Proteomic Approach for Characterization of Hop-Inducible Proteins in *Lactobacillus brevis*[∇]

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Resistance to hops is a prerequisite for the capability of lactic acid bacteria to grow in beer and thus cause beer spoilage. Bactericidal hop compounds, mainly iso- α -acids, are described as ionophores which exchange H^+ for cellular divalent cations, e.g., Mn^{2+} , and thus dissipate ion gradients across the cytoplasmic membrane. The acid stress response of *Lactobacillus brevis* TMW 1.465 and hop adaptation in its variant *L. brevis* TMW 1.465A caused changes at the level of metabolism, membrane physiology, and cell wall composition. To identify the basis for these changes, a proteomic approach was taken. The experimental design allowed the discrimination of acid stress and hop stress. A strategy for improved protein identification enabled the identification of 84% of the proteins investigated despite the lack of genome sequence data for this strain. Hop resistance in *L. brevis* TMW 1.465A implies mechanisms to cope with intracellular acidification, mechanisms for energy generation and economy, genetic information fidelity, and enzyme functionality. Interestingly, the majority of hop-regulated enzymes are described as manganese or divalent cation dependent. Regulation of the manganese level allows fine-tuning of the metabolism, which enables a rapid response to environmental (stress) conditions. The hop stress response indicates adaptations shifting the metabolism into an energy-saving mode by effective substrate conversion and prevention of exhaustive protein de novo synthesis. The findings further demonstrate that hop stress in bacteria not only is associated with proton motive force depletion but obviously implies divalent cation limitation.

Resistance to hops is a prerequisite for the capability of lactic acid bacteria to grow in beer and thus cause beer spoilage. Bactericidal hop compounds, mainly iso- α -acids, are described as ionophores which exchange H^+ for cellular divalent cations, e.g., Mn^{2+} , and thus dissipate ion gradients across the cytoplasmic membrane (26). Consequently, the low intracellular pH interferes with essential enzyme reactions and the proton motive force (PMF)-dependent nutrient uptake is hampered, resulting in cell death (23). Previously, we characterized the acid stress response of *Lactobacillus brevis* TMW 1.465 and adaptation to hop stress in the variant *L. brevis* TMW 1.465A on the level of metabolism, membrane physiology, and cell wall composition (7). The hop resistance that developed during adaptation is a multifactorial dynamic property. Arginine catabolism contributes to energy and PMF generation, and the acquired hop resistance is energy independent and involves an altered composition of the cell wall. Lipoteichoic acids located in the cell wall provide a reservoir of divalent cations, which are otherwise scarce as a result of complexation with hop acids (26). An altered cytoplasmic membrane protects against acid stress. However, these structural improvements were unable to shield the hop-resistant cells from penetration by hop compounds (7). In hop-resistant bacteria, the inability of hop compounds to reach their targets is not responsible for hop resis-

tance (26). Thus, the hop-resistant cells developed a strategy to survive in the presence of hop compounds. High-resolution two-dimensional (2D) gel electrophoresis coupled to Edman sequencing, mass spectrometric (MS) analysis, and newly developed computational possibilities of protein identification (25) provide powerful tools with which to investigate hop resistance in beer spoilage lactobacilli for which no genome sequences are available. Despite the heterogeneity within the species *Lactobacillus brevis*, a species comprising the majority of beer spoilage bacteria (5), the publication of the recently finished genome of *Lactobacillus brevis* ATCC 367 is helpful in hop resistance research. In this work, we studied the mechanisms of hop resistance of beer-spoiling *Lactobacillus brevis* TMW 1.465A on the proteomic level by using these recent innovations in available databases and analytic instrumentation.

MATERIALS AND METHODS

Microorganism, media, and culture conditions. *Lactobacillus brevis* TMW 1.465 and its variant adapted to hop stress, *L. brevis* TMW 1.465A (7), were used. *L. brevis* TMW 1.465 was grown in mMRS4 (29) at pH 6.0 (reference conditions) and in the same growth medium adjusted to a pH of 4.0 with 2 M HCl (acid stress conditions). *L. brevis* TMW 1.465A was grown at pH 4.0 in the presence of isomerized hop extract (hop stress conditions; NateCO₂ GmbH u. Co. KG, Mainburg, Hallertau, Germany) added at an iso- α -acid concentration of 86 μ M. Cultures were grown at 30°C. Cells were harvested by centrifugation ($2,800 \times g$ for 10 min) for each condition at an optical density at 590 nm (OD₅₉₀) of 0.4 in early exponential phase and washed twice with phosphate buffer (50 mM, pH 7). For standardization, a cell mass corresponding to 7 mg cell dry weight was used for all conditions for subsequent protein extraction.

