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Role of Branched-Chain Fatty Acids in pH Stress Tolerance in *Listeria monocytogenes*▽

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In alkaline conditions, *Listeria monocytogenes* cells develop higher proportions of branched-chain fatty acids (FAs), including more anteiso forms. In acid conditions, the opposite occurs. Reduced growth of pH-sensitive mutants at adverse pH (5.0/9.0) was alleviated by the addition of 2-methylbutyrate (an anteiso-FA precursor), suggesting that anteiso-FAs are important in adaptation to adverse pH. The balance between anteiso- and iso-FAs may be more important than changes in the amounts and/or degrees of saturation of FAs in pH adaptation.

Listeria monocytogenes can grow under a wide range of pH stress, i.e., 4.1 to 9.0 (38, 46), increasing its abilities to persist during food processing and attempts to decontaminate food-processing environments (15, 42–45). It also has particularly impressive capacities to modulate its membrane lipids to maintain membrane fluidity and transport functions (10, 40, 41) in response to temperature (1), salt (7), and CO_2/anaerobiotic (21) stress. Such capacities have been suggested to be related to its atypically high iso and anteiso, odd-numbered, branched-chain fatty acid (BCFA) content (1, 20) and its ability to modulate the overall content and proportions of BCFAs, straight-chain FAs (SCFAs), and unsaturated FAs (22, 23). For example, reductions in environmental temperatures lead to increases in the amount of ai15:0 present in *L. monocytogenes* cell membranes, while increases in environmental temperatures lead to reductions in the amounts of ai15:0 and other BCFAs present in membranes (1, 14, 32).

Changes in FA profile have been associated with pH adaptation in *Streptococcus mutans* (16–18), *Escherichia coli* (5, 48), and *Salmonella* (24), *Pseudomonas* (31), and *Bacillus* species (23). However, little is known about pH stress-associated modulation of FAs in *L. monocytogenes* (21), the wider role of FA modulation in its responses to nonthermal stresses, or the cross-protection mechanisms which operate in this hardy pathogen (19, 27, 35, 44).

The aims of this study were to investigate the modulation of the FA profile of *L. monocytogenes* membranes in response to changes in environmental pH, investigate the effects of an exogenous BCFA precursor on the pH stress response of BCFA-deficient mutants (1, 49), and examine possible links between the prevalence of anteiso-BCFAs and the adaptation mechanism(s) of *L. monocytogenes* under adverse pH conditions.

Modified brain heart infusion broth (MBHIB; Difco Laboratories, Sparks, MD), suitable for adverse-pH studies, was prepared to pH 5.0, 5.5, and 6.0 in 2 M disodium phosphate (Sigma Chemical Company, St. Louis, MO) and 0.1 M citric acid (Fisher Scientific, Fair Lawn, NJ) buffer or to pH 7.0, 8.0, 8.5, and 9.0 in 0.1 M solutions of Trizma-hydrochloride and Trizma-base (Sigma) buffer (6). When required, cultures were supplemented with filter-sterilized 100 μM 2-methylbutyric acid (2MBA).

Washed cells from mid-exponential-phase cultures of *L. monocytogenes* 10403S, an isogenic *sigB* null mutant (3), and isogenic non-BCFA-producing *cld-1* and *cld-2* mutants (1, 49) were inoculated into preheated (30°C) 100-ml volumes of the buffered MBHIB and grown (30°C/200 rpm) to an optical density at 600 nm of 0.5 to 0.6. Growth rates of cultures (doubling times per hour of cultures in exponential growth) were calculated (2). Mid-exponential-phase cells were recovered by centrifugation at 8,000 × *g* for 10 min at 4°C and washed three times with distilled water.

