^+-linked ALDH of *Pseudomonas aeruginosa* IH 35342). At pH 7.4, and at ethanol and acetaldehyde concentrations that may be prevalent in the human colon after moderate drinking, ADH activity in four out of five bacterial strains were 10–50 times higher than their ALDH activity. Cyanamide inhibited only NAD^+-linked ALDH activity of *Pseudomonas aeruginosa* IH 35342 at concentrations starting from 0.1 mM. We conclude that ALDHs of the colonic aerobic bacteria are able to metabolize endogenic acetaldehyde. However, the ability of ALDHs to metabolize intracolonic acetaldehyde levels associated with alcohol drinking is rather low. Large differences between ADH and ALDH activities of the bacteria found in this study may contribute to the accumulation of acetaldehyde in the large intestine after moderate drinking. ALDH activities of colonic bacteria were poorly inhibited by cyanamide. This study supports the crucial role of intestinal bacteria in the accumulation of intracolonic acetaldehyde after drinking alcohol. Individual variations in human colonic flora may contribute to the risk of alcohol-related gastrointestinal morbidity.

INTRODUCTION

Excessive alcohol consumption is frequently associated with flatulence and diarrhoea (Fields *et al.*, 1994). Furthermore, marked pathological changes have been observed in the rectal mucosa of heavy drinkers (Brozinsky *et al.*, 1978). A positive association between alcohol intake and the development of colorectal polyps and cancer has been found in epidemiological studies (Pollak *et al.*, 1984; Cope *et al.*, 1991; Kune and Vitetta, 1992; Blot, 1992; Giovannucci and Willett, 1994; Kearney *et al.*, 1995). The mechanisms responsible for this alcohol-related intestinal morbidity, however, are not properly understood.

Under anaerobic conditions, bacteria are capable of producing energy through fermentation (Zeikus, 1980). The end product of alcoholic fermentation is ethanol, which is derived from acetaldehyde in a reductive reaction mediated by bacterial alcohol dehydrogenase (ADH) (Neale *et al.*, 1986). Where there is an excess of ethanol, the reaction catalysed by microbial ADH can run in the opposite direction with acetaldehyde as an end product. Accordingly, the incubation of human colonic contents with increasing ethanol concentrations *in vitro* results in a marked accumulation of acetaldehyde (Jokelainen *et al.*, 1994).

Variable ADH activity and acetaldehyde-producing capacity have been found *in vitro* among the aerobic bacteria representing the normal human colonic flora. The highly signifi-

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cant positive correlation between bacterial ADH activity and acetaldehyde-producing capacity from ethanol suggests the important role of ADH in bacterial acetaldehyde production (Jokelainen *et al.*, 1996*b*). Low apparent Michaelis constant (K_m) values of ADHs of aerobic colonic bacteria predispose them to metabolize ethanol with a near-maximal velocity and to produce marked amounts of acetaldehyde from ethanol concentrations prevailing in the human colon during social drinking (Nosova *et al.*, 1997). Furthermore, diminishing the number of faecal aerobic microbes by treatment with the selective antibiotic ciprofloxacin is associated with a significant decrease of both faecal ADH activity and ethanol elimination rate in rats (Jokelainen *et al.*, 1997).

We have recently proposed the existence of a bacteriocolonic pathway for ethanol oxidation, i.e. intracolonic ethanol is first oxidized by bacterial ADHs and catalase to acetaldehyde, which is then oxidized either by colonic mucosal cells or by bacterial aldehyde dehydrogenases (ALDH) to acetate (Jokelainen *et al.*, 1996*a*; Nosova *et al.*, 1996; Salaspuro, 1996, 1997; Tillonen *et al.*, 1997). Intracolonic acetaldehyde may also be absorbed partially into the portal vein to be metabolized further in the liver (Matysiak-Budnik *et al.*, 1996).

