The production and biochemical characterization of α-carbonic anhydrase from *Lactobacillus rhamnosus* GG

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Abstract

We report the production and biochemical characterization of an α-carbonic anhydrase (LrhCA) from gram-positive probiotic bacteria *Lactobacillus rhamnosus* GG. CAs form a family of metalloenzymes that catalyze hydration of CO2/interconversion between CO2 and water to bicarbonate ions and protons. They are divided into eight independent gene families (α, β, γ, δ, ζ, η, θ, and ι). Interestingly, many pathogens have been identified with only β- and/or γ-CAs, which can be targeted with CA-specific inhibitors (CAIs) acting as anti-pathogen drugs. Since it is important to study the potential off-target effects of CAIs for both the human body and its commensal bacteria, we took *L. rhamnosus* GG as our study subject. To date, only a single α-CA has been identified in *L. rhamnosus* GG, which was successfully produced and biochemically characterized. LrhCA showed moderate catalytic activity with the following kinetic parameters: $k_{cat}$ of 9.86 × 10^5 s⁻¹ and $k_{cat}/K_M$ of 1.41 × 10⁷ s⁻¹ M⁻¹. Moderate inhibition was established with 11 of the 39 studied sulfonamides. The best inhibitors were 5-((4-aminophenyl)sulfonamido)-1,3,4-thiadiazole-2-sulfonamide, 4-(2-hydroxymethyl-4-nitrophenyl-sulfonamidoethyl)-benzenesulfonamide, and benzoamide with $K_i$ values of 319 nM, 378 nM, and 387 nM, respectively. The other compounds showed weaker inhibitory effects. The $K_i$ of acetazolamide, a classical CAI, was 733 nM. In vitro experiments with acetazolamide showed that it had no significant effect on cell growth in *L. rhamnosus* GG culture. Several sulfonamides, including acetazolamide, are in use as clinical drugs, making their inhibition data highly relevant to avoid any adverse off-target effects towards the human body and its probiotic organisms.

Key points

- The α-carbonic anhydrase from *Lactobacillus rhamnosus* GG (LrhCA) is 24.3 kDa.
- LrhCA has significant catalytic activity with a $k_{cat}$ of 9.9 × 10⁵ s⁻¹.
- Acetazolamide resulted in a marginal inhibitory effect on cell growth.

Keywords  Alpha carbonic anhydrase · *Lactobacillus rhamnosus* · Sulfonamide inhibition · Kinetics · In vitro inhibition

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Introduction

To date, we know that there are similar numbers of cells from bacteria and other microorganisms (MOs) as there are individual cells in our body (Sender et al. 2016b); and when comparing nucleated cells, only the ratio reaches roughly 13:1 in favor of bacteria (Sender et al. 2016a). Many of our vital body functions depend on MOs living in our gut, mouth, stomach, skin, and urogenital areas including bacteria, archaea, eukarya, and even bacteriophages (Jalava 2008; Sender et al. 2016b). They affect human physiology, immunity, and nutrition, for example, by aiding us to food digestion, enabling vitamin intake, and reinforcing our immune system (Heikkilä 2018; Smith and Ravel 2017). Our
body is constantly bombarded with numerous opportunistic pathogens. In many areas, much of our body’s natural defense system owes its efficiency to our commensal MOs. These microbes keep us healthy and our bodies functioning normally. The delicate balance of microflora can be easily disturbed through therapeutic applications available today, mainly antibiotics. Paradoxically, antibiotics are both beneficial and detrimental to the homeostasis of MOs in our body. They clear out many infectious agents while at the same time destroying protective commensal MOs. This is one of the reasons that there is an urgent need for medicines with novel mechanisms of action and an improved benefit/risk ratio.

Carbonic anhydrases (CAs) are ubiquitous metalloenzymes present in organisms spanning all kingdoms of life (Supuran and De Simone 2015). CAs belong to eight evolutionary divergent gene families (α, β, δ, γ, ζ, η, θ, and i) that vary in tissue distribution, kinetics, inhibition, and activation profiles (Alterio et al. 2014; Frost and McKenna 2013; Jensen et al. 2019; Kikutani et al. 2016; Modak et al. 2013). These different classes share little amino acid or structural similarities but have convergent functionality. All of them require a metal ion (usually a zinc ion) at their active site, hence the name “metalloenzymes” (Kim et al. 2020). Their catalytic abilities transform CO2 via hydrolysis to bicarbonate and protons — a reaction that is essential for many vital physiological processes in organisms such as acid–base balance, gluconeogenesis, CO2 transport, and photosynthesis (Smith and Ferry 2000; Supuran 2011).

