T**wo component signal transduction systems of *Lactobacillus casei* BL23 influence tolerance to stress conditions**

RUNNING TITLE **Two component systems in stress tolerance of *L. casei***

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ABSTRACT

L. casei BL23 encodes 17 two-component signal transduction systems. Insertional mutations were introduced into each gene encoding the cognate response regulators and their effect on growth under different conditions assayed. Inactivation of systems TC01, TC06 and TC12; (LCABL_02080-LCABL_02090, LCABL_12050-LCABL_12060 and LCABL_19600-LCABL_19610) led to major growth defects under the conditions assayed.

INTRODUCTION

Lactobacillus casei is a facultative heterofermentative lactic acid bacterium used in the food industry as a starter culture for milk fermentation, for maturation of cheeses and as probiotics (4). Probiotic microorganisms must survive the industrial production processes (3,23) and the transit through the gastrointestinal tract (3). Bacteria have evolved sophisticated mechanisms to detect and adapt to environmental changes; and among them, two component systems (TCS) play a central role (25). TCS typically consist of a sensor kinase (HK) and a response regulator (RR) (25). HKs monitor environmental signals and in response to a stimulus, autophosphorylate and subsequently transfer the phosphoryl group to the RR thus modulating its activity.

The role TCS in *Lactobacillus* is not well understood, although they are likely involved in quorum sensing, production of bacteriocins (5,16,22,26) and possibly in stress response (1,18,20). The availability of complete genome sequences of two *L. casei* strains (15,17) enables a more comprehensive study of the role of TCS in stress response of this organism. In this study, we used a broad and operational definition of stress as : any deviation from optimal growth conditions that results in reduced growth rate or lower biomass (7).

The genome sequences of *L. casei* strains BL23 and ATCC 334 harbours 17 putative TCS. For simplicity, we have renamed them numerically from TC01 to TC17 (Table S1 in the supplemental material). TC17 corresponds to the previously characterized system MaeKR (11). Strains and plasmids used in this study are listed in Table S2. Primers used are listed in Table S3. Insertion mutants were obtained by cloning internal DNA fragments of each RR-encoding gene in plasmid pRV300 (14) and introduced in *L. casei* BL23 by electroporation (21). BL23 derivative strains harboring complete deletions of genes LCABL_02080 (RR01),

LCABL_12050 (RR06) and, LCABL_19600 (RR12) were also obtained by insertion of plasmid pRV300 harboring the regions immediately upstream and downstream of each target gene and subsequent internal recombination. Complementation of the RR01 and RR06 deletions was achieved by cloning of the corresponding genes into the expression vector pT1NX (24).

The growth of *L. casei* BL23 and their derivative RR-defective mutants was compared in reference conditions (MRS at 37ºC without shaking) and MRS supplemented with 0.5% bile, MRS supplemented with 0.6 M NaCl, MRS adjusted to pH 3.75 and growth in MRS at 42 ºC. Growth was monitored by changes in optical density at 595 nm in a microtiter plate reader. At least three independent replicates of each growth curve were obtained. Maximal growth rates (μ_{max}) and the increment in O.D. values were considered to compare the performance of *L. casei* BL23 and its derivative mutants. Significant differences in growth parameters in the reference condition between the wild-type strain and each of the mutants were determined by one-way ANOVA. Levene's test was used to assess the equality of error variances. To determine whether the response of the mutant strains to each stress condition assayed was significantly different to that of the wild-type, pairwise two-way ANOVA analyses were performed taking the growth of *L. casei* BL23 and each mutant strain in the reference condition and each of the stress conditions. We considered that a significant difference was detected if the analysis estimated that both the strain variable and interaction were below a P value of 0.01.

Resistance to vancomycin, bacitracin, gramicidin or nisin was determined in MRS using serial dilutions of the antimicrobial agents. The assays were performed in 96 well microtiter plates incubated for 24 h. The Minimal Inhibitory Concentration (MIC, expressed in µg ml^{-1}) was defined as the lowest concentration of antimicrobial agent needed to totally inhibit the growth

of the bacterial strain. IC_{50} was considered as the concentration of antimicrobial agent that diminished the maximal growth rate (μ_{max}) to 50% of its value at reference growth conditions.