We have recently demonstrated that crude extracts of aerobic colonic bacteria possess oxidized nicotinamide adenine dinucleotide (phosphate) NAD(P)$^+$-linked ALDH activities *in vitro* towards acetaldehyde, benzaldehyde, and propionaldehyde. These bacteria were able to metabolize acetaldehyde with subsequent acetate production when incubated *in vitro* (Nosova *et al.*, 1996). The first aim of the present *in vitro* study was to characterize further the ability of the ALDHs of some aerobic bacteria, representing the normal flora of human large intestine, to metabolize acetaldehyde. One strain of each species of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Hafnia alvei* representing the facultative aerobic bacteria of the normal human colonic flora (Finegold *et al.*, 1983) was chosen for the study. The preference to cofactors, the apparent K_m for acetaldehyde and the maximal velocity (V_{max}) of acetaldehyde oxidation in crude extracts were all determined. The second aim was to compare ADH and ALDH activities of the same bacteria under the conditions which are prevalent in the colon after moderate drinking. For this purpose, ADH and ALDH activities from crude extracts of the bacteria were determined with 25 mM ethanol, and with 50 and 200 μM acetaldehyde at pH 7.4. Finally, we studied the effect of cyanamide, a potent mammalian ALDH inhibitor (Deitrich *et al.*, 1976; Cederbaum, 1981), on the ALDH activity of human colonic bacteria *in vitro*.

MATERIALS AND METHODS

Bacteria were obtained from the National Public Health Institute, Helsinki, Finland. Strains were characterized as previously described (see Table 1). Cells were grown on brucella agar plates (BBL, Cockeysville, MD, USA), supplemented with 5% defibrinated sheep blood in air at 35°C for 24 h. The bacterial growth was harvested and first washed three times with 100 mM potassium phosphate buffer (pH 7.4). An aliquot of the bacterial suspension was sonicated five times each for 30 s in an ice bath and then centrifuged at 100000 *g* for 65 min to obtain the crude extract.

ALDH activities in crude extracts were determined spectrophotometrically at 340 nm by measuring, after addition of acetaldehyde, the reduction of NAD$^+$ (final concentration in the reaction mixture 1 mM) or NADP$^+$ (final concentration 1 mM) at 25°C in 60 mM sodium pyrophosphate buffer (pH 8.8) with 10 mM 4-methylpyrazole and

Table 1. Characteristics of the bacterial strains tested

Name	Strain	Isolated from	Reference
Escherichia coli	IH 13369	Urine	Siitonen *et al.* (1993)
Klebsiella pneumoniae	IH 35385	Stool	Siitonen (1992)
Klebsiella oxytoca	IH 35339	Stool	Siitonen (1992)
Hafnia alvei	IH 53227	Minced meat	Ridell *et al.* (1994)
Pseudomonas aeruginosa	IH 35342	Stool	Siitonen (1992)

acetaldehyde concentrations ranging from 6.25 μM to 5 mM.

At neutral pH (7.4) ALDH activities were determined in 0.1 M potassium phosphate buffer with 10 mM 4-methylpyrazole and acetaldehyde concentrations of 50 and 200 μM. Corresponding NAD^+-linked ADH activities (final concentration of NAD^+ 2.5 mM) were determined with 25 mM ethanol at 340 nm at 25°C in the same buffer.

The inhibitory effect of cyanamide (Sigma Chemicals Co., St. Louis, MO, USA) on cytosolic $NADP^+$-linked ALDH activities of *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Escherichia coli* and *Hafnia alvei*, and on NAD^+-linked ALDH activity of *Pseudomonas aeruginosa* were tested using drug concentrations ranging from 0 to 100 mM with 5 mM acetaldehyde and 10 mM 4-methylpyrazole in 60 mM sodium pyrophosphate buffer (pH 8.8).

We have previously shown that four out of five bacteria used in the present study do not possess any $NADP^+$-linked ADH activity. In *Escherichia coli* cytosol, a very low $NADP^+$-linked ADH activity was found, that was nearly 20 times lower than the corresponding NAD^+-linked activity (Nosova *et al.*, 1997). Therefore, $NADP^+$-linked ADH activities in crude extracts of these bacteria could not interfere with the assay of $NADP^+$-linked ALDH activities.