The FAs in washed-cell pellets were saponified, methylated, and extracted as described previously (1, 49, 50). Methyl ester mixtures were separated using an Agilent 5890 dual-tower gas chromatograph with split/splitless injector, flame ionization detector, 25-m by 0.2-mm Ultra 2 capillary column (Hewlett-Packard), and automatic sampler/integrator and analyzed using an FA identification program (MIDI; Sherlock 4.5 Microbial Identification System). Carrier gas (hydrogen) flow was 80 ml/min. The injector and temperatures were maintained at 250 and 300°C, respectively. Samples (2 ml) were injected into the split mode (ratio, 5:3), and the column temperature was ramped from 170 to 270°C at 5°C/min. Individual FAs comprising less than 1% of the FA content were ignored. FA determination was conducted at Microbial ID Inc. (Newark, DE).

All results represent the average means from three independent experiments. Student's *t* test was used to make pairwise comparisons between the acid- and alkaline-adapted cultures and the corresponding controls for each condition tested. The confidence interval for a difference in the mean was set at 95% ($P \leq 0.05$) for all comparisons.

The study established that all samples in all pH conditions contained ai15:0 > ai17:0 > i15:0 > i17:0. Incubation at different pH values induced characteristic and consistent changes in the relative proportions of the above-mentioned major FAs (Table

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TABLE 1. Effects of growth pH on the total fatty acid composition of *L. monocytogenes* 10403S

Growth pH	Buffer	% Total fatty acids (mean ± SD)[a]					ACCL[c] (mean ± SD)
		Anteiso	Iso	Anteiso/iso	Straight	Branched	
7.0	Tris	75.16 ± 3.63	20.2 ± 4.72	3.86 ± 0.95	2.85 ± 0.14	95.37 ± 1.09	15.46 ± 0.16
8.0	Tris	80.77[b] ± 0.26	14.8[b] ± 0.59	5.44[b] ± 0.23	2.04[b] ± 0.21	95.64 ± 0.35	15.39 ± 0.08
8.5	Tris	84.30[b] ± 0.39	12.6[b] ± 0.43	6.69[b] ± 0.22	1.55[b] ± 0.33	96.91[b] ± 0.66	15.52 ± 0.01
9.0	Tris	77.17 ± 0.41	20.8 ± 0.58	3.70 ± 0.12	1.38[b] ± 0.07	98.04[b] ± 0.16	15.64 ± 0.03
7.0	Phosphate	76.69 ± 0.39	21.0 ± 0.36	3.62 ± 0.06	2.13 ± 0.61	97.86 ± 0.61	15.77 ± 0.02
6.0	Phosphate	71.97[b] ± 1.18	25[b] ± 1.29	2.93[b] ± 0.19	3.22[b] ± 0.48	96.56 ± 0.58	15.75 ± 0.02
5.5	Phosphate	72.55[b] ± 0.74	24[b] ± 0.58	3.08[b] ± 0.10	3.44[b] ± 0.16	96.06[b] ± 0.15	15.73 ± 0.03
5.0	Phosphate	80.24[b] ± 0.68	16[b] ± 0.12	5.10[b] ± 0.08	3.02 ± 0.37	95.97 ± 0.55	15.68 ± 0.04

[a] Values are from three independent experiments.
[b] Statistically different ($P < 0.05$) from the control cultures (pH 7.0).
[c] ACCL, average carbon chain length, given by the equation $[\Sigma(\text{FAP} \times C)]/100$, where FAP is the percentage of each fatty acid and C is the number of carbon atoms in the chain.

1); i.e., higher-pH cultures had higher proportions of BCFAs and lower-pH cultures had lower proportions of BCFAs. Figure 1 presents the relative amounts of individual major BCFAs (i15:0, ai15:0, i17:0, and ai17:0) in cells grown at different pH values. Percentages of ai15:0 ranged from 48% (at pH 7.0) to 53% (at pH 8.0 and 8.5) to 47% (at pH 9.0) (Fig. 1A), a pattern also observed for the other three BCFAs examined (Fig. 1B). Cultures grown at pH 8.0 and pH 8.5 (but not at pH 9.0) had higher anteiso/iso ratios than those in control (pH 7.0) samples (Table 1).

