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Rapid Screening Method for Analyzing the Conjugated Linoleic Acid Production Capabilities of Bacterial Cultures▽

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In this paper we describe a rapid method for identifying bacteria which convert free linoleic acid to conjugated linoleic acid (CLA). This method is based on spectrophotometric detection of CLA and compares well with the standard gas-liquid chromatography method. This method should facilitate high-throughput screening of bacterial isolates for the ability to produce conjugated fatty acids.

Conjugated linoleic acid (CLA), a natural component of ruminant milk and tissue fat, is a mixture of positional and geometric conjugated isomers of the essential fatty acid linoleic acid. In recent years, there has been considerable interest in these biogenic isomers due to their potential health-promoting properties and the proposed positive effects that they have on many aspects of human health, most notably their anticarcinogenic, immune modulation, antiatherosclerotic, and antiobesity activities (3, 4, 8, 10, 14–16, 23–25). In addition to the increased interest in the possible physiological effects on humans following CLA consumption, there has been a concomitant increase in interest in the isolation of novel human-derived or dairy starter cultures with the ability to produce the bioactive fatty acid (1, 7, 12, 13, 17, 19, 21). Indeed, bacterial strains belonging to a few genera, such as *Lactobacillus*, *Propionibacterium*, and *Bifidobacterium*, have been reported to produce CLA in either synthetic media or milk (21); however, the ability to produce CLA can vary from strain to strain. The standard gas-liquid chromatography (GLC)-based screening process is laborious and time-consuming and can be a limiting factor when a large number of strains are to be tested. In this study we describe the use of a simple and straightforward spectrophotometric method for screening a large number of culture supernatants for CLA production; this method eliminates the need for GLC during the screening process.

Fifty-eight fecal samples were obtained from a diverse population. Twenty-eight of the samples were obtained from healthy full-term neonates who were 2 to 5 days old. The remaining samples were obtained from adults, 20 of whom were elderly patients infected with the bacterium *Clostridium difficile*. Following sampling, fecal samples were stored at 5°C and were processed in the laboratory within 5 h. Swabs were mixed by vortexing them in maximum-recovery diluents (Oxoid, Ltd., Hampshire, United Kingdom), serially diluted, spread plated on the appropriate medium, and incubated as described below. Fifteen colonies were randomly selected from each fecal sample and were screened spectrophotometrically at a wavelength of 233 nm for CLA production following incubation in medium containing linoleic acid (see below).

Serial dilutions of fecal samples were spread plated onto modified MRS (mMRS) (Difco Laboratories, Detroit, MI) medium supplemented with 0.05% (wt/vol) cysteine hydrochloride (Sigma, St. Louis, MO), 1% (wt/vol) agar (Oxoid), and 100 μg/ml of mupirocin (Oxoid) added as antimicrobial susceptibility disks as previously described (18) to preselect for bifidobacteria. The agar plates were incubated anaerobically (anaerobic jars with Anaerocult A gas packs [Merck, Darmstadt, Germany]) at 37°C for 5 days.

Linoleic acid and the internal standard tridecanoic acid were obtained from Sigma-Aldrich (St. Louis, MO). Isomers of CLA (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) were obtained from Matreya Inc. (Pennsylvania). All other chemicals were obtained from Sigma-Aldrich or Labscan (Dublin, Ireland).

Fecal isolates were incubated anaerobically in mMRS broth containing free linoleic acid (0.5 mg/ml) and 2% (wt/vol) Tween 80 at 37°C for 48 h to determine the ability of strains to convert linoleic acid to CLA, using a modification of a previously described method (17). Following incubation, 1 ml of a culture was centrifuged at 20,800 × *g* for 1 min, the pellet was discarded, and the supernatant was mixed with 2 ml of isopropanol by vortexing and allowed to stand for 3 min. The fatty acids were extracted by vortexing the solution and allowing it to stand for 3 min following the addition of 1.5 ml of hexane. The presence of CLA in the culture supernatant was assayed spectrophotometrically by dispensing 230 μl of the fat-soluble hexane layer into a UV-transparent 96-well plate (Costar, Corning, NY) and determining the absorbance at 233 nm using a 96-well plate spectrophotometer (GENios Plus; Tecan, Medford, MA).