Extraction of whole-cell protein. The cell walls were digested with 318 kU lysozyme in TE-DTT buffer (10 mM Tris-HCl, 0.01 M EDTA, 6 mM dithiothreitol [DTT], pH 8.0) for 45 min at 37°C. Digested cells were centrifuged ($5,000 \times g$ for 5 min), and the supernatant was discarded. The pellet was resuspended in

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FIG. 1. 2D electrophoretic analysis of Coomassie blue-stained total protein from cells of *L. brevis* TMW 1.465A grown under hop stress. HI, proteins detected under hop stress conditions only; AI, proteins detected in acid-stressed cells; HO, proteins overexpressed in hop-stressed cells; HR, proteins repressed in hop-stressed cells.

200 μ l SDS buffer (0.9% sodium dodecyl sulfate [SDS], 0.1% Pefabloc, 100 mM Tris base, pH 8.6) and disrupted by sonication (HD-70/Bandelin, three cycles of 30 s each; power, 90%; cycle, 30%; and intermediate cooling). The suspension was diluted 3.5-fold with thiourea lysis buffer (6.10 M urea, 1.79 M thiourea, 65.06 mM 1-*O*-n-dodecyl- β -D-maltopyranoside (DDM), 1% [wt/vol] DTT, 0.5% [vol/vol] Pharmalyte 3-10) and sonicated again. The proteins were solubilized by shaking for 20 min. The remaining cell wall fragments were removed by centrifugation at $17,500 \times g$ at 4°C for 30 min. The clear supernatants were stored at -80°C (11).

2D electrophoresis. 2D electrophoresis was performed with samples from reference, acid stress, and hop stress conditions as described previously (10). Isoelectric focusing (IEF) was carried out using a Multiphor II DryStrip kit system (Amersham Biosciences Europe GmbH, Freiburg, Germany) with 24-cm immobilized-pH-gradient (IPG) 4.5 to 5.5 strips (Amersham Biosciences) at 20°C. The IPG strips were rehydrated with an excess of rehydration solution (6.10 M urea, 1.79 M thiourea, 8.13 mM DDM, 0.2% [wt/vol] DTT, 0.2% [vol/vol] Pharmalyte 3-10). In total, 300 μ l protein extract was applied by sequential anodic cup loading. Initial IEF was run for 24 h at 50 V and subsequently for 12 h at 150 V. IEF to steady state at 3,500 V was carried out according to the micropreparative IEF protocol (10). SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a vertical system with gels of a total acrylamide concentration of 11% at 15°C. The proteins were visualized by colloidal Coomassie staining (Roti-Blue; Carl Roth GMBH & Co., Karlsruhe, Germany) and quantified as described previously (17). For analysis of reproducibility of protein expression, expression was monitored on 18-cm IPG 3 to 10, 4 to 7, and 4.5 to 5.5 strips by at least two independent experiments. Protein quantification on high-resolution 24-cm IPG 4.5 to 5.5 strips is based on electrophoretic analysis of two independent experiments.

Semidry-blotting and N-terminal-sequence analysis. The transfer of proteins from the 2D gel to a polyvinylidene difluoride membrane (Immobilon-PSQ; Millipore) and their visualization were performed as described previously (6). For N-terminal sequencing of three chosen proteins, the blot was sent to Prosequenz Bioanalytik (Ludwigsburg, Germany) and analyzed with a liquid-phase protein sequencer (model 476; Applied Biosystems, ABI) according to the instructions of the supplier.