FAs in cells grown at pH 5.0 contained significantly higher proportions of ai17:0 (5%) and significantly lower proportions of i15:0 than FAs from pH 7.0 (control) cells (Fig. 1A). This pattern was also observed in relation to total anteiso-FAs; i.e., they were in significantly lower concentrations at pH 6.0 and 5.5. At pH 5.0, the total anteiso-FA content was significantly higher than the control values, and the total iso-FA content was significantly lower ($P < 0.05$) (Table 1). There were no significant differences ($P > 0.05$) among the average lengths (or degrees of saturation) of test and control samples at all pH values examined. There were no significant differences between the results for *L. monocytogenes* 10403S and the isogenic *sigB* null mutant (data not shown).

In the absence of 2MBA, BCFA-deficient mutants (*cld-1* and *cld-2* mutants) grew significantly more slowly (0.28 h^{-1} and 0.20 h^{-1} at pH 9.0 and 0.21 h^{-1} and 0.26 h^{-1} at pH 5.0, respectively) than the parent strain (0.38 h^{-1} at pH 9.0 and 0.47 h^{-1} at pH 5.0) (Fig. 2A and B). In the presence of 2MBA, the growth rates of these BCFA-deficient mutants were almost identical with those of the parent strain.

In the absence of 2MBA at pH 5 or 9, the proportions of anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$ content were lower (Table 2) (and the proportions of iso-$C_{14:0}$, $C_{14:0}$, iso-$C_{16:0}$, and $C_{16:0}$ higher [results not shown]) than in the parent strain. In the presence of 2MBA, the proportions of anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$ were significantly higher than in the absence of 2MBA. In the presence of 2MBA, the proportions of SCFAs were significantly lower (and the proportions of BCFAs were significantly higher) than in the absence of 2MBA.

This study observed that *L. monocytogenes* 10403S exhibited qualitatively and quantitatively different membrane FA contents at different pH values. Growth at pH 8.0 or 8.5 resulted in higher proportions of BCFAs, especially anteiso forms, changes that have been suggested to increase membrane fluidity (41) and limit alkali and detergent damage (30, 33, 34). Increased BCFA content has been associated with alkali tolerance in alkalophilic species (9, 47). Growth at pH 5.5 or 6.0

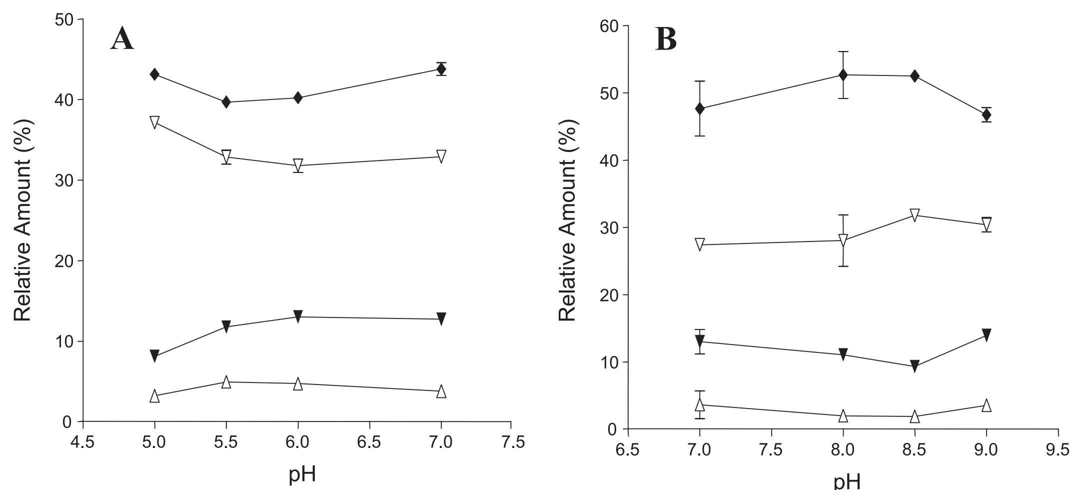

FIG. 1. Effect of growth pH on fatty acid composition of *L. monocytogenes* 10403S cells grown in BHI broth at acid (A) and alkaline (B) pHs. The data represent the means ± standard deviations of three independent experiments. ▼, $iso_{15:0}$; ◆, $anteiso_{15:0}$; △, $iso_{17:0}$; ▽, $anteiso_{17:0}$.