The activity of fructose-6-phosphate phosphoketolase (F-6-PPK), an enzyme that indicates bifidobacterial carbohydrate metabolism, of each CLA-producing isolate was determined based on an assay described previously (5). 16S rRNA gene sequencing was performed by previously described methods (20, 22). An approximately 1.5-kb 16S rRNA gene fragment was generated using two 16S rRNA gene primers, CO1 (5′-AGTTTGATCCTGGCTCAG-3′) for the 5′ end and CO2 (5′-TACCTTGTTACGACT-3′) for the 3′ end or, alternatively, Im26-f (5′-GATTCTGGCTCAGGATGAACG-3′) for the 5′

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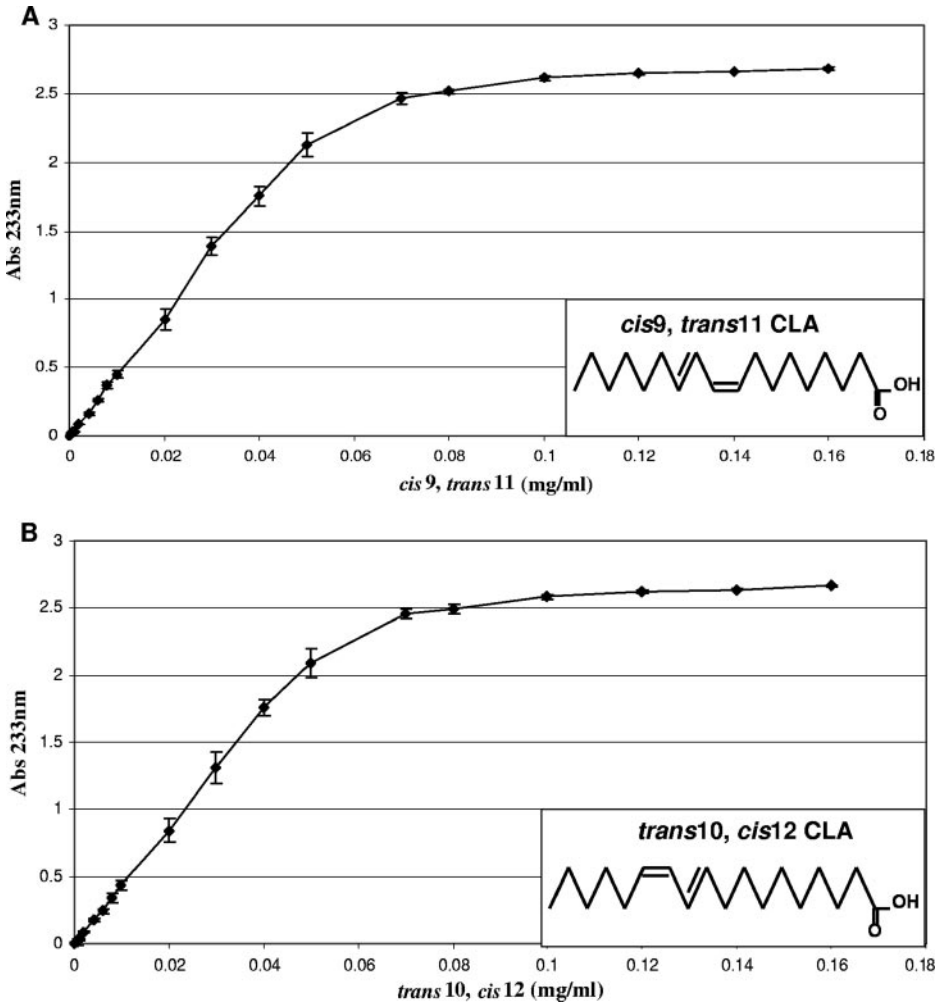

FIG. 1. Standard curves for the absorbance of the fat-soluble hexane layer versus CLA concentration. (A) Absorbance at 233 nm versus concentration of the *cis*-9,*trans*-11 CLA isomer. The chemical structure of the *cis*-9,*trans*-11 CLA isomer is also shown. (B) Absorbance at 233 nm versus concentration of the *trans*-10,*cis*-12 CLA isomer. The chemical structure of the *trans*-10,*cis*-12 CLA isomer is also shown.

