CATABOLISM OF LEUCINE BY *STAPHYLOCOCCUS CARNOSUS* 833 STRAIN

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Background

Staphylococcus strains have been shown to influence the composition of volatile compounds in the sausages and consequently their aroma [1,2]. Sausages inoculated with *Staphylococcus carnosus* were distinguished by their high desorption of branched-chain aldehydes (3-methyl butanal) and their corresponding alcohols, acids and esters. Among these volatile compounds, 3-methyl butanal and 3-methylbutanoic acid arising from leucine catabolism have a strong impact on sausage aroma [2,3].

Objectives

Our objective was to determine the role of *S. carnosus* 833 strain in the formation of aroma compounds derived from leucine catabolism. The initial step of the catabolic pathway for the branched-chain amino acids such leucine is transamination, a reversible reaction that yields the corresponding branched-chain α-ketoacid. This study focused on the molecular characterization of the *S. carnosus* 833 strain gene coding this branched-chain aminotransferase (BCAT).

Methods

Preparation of cell extract (CE)

S. carnosus 833 strain was grown in LB medium until $OD_{600\ nm}$ of 1.5. Sixty ml of this culture were harvested by centrifugation. The pellet was resuspended in 2ml of potassium phosphate buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.5) contained 120 µg of lysostaphin and 40 µg of DNAse. It was incubated 30 min at 37°C. After centrifugation the supernatant was collected and used as CE for the enzyme assays.

Aminotransferase enzyme assays

CE were incubated overnight at 37°C with 2 mM amino acid (Leu, Ile, Val, Trp, Tyr, Phe, Asp, Ala, Met, Cys or Lys), 10 mM α ketoglutarate, 2 mM pyridoxal phosphate and 2 mM thiamine pyrophosphate in phosphate buffer (67 mM Na_2HPO_4, 67 mM KH_2PO_4, 2 mM EDTA, pH 7.4). Aminotransferase activities were quantified using the colorimetric L-glutamic acid assay kit of Roche. Aminotransferase activities were calculated as µmoles of formazan formed min^{-1} mg of protein $^{-1}$, using the extinction coefficient of formazan at 492 nm. The protein concentration was determined by the method of Bradford with bovine serum albumin as the standard.

Branched-chain aminotransferase (BCAT) gene cloning, sequencing, and inactivation

All DNA manipulations were performed according to standard procedures [4]. Chromosomal DNA of *S. carnosus* 833 strain was amplified by PCR with the primers 1:GCAGGWAACTATGCKKCAAG, 2:GAAATAACWGCTGCWGTACC and 3:GTGGTTTTATTCAGACC AGACC designed from DNA sequences of homologous genes of *Bacillus subtilis* and *Staphylococcus aureus*. PCR amplification was performed by using a GeneAmp® PCR System 9700 (PE Applied Biosystems) and Taq DNA polymerase (Roche). Reaction conditions were an initial denaturation at 94°C, 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min and elongation at 72°C for 1 min, and a final elongation at 72°C for 7 min. The two fragments amplified (300 and 600-bp) were cloned into the pGEM-T vector (Promega). These vectors were used as a sequencing template. Sequencing reactions were performed using BigDye® terminator cycle sequencing kit and were run on an ABI PRISM 310 DNA sequencer (PE Applied Biosystems). GCG programs were used to assemble, analyse sequence data and search for homologous sequences in databases.

Southern blot was performed after digestion of chromosomal DNA of 833 strain with *Pst*I, *Xba*I, *Sph*I, *Eco*RI and *Hind*III. The 300-bp fragment amplified by PCR with primers 1 and 2 was labelled with the Dig-High Prime kit (Roche) and was used as probe. Southern blot hybridisation was performed as described by the supplier of the labelling system (Roche).

Inverse PCR was used to generate DNA products flanking *bcat* fragment. Chromosomal *Hind*III, *Eco*RI, *Dra*I, *Rsa*I or *Hinf*I self-ligated fragments were used as template. *Hind*III and *Eco*RI enzymes do not cut in the *bcat* fragment and the others cut in the 3'-extremity of this one. The amplified fragments were cloned, sequenced and analysed as described above.

The temperature sensitive shuttle vector pBT2 [5] was used for chromosomal inactivation of *bcat* gene in *S. carnosus*. The *ermB* cassette from plasmid pEC7 [5] was used to interrupt this gene. Plasmid DNA was introduced into 833 strain by electroporation with glycerol-treated electrocompetent cells [6]. By a double-crossover event, the inactivated copy of gene was introduced into the genome as described by Brücker [5] with some modifications.

Results and discussion

Aminotransferase activity

Aminotransferase activities were measured in CE of *S. carnosus* 833 strain with different amino acids (Leu, Ile, Val, Trp, Tyr, Phe, Asp, Ala, Met, Cys or Lys) as substrates. No aminotransferase activity was detected against Ala, Met, Cys or Lys (Figure 1). A similar specific activity (SA) was measured with the three-branched-chain amino acids. With aromatic amino acids, the SA was twice lower than with branched-chain amino acids. The SA_{asp} is 1.5 fold higher than the SA_{leu}. The branched chain aminotransferase could catalyse the transamination of Leu, Ile, Val and all the aromatic amino acids. On the contrary, the aromatic aminotransferase can only used Leu, and not Ile or Val [7]. So, in our experimental conditions, at least two aminotransferases were active in *S. carnosus* 833 strain: a Asp-aminotransferase and a branched-chain aminotransferase (BCAT). The branched-chain aminotransferase activity for leucine transamination is very interesting since its substrates are precursors of aroma compounds of sausage, such as 3-methyl butanal and 3-methylbutanoic acid.