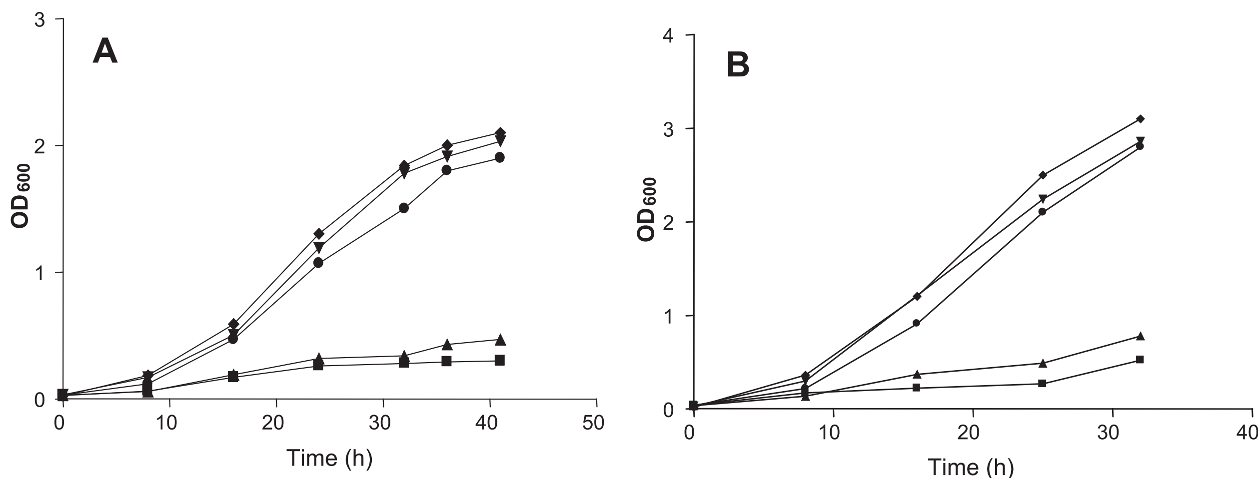

FIG. 2. Influence of 2-methylbutyric acid on growth of the *cld* mutants at pH 5.0 (A) and pH 9.0 (B). Cultures were grown at 30°C in the absence and presence of 2MBA in MBHIB. OD_{600}, optical density at 600 nm; ◆, 10403S; ■, *cld-1* mutant; ●, *cld-1* mutant in the presence of 2MBA; ▲, *cld-2* mutant; ▼, *cld-2* mutant in the presence of 2MBA.

resulted in higher proportions of SCFAs. This is different from the patterns of change in other organisms, where such conditions induce increases in monounsaturated long-chain FAs (5, 16–18) and alterations in cyclopropane FA content (11, 39).

pH stress did not induce gross changes in the total amounts of unsaturated FAs. This is interesting because pH stress induces considerable changes in total unsaturated FA content in other bacteria, and *L. monocytogenes* does make such changes in response to other environmental stresses (4, 13, 25, 26, 29, 32, 49). The absence of significant differences between the responses of *L. monocytogenes* 10403S and the isogenic *sigB* null mutant suggests that *sigB* does not have a major role in pH-induced fatty acid modulation in *L. monocytogenes*.

The study observed clear and different patterns in *L. monocytogenes* responses to moderate pH stress (pH 5.5, 6.0, 8.0, or 8.5), although these patterns were not observed at the most extreme pH values studied (i.e., pH 5.0 and pH 9.0). Such discontinuity may reflect a general disruption of membrane fluidity as conditions move beyond the range of compensation of FA modulation-based homeostasis to a state where the imperative is to maintain cytoplasmic pH. Alternatively, more-extreme stress may activate one or more additional "extreme" stress responses, redirecting cellular investment away from moderate stress adaptation mechanisms to more drastic emergency responses.