end and Im3-r (5′-CGGGTGCTCCCACTTTCATG-3′) for the 3′ end. Alternatively, a segment of the gene coding for the 60-kDa heat shock protein HSP60 was used to characterize the strains (11). DNA sequencing was performed by Lark Technologies (United Kingdom). Strains were assigned to species following comparison of the 16S rRNA gene and HSP60 sequences using the BLAST program (2). The species identification results obtained for strains using the 16S rRNA gene and HSP60 sequences were confirmed using a modification of a previously described species-specific primer identification method for bifidobacteria (9). The only modification involved using an annealing temperature of 70°C when both primer mixtures were used.

High-molecular-weight chromosomal DNA was isolated from stationary-phase cultures by a previously described method (22). Restriction enzyme XbaI (New England Biolabs) was used to cleave chromosomal DNA, and the fragments were separated using a contour-clamped homogeneous electric field CHEF-DR III pulsed-field system (Bio-Rad Laboratories). Fragments were resolved with a linear ramp pulse time of 1 to 15 s for 18 h at 6 V/cm in a running buffer containing 0.5× Tris base-borate-EDTA maintained at 12°C. DNA fragment sizes were estimated by comparison with the lambda ladder PFG marker (New England Biolabs).

The ability of isolates to convert free linoleic acid to CLA was assayed by incubating cultures in mMRS broth containing free linoleic acid (0.5 mg/ml) at 37°C for 72 h and subsequently assessing the fatty acid profile of the culture supernatant by GLC as previously described (7).

The rapid screening method employed in this study involved the use of a UV-transparent and colorless 96-well plate to detect CLA production by bacterial cultures at a wavelength of 233 nm. In order to verify the suitability of this method, a standard curve was constructed for the absorbance at 233 nm versus the CLA concentration (Fig. 1), using pure *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers. The graph demonstrated that an increase in the CLA concentration (from 0 to 0.05 mg/ml) coincided with a linear increase ($R^2 = 0.9985$) in absorbance for the *cis*-9,*trans*-11 CLA isomer up to an absorbance of 2.1. Therefore, the CLA concentrations in culture supernatants with an absorbance at 233 nm less than or equal to 2.1 could be calculated from the linear trend line of the

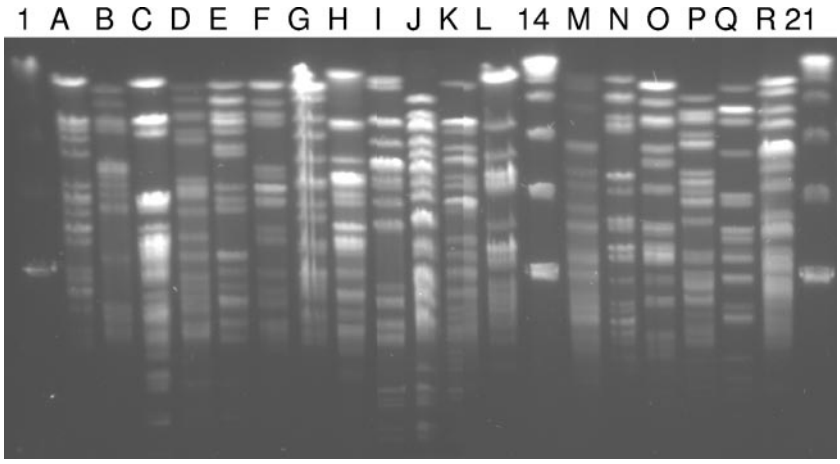

FIG. 2. PFGE macrorestriction patterns following genomic DNA digestion with restriction enzyme XbaI. Lanes A through L, PFGE types A to L, respectively; lanes M through R, PFGE types M to R, respectively. Lanes 1, 14, and 21 contained molecular size markers (lambda ladder PFG marker).

standard curve using the equation $y = 43.431x + 0.0053$. The standard curve constructed using the *trans*-10,*cis*-12 CLA isomer displayed similar results (Fig. 1). The assay was extremely sensitive and could detect *cis*-9,*trans*-11 CLA isomer concentrations as low as 0.002 mg/ml in the hexane layer with a corresponding absorbance at 233 nm of 0.0871 ± 0.004.