For the NAD^+-linked ADH activities of the bacteria tested, the inhibition constants (K_i) using 4-methylpyrazole were determined, with apparent K_i of 6.4 mM for *Klebsiella oxytoca*, 0.47 mM for *Klebsiella pneumoniae*, 6.6 mM for *Pseudomonas aeruginosa*, and 18.2 mM for *Escherichia coli*. The ADH activity of *Hafnia alvei* was not inhibited by 4-methylpyrazole (Nosova *et al.*, 1997). In a preliminary study, no difference in NAD^+-linked ALDH activity of *Klebsiella oxytoca* and *Pseudomonas aeruginosa* with final 4-methylpyrazole concentrations of 10 and 25 mM were found. Furthermore, no difference in the NAD^+-linked ALDH activity of *Escherichia coli* was found with either 10 or 40 mM 4-methylpyrazole. Accordingly, the 4-methylpyrazole concentration used in the present study seems to be quite sufficient for the inhibition of NAD^+-linked ADH activities of *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*.

The ALDH assay was performed after a reaction time of 300 s and the concentrations of protein in crude extracts were chosen individually for each bacterial strain. Crude extracts were diluted 1:5 in order to obtain linear NAD(P)H formation with time. Protein contents were determined according to Lowry *et al.* (1951), using bovine serum albumin as a standard. ADH and ALDH activities were calculated as nmol of NAD(P)H produced/mg of protein/min, based on an absorption coefficient of 6.22 l/mM/cm at 340 nm.

Results are expressed as means ± SEM of at least three different determinations, unless otherwise stated. Statistical significance of the differences between enzyme activities was evaluated by analysis of variance (ANOVA) and Tukey multiple comparison tests. Values for apparent K_m and V_{max} were determined by Michaelis–Menten plots using a computerized data analysis program (Brooks, 1992).

RESULTS

At alkaline pH (8.8), four out of five bacteria possessed notable $NADP^+$-linked ALDH activity, with the $NADP^+$-linked ALDH activity of *Escherichia coli* being the highest (43 nmol/min/mg of protein at 25 μM acetaldehyde) (Fig. 1). $NADP^+$-linked ALDH activity of *Escherichia coli*

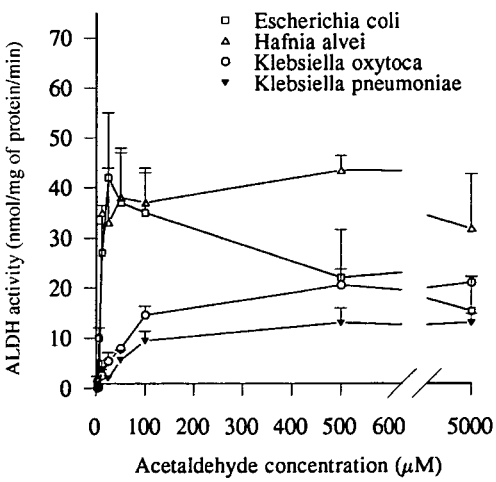

Fig. 1. Effects of acetaldehyde on the cytosolic $NADP^+$-linked aldehyde dehydrogenase (ALDH) activities of the aerobic bacteria representing human colonic flora. Results are means ± SEM of at least three determinations with each bacterium at pH 8.8.

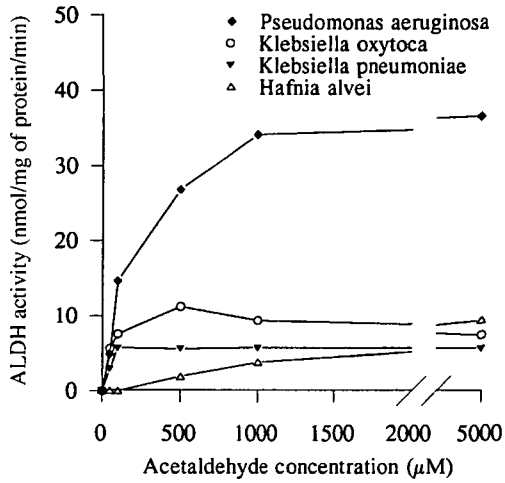

Fig. 2. Effects of acetaldehyde on the cytosolic NAD^+-linked aldehyde dehydrogenase (ALDH) activities of the aerobic bacteria representing normal human colonic flora at pH 8.8.
Results are means of two different determinations with each bacterium.

first increased with increasing acetaldehyde concentration from 3.12 to 25 μM. Thereafter, activity decreased and with 5 mM acetaldehyde ALDH activity was about 35% of that determined with 25 μM acetaldehyde. This may be explained by substrate inhibition of the ALDH activity of *Escherichia coli* by acetaldehyde. *Pseudomonas aeruginosa* did not show any ALDH activity with $NADP^+$ (results not shown).