Insertion mutants were obtained for each RR thus indicating that none of the TCS is essential for growth. RR16 is homologous to RR YycF/VicR which is essential for growth in other low-G+C Gram-positive (27). Inactivation of the YycF homolog-encoding gene (*rrp-3*) in *Lactobacillus sakei* did not result in any significant difference with the parental strain under a number of stress conditions (18). This suggest that the YycFG TCS is not essential in lactobacilli although it is so in the closely related enterococci and streptococci.

The growth rates of the different mutants were similar to that of the wild type strain in reference conditions except TC04 and TC11 mutants, which were significantly reduced in growth rate and TC12 in which the maximum cell density was significantly lower than in the wild-type strain (Fig 1A and Table S4). The inactivation of systems TC01, TC06 and TC12 led to major growth defects under stress conditions (Fig. 1, Table 1 and Tables S5 A and S5 B).

The effect of the inactivation of *L. casei* TCS to tolerance against antibiotics targeted to the cell envelope was also investigated. BL23 and its derivative mutants were resistant to bacitracin and vancomycin but IC_{50} values for vancomycin in mutants TC01, TC06 and TC12 were lower than in the parental strain (Table S6). In contrast, *L. casei* BL23 was sensitive to bacitracin and nisin (Table S6) and the responses of the TCS mutants varied. Mutants TC01, TC09 and TC10 were more sensitive than BL23 to both antimicrobials, whereas three other mutants (TC06, TC11 and TC12) were, more sensitive only to nisin. Three mutants were more resistant to bacitracin (TC15, TC16 and TC17), and one mutant was more resistant to both antimicrobials (TC04).

To determine possible polar effects of the insertional inactivation of systems TC01, TC06 and TC12, strains carrying deletions of RR01, RR06 and RR12 (ΔRR01, ΔRR06 and ΔRR12) and the corresponding complemented strains (except for ΔRR12 which resulted impervious to transformation) were obtained. Strains BL23 and ΔRR01 grew similarly under reference conditions. The growth of ΔRR01 under different stress conditions was similar to that observed for the insertional mutant (Fig. S1 A to D and Table S6) and the effects of the mutation were relieved in the complemented strain ΔRR01-c except in the presence of 0.6M NaCl where ΔRR01 was able to grow (Fig. S2 B). Therefore, the growth defect observed with salt was possibly due to a polar effect on the expression of some of the genes located downstream. System TC01 is homologous to the *rrp-31hpk-31* (LSA0277-78) system of *L. sakei* and the CroRS system of *Enterococcus faecalis* (2,12,13). Inactivation of the cognate RR in *L. sakei* led to premature arrest of growth in reference conditions (MRS at 30° C), poor growth at high temperature (39° C), sensitivity to heat shock, aeration and, H_2O_2 and, higher resistance to vancomycin (18). These results contrast with our observations of the ΔRR01 mutant thus suggesting that these homologous TCS have different physiological roles. The sensitivity of the ΔRR01 mutant to bile and the cell envelope-targeted antimicrobials bacitracin and vancomycin suggests that TC01 is involved in the cell envelope stress tolerance.

Mutants ΔRR06 and ΔRR12 showed very similar phenotypes to that of their corresponding insertional mutant strains (Fig. S2 and Table S6). Complementation of ΔRR06 with pT1-RR06 relieved the effects of the mutation, and we conclude that the effects observed were due to the inactivation of RR06 and not to polar effects on downstream genes. Complementation

of ΔRR12 was not achieved, notwithstanding, the similarity of the phenotypes of strains TC12 and ΔRR12 suggests that the observed effects are mainly due to inactivation of RR12.