In order to determine whether this method could be used to test a large number of strains, 15 isolates were randomly selected from each of the 58 fecal samples. All of these isolates were then screened spectrophotometrically for CLA production following growth in the presence of linoleic acid. Using this approach, a total of 88 CLA-producing isolates (~10% of the 870 isolates screened) were identified for 15 of the 58 fecal samples, while no CLA-producing isolates were detected in the remaining 43 samples using the 96-well plate assay. Pulsed-field gel electrophoresis (PFGE) was performed for all of the CLA-producing isolates following genomic restriction with the enzyme XbaI, and a total of 18 distinct CLA-producing strains were identified among the 88 initial isolates (Fig. 2). Subsequently, each strain was identified to the genus and species levels by partial sequencing of either the 60-kDa heat shock protein HSP60 or the 16S rRNA gene (Table 1). All of the CLA-producing bacteria exhibited levels of homology of ≥97% with different species belonging to the genus *Bifidobacterium*, and all of them were found to be F-6-PPK positive, a characteristic biomarker for the genus (Table 1). Nine of the CLA-producing strains belonged to *B. longum*, six strains belonged to *B. breve*, and a single strain each belonged to *B. infantis*, *B. dentium*, and *B. catenulatum*. These results were

TABLE 1. Description, PFGE patterns, 16S rRNA gene and HSP60 partial sequencing, F-6-PPK detection, subject age, and CLA conversion by the strains

% Conversion	A_{233}	PFGE type	16S rRNA gene-HSP60 partial sequencing and *Bifidobacterium* species-specific PCR identification	F-6-PPK assay	% Homology	Age of donor	Sample(s)	Subject description
76.65 ± 1.75	2.39	A	*B. breve*	Positive	98	81 yr	1, 3, 43	*C. difficile* positive
61.12 ± 3.85	2.38	C	*B. breve*	Positive	99	50 yr	16	*C. difficile* positive
60.12 ± 5.14	2.40	E	*B. longum*	Positive	99	25 yr	22	Healthy adult
53.08 ± 2.51	2.38	B	*B. longum*	Positive	99	64 yr	7	*C. difficile* positive
44.65 ± 2.57	2.41	H	*B. breve*	Positive	99	63 yr	26	*C. difficile* positive
38.50 ± 0.96	2.41	I	*B. longum*	Positive	99	3 days	30	Healthy baby
27.20 ± 8.81	2.37	G	*B. breve*	Positive	97	73 yr	23	*C. difficile* positive
21.53 ± 2.37	2.30	O	*B. breve*	Positive	99	81 yr	43	*C. difficile* positive
20.16 ± 3.77	2.27	J	*B. longum*	Positive	100	3 days	30, 35	Healthy baby
18.66 ± 0.26	2.27	K	*B. longum*	Positive	99	5 days	34	Healthy baby
18.11 ± 1.40	2.23	D	*B. infantis*	Positive	98	31 yr	17	Healthy adult
12.55 ± 0.42	2.05	L	*B. dentium*	Positive	99	4 days	35	Healthy baby
6.31 ± 0.92	1.19	R	*B. longum*	Positive	100	4 days	52	Healthy baby
5.24 ± 4.79	1.42	N	*B. longum*	Positive	99	4 days	36	Healthy baby
4.12 ± 0.68	0.86	P	*B. breve*	Positive	99	81 yr	43	*C. difficile* positive
3.98 ± 2.64	0.54	F	*B. longum*	Positive	99	25 yr	22	Healthy adult
3.68 ± 0.41	0.57	Q	*B. longum*	Positive	100	37 yr	47	Healthy adult
2.60 ± 1.00	0.55	M	*B. pseudocatenulatum*	Positive	99	4 days	36	Healthy baby