NAD^+-linked ALDH activity also was found in four out of five bacteria, with NAD^+-linked ALDH activity of *Pseudomonas aeruginosa* being the highest (36.6 nmol/min/mg of protein at 5 mM acetaldehyde) (Fig. 2). However, *Escherichia coli* did not show any NAD^+-linked ALDH activity (data not shown) and ALDH of *Hafnia alvei* was not saturable up to 5 mM acetaldehyde (Fig. 2). The apparent K_m and V_{max} values for acetaldehyde of NAD^+- and $NADP^+$-linked ALDH activities are shown in Table 2.

At neutral pH, significant differences between ADH (with 25 mM ethanol) and ALDH activities (with 50 and 200 μM acetaldehyde) for *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Escherichia coli* ($P < 0.0001$), and for *Pseudomonas aeruginosa* ($P < 0.02$) were observed (Fig. 3). In these bacteria, ADH activities were as much as 10–50 times higher than ALDH activities. In contrast, cytosolic ADH and ALDH activities of *Hafnia alvei* did not differ significantly (Fig. 3). No significant differences in ALDH activity with 50 or 200 μM acetaldehyde were detected in any of the bacteria.

Cyanamide significantly inhibited ($P < 0.05$) only the NAD^+-linked ALDH activity of *Pseudomonas aeruginosa* at concentrations starting from 0.1 mM (Fig. 4). $NADP^+$-linked ALDH activities of the other bacteria were not significantly inhibited by cyanamide up to 100 mM (data not shown).

DISCUSSION

Taken orally, alcohol is rapidly absorbed from the upper gastrointestinal tract and then distributed via blood circulation to the water phase of the large intestine and ethanol concentrations in the colon are equal to those in the blood (Halsted *et al.*, 1973; Levitt *et al.*, 1982). Intracolonic ethanol can be further metabolized both by the mucosal cells of the colorectum and by intracolonic bacteria (Salaspuro, 1996, 1997).

Colonic mucosal ADH activity has been found

Table 2. Apparent K_m and V_{max} values for acetaldehyde determined in crude extracts of some aerobic colonic bacteria

Bacteria		$NADP^+$-linked (mean ± SEM)		NAD^+-linked (mean)	
Name	Strain	K_m (μM)	V_{max} (nmol/min/mg of protein)	K_m (μM)	V_{max} (nmol/min/mg of protein)
Klebsiella oxytoca	IH 35339	67.4 ± 5.3	20.8 ± 1.2	27.8	9
Klebsiella pneumoniae	IH 35385	44.4 ± 11	13.2 ± 1	31.1	6
Escherichia coli	IH 13369	6.8 ± 0.9	29.9 ± 5	0	0
Hafnia alvei	IH 53227	7.1 ± 0.6	32.0 ± 2	n.s.	n.s.
Pseudomonas aeruginosa	IH 35342	0	0	205.1	39

n.s. Denotes not saturated under the experimental conditions of this study.

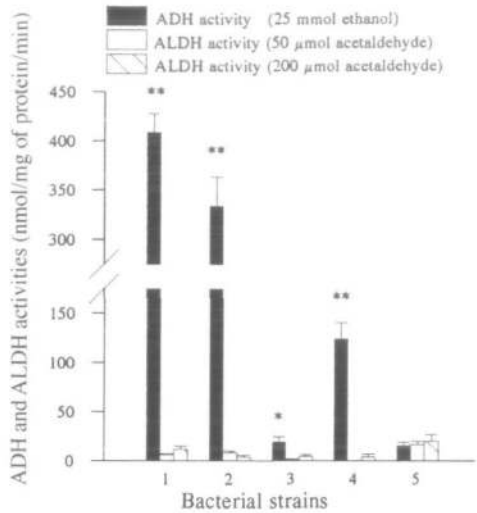

Fig. 3. Cytosolic alcohol dehydrogenase (ADH) activities and corresponding aldehyde dehydrogenase (ALDH) activities of the aerobic bacteria representing human colonic flora determined at pH 7.4.