MS protein characterization. The colloidal Coomassie (Roti-Blue; Roth)-stained proteins were excised from the gel and sent to the Zentrallabor für Proteinanalytik (Ludwig-Maximilians-Universität München, Munich, Germany) for matrix-assisted laser desorption/ionization-time-of-flight MS, liquid chromatography electrospray ionization (LC-ESI) tandem MS (MS/MS), and ESI MS/MS analysis.

Protein identification. For identification of proteins from non-genome-sequenced *L. brevis* TMW 1.465A, a protein database was created as described previously (15). All available protein sequences from *Lactobacillus* and *Lactococcus* species, especially from *L. brevis* ATCC 367, were obtained from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). The coding sequence translations of the genome draft sequences from *Lactobacillus casei* ATCC 334, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *Lactobacillus gasseri* ATCC 33323, *Lactobacillus reuteri* JCM 1112, *Lactobacillus reuteri* 100-23, and *Lactococcus lactis* subsp. *cremoris* SK11, which were produced by the U.S. Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes/), were downloaded. Three hundred contaminant proteins, including keratins and proteases, were added into the database (37). For protein identification, the NCBI database and the created database, which contained a total of 34,401 sequences, were searched by using the Matrix Science Mascot software (21) and the MS-BLAST version (25) of the WU-

FIG. 2. Induction factors of proteins overexpressed under hop and acid stress conditions. The normalized volume of each protein spot (HO Protein nr.) was calculated using the Image Master2D Elite software. The induction factors were calculated by dividing each stress-regulated protein spot volume by the corresponding spot volume of the reference condition. Shown are the means of two independent experiments.

BLAST2 program (<http://blast.wustl.edu>). For the latter, "Sequest dta formatted" (35) files derived from LC-ESI MS/MS analysis were processed to peptide sequences with LutfiskXP (31). A program written in the C programming language generated and submitted multiple permutations and catenations of peptide sequences to MS-BLAST.

RESULTS

Optimization and control experiments for reproducible 2D analysis of hop-regulated proteins. A comparison of sequential protein extraction, as described by Molloy et al. (19), to detergent-based whole-cell protein extraction, as specified above by 2D-PAGE (IPG 3 to 10), determined the latter as more robust and reproducible. The main reason for this observation resides in the tough cell walls of *L. brevis* TMW 1.465, which are variable in composition under different growth and stress conditions and very resistant to lysis (7). Accordingly, an extraction protocol was chosen that ensures high cell lysis efficiencies under all conditions investigated. Consecutively, the experimental error of protein spot quantification was determined principally as described previously (17). Total cell protein extracts were diluted in steps of 10% with lysis buffer in the range from 10% to 90% and separated by 2D PAGE (IPG 4 to 7). A linear behavior of individual protein volumes was observed, with a maximum relative standard deviation of 16%. To determine the pI range of interest, within which most of the stress-regulated proteins are located, the protein extracts from all three growth conditions were focused on IPG 3-to-10, 4-to-7, 4.5-to-5.5, and 6-to-11 strips. Changes in the protein patterns were noticed mainly in the range from pI 4.8 to 5.4 and to a lesser extent from pI 9.8 to 11. However, the patterns in the range from 9.8 to 11 were difficult to reproduce and showed very small protein quantities on the gel. Higher protein loads did not separate properly. Thus, the IPG 4.5 to 5.5, which covered 86% of all stress-regulated proteins, was chosen for incipient analysis. The dependence of the growth phase on the protein yield and the expression level of stress-inducible proteins was determined from exponential growth phases at

OD₅₉₀ of 0.3, 0.4, and 0.5 and that of the transient growth phase at an OD₅₉₀ of 0.9. Hop stress-regulated proteins were detected with similar expression levels in all growth phases, and the extractable protein yield was maximal at an OD₅₉₀ of 0.4. As a control for possible carryover of hop proteins, isomerized hop extract (860 μ M iso- α -acid in lysis buffer) was separated by 2D PAGE. The isomerized hop extract showed two rows of four proteins with a pI of 4.76 to 4.94 and mass of 69 kDa and a pI of 4.99 to 5.10 and mass of 57 kDa in each case, which were detectable only by sensitive silver staining. Hence, there was no detectable contamination in the protein samples derived from the addition of isomerized hop extract to the growth medium.