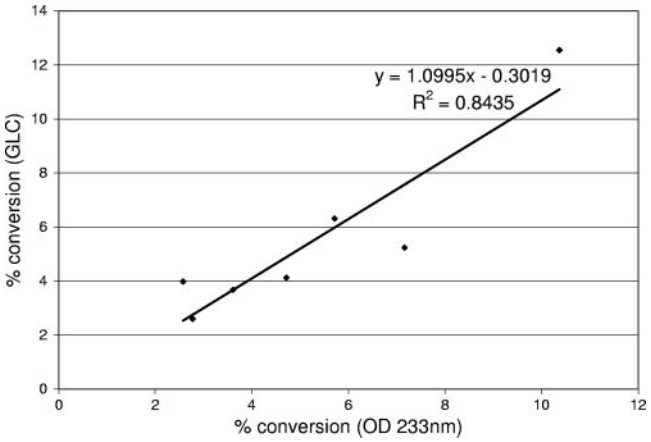

FIG. 3. Plot of CLA conversion as determined by GLC versus CLA conversion calculated from the standard curve for bacterial samples with an absorbance at 233 nm (OD 233nm) of less than 2.1.

confirmed by *Bifidobacterium* species-specific PCRs using 16S rRNA gene-based primers (data not shown).

In addition to the standard curve, the suitability of the 96-well plate assay for determining the CLA content of a culture broth was compared with the suitability of GLC. The percentage of linoleic acid converted to CLA by each individual stain, as determined by GLC, was compared with the absorbance at 233 nm obtained by analysis of the hexane layer during the initial step of fatty acid extraction prior to drying under nitrogen, and the data are shown in Table 1. Strains are listed in Table 1 in descending order based on the percentage of linoleic acid converted to CLA. The CLA conversion values for the seven strains with an absorbance at 233 nm of less than 2.2 (Table 1) were calculated from the linear trend line equation ($y = 43.431x + 0.0053$) of the standard curve for the *cis*-9,*trans*-11 CLA isomer. The conversion values calculated from the standard curve were plotted against the conversion values determined by GLC (Fig. 3).

In all cases, the *cis*-9,*trans*-11 CLA isomer was the predominant isomer in the culture supernatant; the *trans*-9,*trans*-11 CLA isomer was also generated, however, at much lower levels, as determined by GLC (data not shown). Interestingly, we have recently shown that the latter isomer also has very potent antiproliferative activity against a human colonic cancer cell line (6). A number of non-CLA-producing bacteria were also assayed to determine their CLA production; using both GLC and the spectrophotometric assay, no CLA production was detected in these strains (data not shown). Importantly, it should be emphasized that the spectrophotometric method does not distinguish between isomers of CLA since it is based on measurement of the conjugated double bond in the fatty acid.

Altogether, nine strains converted more than 20% of the linoleic acid to CLA in the culture supernatant, and one of these strains converted more than 75% of the linoleic acid to CLA. Five of the CLA-producing strains which converted more than 20% of the linoleic acid to CLA belong to the species *B. breve*, and the remaining four strains were identified as *B. longum* strains.

The source of the CLA-producing bifidobacteria in this study was a further point of interest. In previous studies workers have isolated CLA-producing bifidobacteria from the fecal material of neonates (17, 19); however, the best source of CLA-producing bifidobacteria in this study, in terms of both conversion of linoleic acid to CLA and prevalence of CLA-producing strains within a group, was the fecal material of elderly patients infected with the bacterium *C. difficile*. In this study only 5 of the 28 healthy infants screened for CLA-producing bifidobacteria harbored CLA-producing strains, while 7 of the 20 *C. difficile* patients screened harbored CLA-producing strains and 3 of the 10 healthy adults harbored CLA-producing bifidobacteria. Indeed, in the diverse population screened in this study, one in four subjects harbored a CLA-producing *Bifidobacterium* strain.

Currently, there is a need in intestinal microbiology to develop high-throughput methods to identify specific bioactivities in certain subpopulations. Therefore, a high-throughput spectrophotometric method for analyzing the CLA production capabilities of gut microfloras was developed. This method allows rapid screening of large numbers of bacterial isolates for CLA production. Using the method which we developed, in this study the prevalence and diversity of CLA-producing *Bifidobacterium* species in the gastrointestinal tracts of human subjects were highlighted.

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