Shifts between moderate and extreme stress responses have been associated with shifts between metabolic pathways and changes in the concentrations of key enzymes such as β-keto-acyl-acyl carrier protein synthases (32). In *L. monocytogenes* (49, 50) (and also in *Bacillus subtilis* [12]), such enzymes and their products have vital roles in membrane adaptation to high- and low-temperature stress. BCFA synthesis involves the transamination of branched-chain amino acids such as isoleucine, valine, and leucine by a branched-chain amino acid transaminase (BcaT) (12, 23) and subsequent oxidative decarboxylation by the branched-chain α-keto acid dehydrogenase (Bkd) (8, 28, 36, 37). Thus, these two enzymes, BcaT and Bkd, are critical for BCFA biosynthesis in *L. monocytogenes* and

TABLE 2. Fatty acid compositions of parent strain 10403S and *cld-1* and *cld-2* Bkd mutants in BHIB with or without 2-methylbutyrate at 30°C[a]

Strain and growth conditions	% Total fatty acids[b]									ACCL[d]
	i15:0	ai15:0	i17:0	ai17:0	Ante	Iso	Ante/iso	Straight-chain	Branched-chain	
10403S; pH 9.0	13.94	46.73	3.53	30.4	77.17	20.87	3.70	1.463	98.04	15.680
cld-1; pH 9.0	8.10[c]	27.17[c]	5.36[c]	9.11[c]	39.33[c]	29.81[c]	1.32[c]	30.85[c]	69.14[c]	16.279
clc-1; pH 9.0, 2MBA	1.26[c]	40.84	ND[c]	25.84	66.68	15.45	4.32	17.88[c]	82.13[c]	15.702
cld-2; pH 9.0	3.63[c]	17.55[c]	1.40[c]	7.75[c]	25.30[c]	29.74[c]	0.85[c]	44.98[c]	55.04[c]	15.454
cld-2; pH 9.0, 2MBA	1.22	39.54	ND[c]	26.84	66.38	16.19	4.10	17.36[c]	82.57[c]	15.712
10403S; pH 5.0	8.11	43.12	3.18	37.12	80.24	16.00	5.10	3.02	95.97	15.68
cld-2; pH 5.0	4.12[c]	15.66[c]	ND[c]	5.70[c]	21.36[c]	35.52[c]	0.60[c]	43.12[c]	56.88[c]	15.285
cld-2; pH 5.0, 2MBA	1.33[c]	40.03	ND[c]	33.02	73.05	10.41[c]	7.01	16.53[c]	83.46	15.759
cld-2; pH 5.0	3.24[c]	15.57[c]	0.93[c]	5.30[c]	20.87[c]	35.91[c]	0.58[c]	43.22[c]	56.78[c]	15.307
cld-2; pH 5.0, 2MBA	1.79[c]	39.12	ND[c]	35.25	74.37	13.52[c]	5.50	12.10[c]	87.89	15.765

[a] Minor fatty acid components are not included in this table.
[b] Values are from three independent experiments. ND, not detected.
[c] Statistically different ($P < 0.05$) from the control cultures (10403S; pH 9.0 and pH 5.0)
[d] ACCL, average carbon chain length, given by the equation $[\Sigma(\text{FAP} \times C)]/100$, where FAP is the percentage of each fatty acid and C is the number of carbon atoms in the chain.

represent a possible mechanism for stress regulation and modification of FA profiles in this pathogen (8, 28, 36, 37).

The differences between the growth rates of BCFA-deficient mutants and the BCFA-competent parent strain under pH stress and the resolution of such differences by the provision of exogenous 2MBA, bypassing the branched-chain α-keto acid dehydrogenase step in the biosynthesis of BCFA (49, 50), demonstrated the close correlation between membrane BCFA content and the ability of *L. monocytogenes* to grow under such adverse environmental conditions.

The results of this study suggest that *L. monocytogenes* uses subtle manipulation of BCFA content, and of the relative proportions of anteiso and iso FAs, as a very sensitive and effective means of adaptation to mild or moderate pH stress.

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