System TC06 is homologous to the *Bacillus subtilis* YclJ-YclK TCS, which is activated in oxygen limitation conditions (10) and the *E. faecalis* system Err06-Ehk06. Inactivation of this system in *E. faecalis* V583 resulted in a heat and SDS sensitive phenotype (6). Furthermore, it has been shown that Err06 is involved in resistance to H_2O_2 in *E. faecalis* JH2-2 (19). *L. casei* TC06 was very sensitive to most stress conditions assayed. In this sense, it is worth noting the presence of a putative tmRNA-encoding gene located upstream of the TC06 encoding genes. Interestingly, the involvement of an homologous tmRNA of *E. coli* in cell envelope stress response has been recently demonstrated (8) although it remain to be established both the involvement of TC06 in the regulation of the tmRNA expression and the actual role of this tmRNA.

System TC12 is paralogous to system TC09 but in contrast to TC12, inactivation of TC09 only resulted in higher sensitivity to bacitracin and nisin than BL23 (Table S6). Systems TC09 and TC12 are homologous to the three paralogous TCS of *B. subtilis*, BceRS, YvcPQ and YxdJK, involved in the cell envelope stress response (9). The three of them are located next to genes encoding ABC transporters. Similarly, both TC09 and TC12 are located next to genes encoding putative ABC transporters. The lower resistance of TC09 against bacitracin and nisin and of TC12 against nisin suggests that these systems may also be involved in cell envelope stress response. However, the growth defects of the TC12 mutant, particularly at low pH, suggest that the functional role of this system is quite different in *L. casei* of that of its homolog BceRS in *B. subtilis*.

In summary, this study shows that some TCS play a major role in the physiology of *L. casei* and its adaptation to changing environmental conditions. The detailed study of these systems should provide valuable insight to understand the performance of this organism in conditions of industrial production and the gastrointestinal habitat.

ACKNOWLEDGMENTS

This work was financed by funds of the AGL2007-60975/ALI and Consolider Fun-*C*-Food CSD2007-00063 from the Spanish Ministerio de Ciencia e Innovación. A. Revilla-Guarinos is recipient of a FPI grant (BES-2008-004527) from the Spanish Ministerio de Ciencia e Innovación.

We thank Laura Barrios and Fernando López for their help with statistical analyses, Amalia Blasco for technical assistance and Vicente Monedero for critical reading of this manuscript.

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TABLE 1. Summarized results of the growth assays carried out with *L. casei* BL23 and selected TC-defective mutants under different growth conditions (see Tables S4A and S4B for data of all mutants and the results of the ANOVA analysis).