Differential proteome analysis. Whole-cell protein was isolated from *L. brevis* TMW 1.465 under reference and acid stress conditions and under hop stress conditions from *L. brevis* TMW 1.465A, its highly hop-resistant variant (7). High-resolution IPGs with 24-cm separation distances in the range from pH 4.5 to 5.5 were used in the first dimension. The second dimension exhibited a separation range from 10 to 250 kDa. This combination resulted in the separation of high protein loads in the range of 4.74 to 5.46. The anodic edge was not exploitable for protein pattern comparison, because it showed vertical streaks at the cup loading position. In total, 12 hop stress-inducible proteins, two acid stress-inducible proteins, 17 hop stress-overexpressed proteins, and 1 hop stress-repressed protein were identified (Fig. 1). Acid stress-inducible proteins were expressed under acid stress but not overexpressed under the hop stress condition. For expression analysis, a reference map with proteins of *L. brevis* expressed at pH 6 (reference condition) was established and compared to those proteins expressed by the same strain under acid stress (pH 4.0) and the hop-adapted variant under hop stress (pH 4.0 and 86 μ M iso- α -acids). The expression values of hop-overexpressed proteins are depicted in Fig. 2.

Protein identification. The N-terminal sequence of HO13 was determined by Edman degradation to be A(G/T)SNGKV

FIG. 3. Protein HI10 expression of *L. brevis* TMW 1.465 under reference conditions (A) or acid stress (B) or of *L. brevis* TMW 1.465A under hop stress conditions (C).

AMVTGGXQ, which identified HO13 as member of the oxidoreductase group. Subsequent protein analysis was performed by mass spectrometry. All differentially expressed proteins were subjected to a matrix-assisted laser desorption ionization–time-of-flight MS and LC-ESI MS/MS analysis. MS data were interpreted by the Matrix Science Mascot software and searched against the NCBI and custom databases (see above). In parallel, LC-ESI MS/MS data were exported to Sequest dta files, which were processed by the de novo sequencing program LutefiskXP and MS-BLAST as described above. The two methods provided consistent results if good-quality MS/MS spectra were obtained. However, the latter method was very sensitive (14) and enabled a higher degree of reliability by a higher sequence coverage than the Mascot analysis. For iden-

tification of HI10, which was strongly induced by hop stress (Fig. 3), nanospray sequencing ESI MS/MS was used. The spectra were manually interpreted and led to the identification of HI10 as a pyruvate carboxylase. Five proteins (HO2, HI1, HI5, HI6, and HI10) exhibited a different molecular weight in comparison to the corresponding database stored sequence. Eighty-four percent of proteins investigated from non-genome-sequenced *L. brevis* TMW 1.465A were identified by these techniques and are displayed in Table 1.

The acid shift induced a cysteine sulfurase-related enzyme and the cyclopropane-fatty-acyl-phospholipid synthase. The proteins regulated under the hop stress condition were enzymes from glycolysis, the reversed tricarboxylic acid cycle pathway, glycerol metabolism, amino acid metabolism, and

TABLE 1. Molecular weights and pIs of proteins identified from 2D electrophoresis^a