Genetic characterization of S.carnosus 833 strain bcat gene

To identify a part of the branched-chain aminotransferase gene (*bcat*) of *S. carnosus* 833 strain, two fragments of 300-bp and 600-bp were amplified and sequenced. The deduced amino acid sequence showed 77% of similarity with *B. subtilis* branched-chain aminotransferase. *S. carnosus* 833 chromosomal DNA digested with different restriction enzymes was probed with the 300-pb amplified fragment of *bcat* gene (Figure 2). Since the probe hybridised with a single fragment in all digests, it is likely that *S. carnosus* 833 strain possesses a single gene encoding for branched-chain aminotransferase. To identify the branched-chain aminotransferase gene (*bcat*), flanking DNA products of identified *bcat* fragment were amplified by inverse PCR. A total of 2061-bp were identified. There was an open reading frame (ORF) of 1,077 nucleotides coding for 359-residue protein (BCAT) with a theoretical M_r of 40.5 kDa and a pI of 4.54. BLAST searches indicated that

the deduced amino acid sequence has 58 % identity to branched-chain aminotransferase of *B. subtilis* and 80% to the one of *S. aureus*. The *S. carnosus* BCAT also has an amino acid sequence, which matches the PROSITE aminotransferase class-IV pyridoxal-phosphate binding site. Downstream of BCAT a putative *rho*-independant transcriptional terminator was identified. Putative –10 and –35 promoter regions and a ribosome-binding site were also identified upstream of the ORF. No signal peptide sequence was found in the N-terminal region of BCAT. To investigate the physiological role of the *bcat* gene, a *bcat* mutant of *S. carnosus* 833 strain was constructed by allelic exchange. The characterization of this mutant is in progress.

Conclusion

S. carnosus 833 strain has enzymatic potential to transform amino acids to aroma compounds. In particular, this strain can transform leucine to aroma compounds that have been identified as major aroma components in sausage. We have characterised the gene coding for the first enzyme involved in leucine catabolism of *S. carnosus* strain 833, the branched-chain aminotransferase. The characterization of the *bcat* mutant of *S. carnosus* 833 strain and of the enzyme will help to understand the metabolic pathway involved in the degradation of leucine by *S. carnosus* and then a better control of this pathway in sausage will be done.

Pertinent literature

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[5] Brückner R. (1997) FEMS Micobiol. Lett. 151, 1-8.
[6] Augustin J. and Götz F. (1990) FEMS Micobiol. Lett. 66, 203-208.
[7] Yvon M., Thirouin L., Rijnen L., Fromentier D., and Gripon J.C. (1997) Appl. Environ. Microbiol. 63, 414-419.

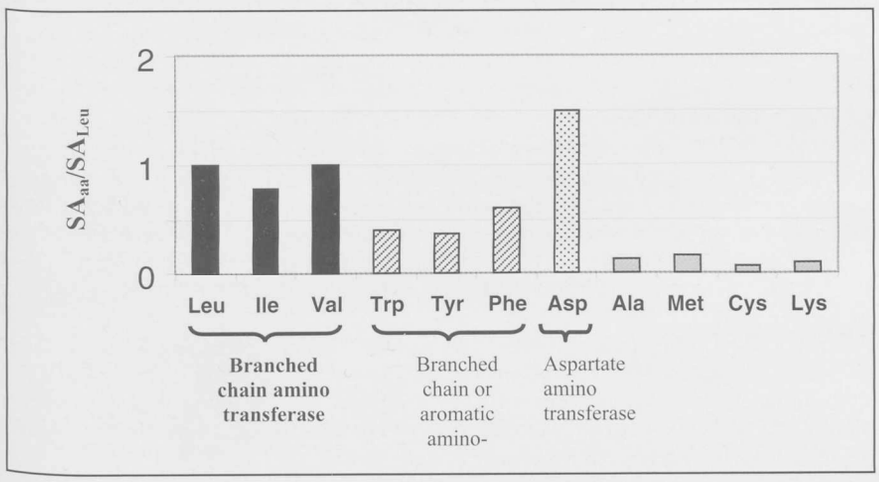

Figure 1: Aminotransferase activities of *S. carnosus* 833 stain with different amino acids
SA : specific activity

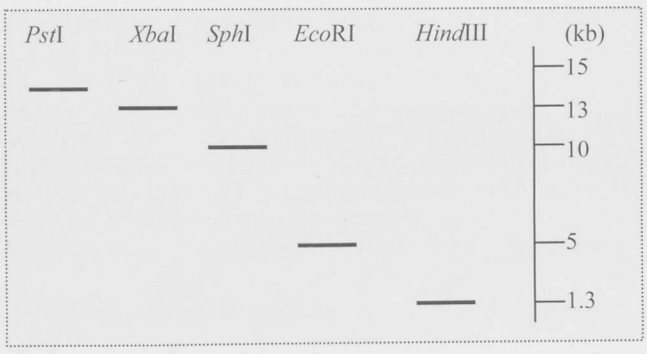

Figure 2: Schematic representation of Southern blot analysis of *S. carnosus* chromosomal DNA with DIG-labelled *bcat* 300-pb probe