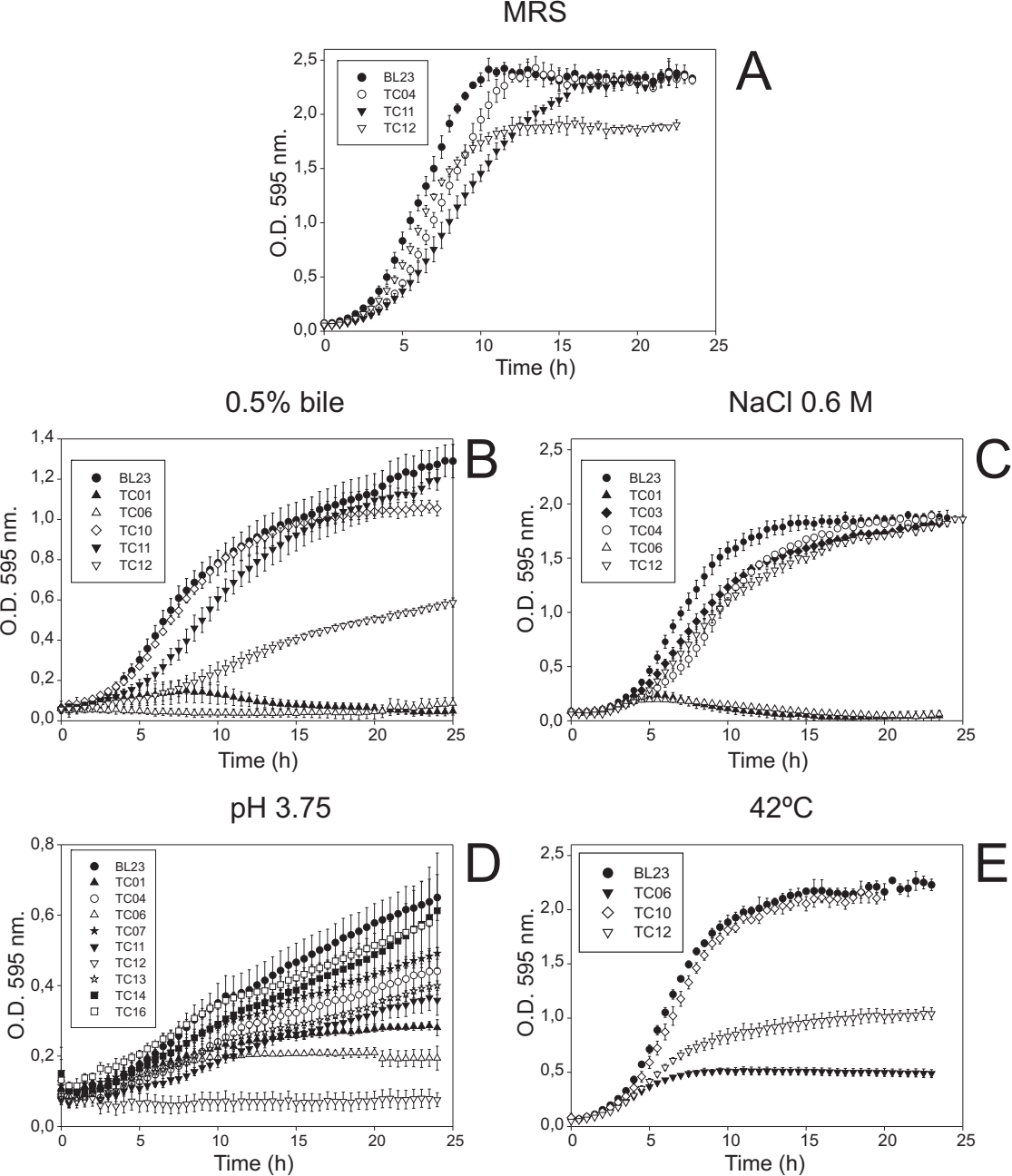
Condition	Growth parameters[a]	Strain			
		BL23	TC01	TC06	TC12
Bile 0.5%	μ_{max}	$0.17\pm<10^{-2}$	NG[b]	NG	$0.08\pm<10^{-2}$
	Δ O.D.	1.15±0.14	NG	NG	0.52±0.03
0.6 M NaCl	μ_{max}	0.23±0.01	NG	NG	0.21±0.01
	Δ O.D.	1.83±0.04	NG	NG	1.81±0.03
pH 3.75	μ_{max}	$0.07\pm<10^{-2}$	$0.03\pm<10^{-2}$	0.03±0.01	NG
	Δ O.D.	0.56±0.07	0.19±0.06	0.14±0.01	NG
T 42°C	μ_{max}	0.26±0.01	$0.24\pm<10^{-2}$	0.17±0.01	0.22±0.02
	Δ O.D.	2.19±0.10	2.07±0.15	0.46±0.03	1.02±0.05

[a] Data shown are the mean and standard deviation.

[b] No growth.

Figure legends

Fig. 1. Growth of *L. casei* BL23 and TC-defective mutants under different conditions (only strains that displayed significant differences with strain BL23 are shown). Error bars indicate SD (at least three replicates).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O.D. 595 nm.
Time (h)
0.5% bile
B
BL23
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TC11
TC12
NaCl 0.6 M
C
BL23
TC01
TC03
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TC06
TC12
pH 3.75
D
BL23
TC01
TC04
TC06
TC07
TC11
TC12
TC13
TC14
TC16
42ºC
E
BL23
TC06
TC10
TC12