Results are means ± SEM of at least three determinations. 1, *Klebsiella oxytoca* IH 35339; 2, *Klebsiella pneumoniae* IH 35385 (both $NADP^+$-linked ALDH activity); 3, *Pseudomonas aeruginosa* IH 35342 (NAD^+-linked ALDH activity); 4, *Escherichia coli* IH 13369; and 5, *Hafnia alvei* IH 53227 (both $NADP^+$-linked ALDH activity); $^*P < 0.02$; $^{**}P < 0.0001$ when ADH and ALDH activities are compared in the same bacteria. Differences between ALDH activities with 50 and 200 μM acetaldehyde were not significant for all tested bacteria.

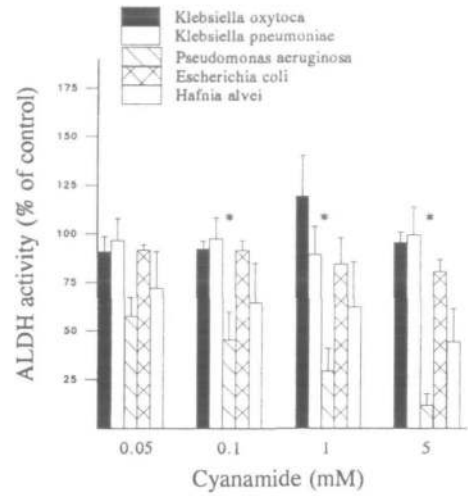

Fig. 4. Inhibition of cytosolic aldehyde dehydrogenase (ALDH) activities of the bacteria representing human colonic flora by cyanamide.

ALDH activity was determined with 5 mM acetaldehyde at pH 8.8. Results are means ± SEM of at least three different determinations of two bacterial growths. $^*P < 0.05$ when compared with control.

in humans and rodents (Pestalozzi *et al.*, 1983; Yin *et al.*, 1994), but it is markedly lower than hepatic ADH activity (Yin *et al.*, 1994; Koivisto and Salaspuro, 1996). An enhanced ability of human rectal mucosa to produce acetaldehyde, in comparison with colonic mucosa has been recently reported (Seitz *et al.*, 1996).

The large intestine is the most richly colonized site of the digestive tract. More than 400 different bacterial species and 10^{14} individual bacteria inhabit a single human colon at a given time (Luckey, 1977). The colonic flora can be considered as an important organ, with a flexibility and potential for metabolic transformations at least as great as that of the liver (Bingham, 1988) or even exceeding that of the whole human body (Cummings, 1983).

The results of our study demonstrate that ALDHs of the crude extracts of aerobic colonic bacteria possess variable apparent K_m values for acetaldehyde ranging from 6.8 ± 0.9 μM ($NADP^+$-linked activity of *Escherichia coli*) to 205 μM (NAD^+-linked activity of *Pseudomonas aeruginosa*). Accordingly, these ALDHs are able to metabolize colonic endogenous acetaldehyde at concentrations of up to 120 μM (Jokelainen *et al.*, 1996*a*), with a velocity close to the maximum. The ability of ALDHs to metabolize higher intracolonic acetaldehyde levels (Jokelainen *et al.*, 1996*a*) associated with alcohol drinking is rather low.

At near-physiological conditions, i.e. at neutral pH, and at ethanol and acetaldehyde concentrations that may prevail in the colon after moderate drinking (Halsted *et al.*, 1973; Jokelainen *et al.*, 1996*a*), ALDH activities (both at 50 and 200 μM acetaldehyde) in four out of five bacterial strains were significantly lower than the corresponding ADH activities. Therefore, the large differences in ADH and ALDH activities found in our study may explain, at least in part, the intracolonic accumulation of acetaldehyde found previously *in vitro* and *in vivo* (Jokelainen *et al.*, 1996*a*,*b*).

The results of our study demonstrate that cyanamide only inhibited the NAD^+-linked

ALDH activity of *Pseudomonas aeruginosa*, starting from decimolar cyanamide concentrations. This is a hundred times higher than the cyanamide concentration that inhibits the ALDH activity of the liver homogenates of rodents and dogs *in vitro* (Sanny and Rymas, 1993). Thus, this classic mammalian ALDH inhibitor is not effective in the case of bacterial ALDHs. Cyanamide is currently used as a drug for the treatment of alcoholism (Ferguson, 1956) in amounts of 35–50 μM. None of the bacterial ALDHs in our study could be significantly inhibited by these concentrations and, accordingly, therapeutic doses of cyanamide are unlikely to be associated with increased intracolonic endogenous acetaldehyde levels *in vivo*.