Protein	Mol wt (10 ³)	pI	Accession no.	Function
HO1	66	4.83	gi 116333412	Pyruvate kinase
HO2	66	4.84	gi 116333309	3-Phosphoglycerate kinase
HO3	65	4.92	gi 116333412	Pyruvate kinase
HO4	65	4.89	gi 116333412	Pyruvate kinase
HO5	45	5.04	gi 116333309	3-Phosphoglycerate kinase
HO6	45	5.05	gi 116334175	Phosphopentomutase
HO7	44	5.08	gi 116334720	Glycerol dehydrogenase
HO8	38	4.82		Negative
HO9	38	5.10	gi 116332816	2-Hydroxyacid dehydrogenase
HO10	37	5.08	jgi1730_lcasATCC334	Transcriptional regulatory protein
HO11	33	4.81	gi 62463721	Malate dehydrogenase, NAD dependent
HO12	31	5.05	gi 116333251	RecR protein
HO13	29	4.87	gi 116332857	Acetoin dehydrogenase
HO14	29	5.18	gi 28379560	Phosphoglyceromutase
HO15	23	5.36	gi 116334174	Purine-nucleoside phosphorylase DeoD
HO16	21	5.40		Negative
HO17	15	4.97	gi 116333447	ATP-dependent protease HslVU (ClpYQ)
HI1	159	5.18	jgi3841_lcreSK11	Formamidopyrimidine-DNA glycolase
HI2	140	5.30		Negative
HI3	140	5.39		Negative
HI4	140	5.41		Negative
HI5	140	5.42	gi 116334767	Pyridoxal-dependent decarboxylase
HI6	65	5.39	gi 116333104	3-Hexulose-6-phosphate synthase related
HI7	65	4.86	gi 116333412	Pyruvate kinase
HI8	65	4.87	gi 116333412	Pyruvate kinase
HI9	65	4.94	gi 116333334	Glucosamine-6-phosphate synthetase
HI10	37	4.93	gi 42519197	Phosphoenolpyruvate carboxylase
HI11	37	5.33	gi 116334587	Ornithine carbamoyltransferase
HI12	19	5.29	gi 28378831	Peptidylprolyl isomerase
HR1	40	4.80	gi 116333957	D-Lactate dehydrogenase
AI1	43	5.00	gi 116334038	Cysteine desulfurase-related enzyme
AI2	39	5.33	gi 116333366	Cyclopropane-fatty-acyl-phospholipid synthase

^a The proteins designated negative could not be identified by a peptide mass fingerprint and an LC-ESI MS/MS analysis. Accession numbers designated gi refer to NCBI database entries and jgi to the U.S. Department of Energy Joint Genome Institute database, which is followed by the designation of the strain. HI, proteins detected under hop stress conditions only; AI, proteins detected in acid-stressed cells; HO, proteins overexpressed in hop-stressed cells; HR, proteins repressed in hop-stressed cells.

(5). Its corresponding gene might be a possible additional target gene for discrimination of hop-resistant and -sensitive strains beyond previously described ones, including, e.g., *hitA*, *horA*, or *orf5* (12, 24, 30). The use of the reductive tricarboxylic acid cycle and concomitant formation of malate by HO11 contribute to manganese homeostasis based on the manganese complexing properties of malate (20). HO9, which is the 2-hydroxyacid dehydrogenase, may also catalyze the conversion from oxaloacetate to malate (3). The formation of neutral acetoin prevents acidification, and acetoin diacetyl interconversions are known to function as redox balancers (28). In heterofermentative lactobacilli, NADH recycling doubles the ATP yield from hexoses and hence contributes to an enhanced energy yield despite a reduced metabolic flux in central carbon metabolism. In agreement with the overexpression of several oxidoreductases involved in NADH recycling (Table 1; Fig. 4), we previously reported that hop adaptation induced a shift from fructose utilization as a carbon source to fructose utilization as an electron acceptor (7).

Another cluster of hop-regulated enzymes is involved in nucleotide metabolism. The phosphopentomutase DeoB (HO6) and the purine-nucleoside phosphorylase DeoD (HO15) are part of the ribonucleoside and desoxyribonucleoside catabolism. These enzymes can establish an additional source of energy, which exploits the carbon moiety of the degraded nucleotides. The coexpression of these two genes is consistent with their regulation in *Escherichia coli* (1). Interestingly, phosphopentomutase and phosphoglycerate mutase (HO14; see above) are members of a superfamily of metalloenzymes and require two Mn atoms for activity (9). For purine-nucleoside phosphorylase, a stimulatory effect of Co^{2+} in a 1 mM concentration is reported, while Mg^{2+} had no effect at the 100 μM level (22). This suggests a stimulation by a high level of divalent cations, as described for the pyruvate-converting enzymes of the intermediate carbon metabolism; however, the influence of manganese was not yet tested.

RecR (HO12) is a DNA-binding protein which is involved in DNA repair and recombination with binding affinity for damaged DNA. It does not recognize damaged bases, although unusual local deformability could be its target. It was concluded that maximal activity and binding requires ATP and divalent cations, such as Mn^{2+} , and divalent cations may play a decisive role in the structure of the RecR protein (2). Specific binding was established only in the presence of divalent cations. Thus, this enzyme can account for the fidelity of genetic information in bacteria. However, full functionality can be achieved only in the presence of millimolar (optimal, 2 mM) levels of divalent cations (2).

Manganese is a scavenger of toxic oxygen (4), and manganese depletion by hop stress (26) can result in accumulation of hydrogen peroxide and superoxide that cause oxidative damage. A known oxidative DNA damage is formed by imidazole ring-opening of adenine and guanine, leading to the formation of formamidopyrimidines. The formamidopyrimidine-DNA glycolase (HI1) excises formamidopyrimidine from DNA and contributes to DNA fidelity (33).

Enzymes involved in amino acid metabolism are a cysteine sulfinate desulfinate/cysteine desulfurase-related enzyme (AI1) and a pyridoxal phosphate-dependent decarboxylase (HI5). The former is known to be pyridoxal phosphate dependent and is

associated with the mobilization of sulfur required for metallocluster formation in proteins. In *Azotobacter vinelandii*, the cysteine desulfurase NIFS is involved in activation of nitrogenase component proteins that have metallocluster prosthetic groups (36). Since AI1 was found to be upregulated in response to acid stress, a function as a homocysteine desulfurase, which catalyzes the reaction $\text{l-homocysteine} + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{S} + \text{NH}_3 + 2\text{-oxobutanoate}$, is also likely, due to the elevation of extracellular pH by ammonium production. A member of the pyridoxal phosphate-dependent decarboxylase enzymes, notably the glutamate decarboxylase (Gad), confers the ability to survive in extremely acidic environments via production of γ -aminobutyrate and NH_3 , comparable to the arginine deiminase pathway (see above). These metabolites increase the intra- and extracellular pH (32).

The hop stress-inducible peptidylpropyl isomerase (HI12) is involved in protein refolding and repair. Since the hop stress condition evokes a low intracellular pH (7) and reduces the amount of intracellular divalent cations (26), it is likely that proteins could be rendered nonfunctional by conformational changes. The concomitant nutrient limitation during growth in hopped media (23) requires mechanisms for avoiding exhaustive protein de novo synthesis by adequate protein repair mechanisms. If protein damage is irreversible, the cell's last resort is degradation (34). ATP-dependent proteases (HO17) can catalyze the recycling of proteins. The activity is dependent on nucleotide or divalent cations binding. It is concluded that the rapid protein degeneration is achieved by ATP hydrolysis; however, MgCl_2 , MnCl_2 , and CaCl_2 allow some peptidase activity in the absence of any nucleotide. The absence of divalent cations abolishes all activity (13).

Finally, a transcription regulation protein was identified. This might be an interesting target for detection of beer spoilage lactobacilli. Since hop stress results in a huge amount of regulated proteins, it is likely that exact regulation of all enzymes principally enables the adaptation to hop stress.

Taken together, hop stress resistance mechanisms imply mechanisms to cope with intracellular acidification and mechanisms for energy generation, economy, genetic information fidelity, and enzyme functionality. In addition to the dissipation of the PMF, the depletion of the intracellular divalent cation reservoir (26) is a triggering factor in hop stress, resulting in a loss of short-term regulation potential of enzyme activity. Hop-resistant cells can bypass or compensate for the deficit in regulatory potential and even misregulation by the means of a complex change in protein expression. This results in a change in metabolism, as well as structural improvements of the wall components (7). Our results explain previous findings that beer isolated cells sometimes fail to grow upon reinoculation into hopped medium unless they have first been exposed to subinhibitory concentrations of hop compounds (27), since the gain of resistance apparently requires an exact response. Additionally, the growth phase at the time of first contact with hop compounds could influence the acquisition of hop resistance, since cellular divalent cation levels are altered during the growth cycle. Once the culture has acquired hop resistance, protein expression is independent of growth phase (from early exponential to transient growth phase). This indicates that only a strict adherence to a hop resistance protein profile enables long-term survival. The bottom line is a metab-

olism in economic mode by effective substrate conversion and prevention of exhaustive de novo protein synthesis. Our findings demonstrate with multiple evidence that hop stress in bacteria not only is associated with PMF depletion but obviously implies divalent cation limitation. A closer look at manganese-dependent enzymes and regulators should be useful for detection of beer spoilage lactobacilli and the preservation of beer.

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