The anaerobic conditions prevailing in the colon may not, in general, favour the oxidation of ethanol and acetaldehyde by the bacterial enzymes. However, due to the diffusion of oxygen from the colonic mucosa, the mucosa-associated flora may contain even more aerobes than anaerobes (Hill, 1995). Therefore, the higher oxygen tension of the colonic mucosal surface and its flora may favour the oxidation of acetaldehyde to acetate in the large intestine *in vivo*.

Aldehyde oxidases can also use molecular oxygen as an electron acceptor and are therefore able to oxidize acetaldehyde *in vitro* (Johns, 1967; Shaw and Jayatilleke, 1990) with the apparent K_m for acetaldehyde determined to be 1 mM (Shaw and Jayatilleke, 1990). The physiological role of aldehyde oxidases is considered to be that of catalysing the oxidation of nitrogen-containing heterocyclic compounds (Beedham *et al.*, 1990), certain drugs, and xenobiotics (Clarke *et al.*, 1995). Hepatic aldehyde oxidases do not actively participate in the metabolism of acetaldehyde arising from the oxidation of ethanol (Richert and Westerfeld, 1957).

We have not found data about the presence of aldehyde oxidases in aerobic colonic bacteria in the current literature. However, because of the rather high K_m for acetaldehyde, bacterial aldehyde oxidases, if present, are unlikely to participate *in vivo* in the removal of intracolonic acetaldehyde resulting from ethanol oxidation.

Data concerning the inducibility of ADH and ALDH activities in various species and tissues is quite variable. It is commonly agreed that hepatic ADH activity is not induced by chronic alcohol consumption (Nuutinen *et al.*, 1983; Vidal *et al.*, 1990). However, the ADH activities of *Acetobacter pasteurianus* (Takemura *et al.*, 1993) and *Drosophila melanogaster* (Geer *et al.*, 1988) were enhanced *in vitro* by the addition of ethanol to the growth medium. Cytosolic ALDH, inducible by xenobiotics, has been isolated from rat liver (Lindahl and Evces, 1984; Pappas *et al.*, 1995). However, chronic alcohol treatment resulting in increased hepatic or blood acetaldehyde levels has been shown to decrease hepatic mitochondrial ALDH activity (Lebsack *et al.*, 1981; Nuutinen *et al.*, 1983) and either to increase (Tomita *et al.*, 1992) or to decrease (Nuutinen *et al.*, 1983) cytosolic ALDH activity.

It is still to be established how the increased levels of ethanol and acetaldehyde resulting from chronic alcohol consumption influence the ADH and ALDH activities of colonic bacteria. A study investigating the possible induction of bacterial ADH activity by excess ethanol is currently in progress in our laboratory.

In conclusion, ALDHs of the aerobic colonic bacteria, according to their apparent K_m values, are able to metabolize endogenous acetaldehyde. However, the ability of bacterial ALDHs to oxidize the higher concentrations of intracolonic acetaldehyde associated with alcohol consumption is rather low. At ethanol and acetaldehyde concentrations found in the colon after moderate drinking, the ALDH activities of most of the bacteria were markedly lower than the ADH activities. These findings offer an additional explanation for the mechanism of the accumulation of ethanol-derived acetaldehyde in the colon after ethanol intake. The ALDH activities of colonic bacteria are poorly inhibited by cyanamide at concentrations that are used in the treatment of alcoholism. Individual variations in human colonic flora may contribute to the accumulation of intracolonic acetaldehyde and to the risk of alcohol-related gastrointestinal morbidity, such as diarrhoea, colonic polyps and cancer, and liver injury.

Acknowledgements — This work was supported financially by grants from the Yrjö Jahnsson Foundation, the Finnish Foundation for Gastroenterological Research, the Finnish-Norwegian Foundation for Medicine, the Finnish Foundation for Alcohol Studies, and from the Helsinki University Central Hospital Research Funds. The authors also thank Dr

Anja Siitonen, National Public Health Institute, for providing the bacterial strains.

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