Excluding alpha CAs, other CA groups have emerged as new and promising anti-infective targets. This drives us towards designing CA-specific inhibitors (CAIs) that bind to the target CA and block its function, ultimately leading to the elimination of pathogens (Aspatwar et al. 2020; Kaur et al. 2020; Supuran et al. 2003). Current therapeutics, mainly antibiotics, have the unfortunate side effect of affecting not only target pathogens but also the probiotic bacteria in our bodies. In the same way, as the design of specific CAIs can reduce off-target effects against human CAs, it is equally important not to affect the CAs of commensal bacteria. From the eight known CA groups, humans, and some commensal lactobacilli — only possess CAs belonging to the α-group. Simultaneously, many pathogens have been discovered with only β- and/or γ-CA-encoding genes in their genomes (Supuran 2011). Based on this finding, CAIs designed to target β- and/or γ-CAs can be considered an intriguing option for antimicrobial drugs compared to classical antibiotics. By affecting only the CAs of target pathogens in the body, both the CA functions of human and the probiotic bacteria like lactobacilli will remain undisturbed.

Lactobacillus species are a normal part of the flora of human mouth, gastrointestinal system, and female genital tract (Bratcher 2018). In the latter, lactobacilli are known to produce lactic acid to create acidic conditions against pathogenic invasion (Witkin et al. 2007). In the gut and vagina, lactobacilli form biofilms that make them more resilient in harsh environmental conditions and maintain ample populations (Salas-Jara et al. 2016). Lactobacilli do not cause human disease, and some Lactobacillus species are used as consumable human probiotics (Barrons and Tassone 2008; Doron et al. 2005; Falagas et al. 2007; Reid 2001).

Lactobacillus rhamnosus is one example of probiotic bacteria which are used in dairy products, such as yogurt and semihard cheese. It was initially thought to be a subspecies of L. casei but was later confirmed as an individual species (Avlamis et al. 2001). These short, gram-positive, facultative bacteria can grow either in the presence or absence of oxygen. L. rhamnosus has a broad spectrum of strains isolated from various environments such as the human gastrointestinal tract and vagina (Ceapa et al. 2016). L. rhamnosus GG is a strain isolated in 1983 from human intestinal tract by Sherwood Gorbach and Barry Goldin. Thereafter, L. rhamnosus GG has been studied extensively and is currently the world’s most studied probiotic bacterial species (Avlamis et al. 2001; Silva et al. 1987).

In this study, we have produced and characterized a novel α-CA from L. rhamnosus GG. We also tested the inhibition properties of this enzyme using several sulfonamide compounds. Finally, we also investigated the effect of the clinical drug, acetazolamide, on the growth and survival of L. rhamnosus GG bacteria.

Materials and methods

Protein expression and recombinant production

We used a pBVboostFG-based plasmid (Laitinen et al. 2005) as protein expression vector to produce L. rhamnosus GG α-CA (UniProt (Hunter et al. 2009) protein entry: C8URX6) (UniProt Consortium 2002-2017) in BL21 Star Escherichia coli (OneShot® BL21 Start™ (DE3) chemically competent cells, #C601003, Thermo Fisher Scientific, Waltham, USA). The subcloned insert was designed to contain Gateway-compatible recombination sites (attL1, attL2), Shine-Dalgarno and Kozak sequences, 6xHis-tag with surrounding spacer regions (MSTT and ATAIPPT (Piao et al. 2008)), LrhCA, and a thrombin cleavage site (LVPRGS (Hilvo et al. 2008)) (Fig. 1).

GeneArt (Thermo Fisher Scientific, Waltham, USA) was responsible for gene synthesis and subcloning. Transformation — following the guidelines of Thermo Fisher Scientific OneShot® BL21(DE3) competent cells manual (part no. 28–0182). Cells were cultured at 37 °C in Luria–Bertani (LB) medium supplemented with 10 mg/mL gentamicin (1:1000, v/v) until optical density (ODs595) of 0.4–0.6 was reached. Expression of the protein was
induced by adding 1 M isopropyl β-D-1-isopropyl-thiogalactopyranoside (IPTG) 1:1000 (v/v), and afterwards, the culturing was continued overnight at 37 °C. Harvesting of the cells was performed by centrifugation at 5000 g for 15 min at 4 °C.

Harvested cells were mechanically disrupted with EmulsiFlex-C3 homogenizer (AVESTIN, Ottawa, Canada), and the lysate was centrifuged at 20,000 g for 15 min at 4 °C. Supernatant was combined with Protino® nickel-nitrilotriacetic acid (Ni2+-NTA) agarose affinity chromatography resin (Macherey–Nagel GmbH Co., Düren, Germany) and 50 mM Na2HPO4, 0.5 M NaCl, and 50 mM imidazole (binding buffer [BB]; pH 8.0, 1:3 [vol/vol]) for 2 h at room temperature (RT) on a constant agitation. After incubation, the resin was packed into a chromatography column with an EMD Millipore™ vacuum filtering flask (Merck, #XX1004705, Darmstadt, Germany). Prior to elution, the resin was washed generously with BB. The protein was eluted with 50 mM Na2HPO4, 0.5 M NaCl, and 350 mM imidazole (pH 7.0). The eluted fractions were pooled and concentrated on a 5000 MWCO polyethersulfone membrane (Vivaspin 2, Vivascience Sartorius group, VS0211) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The yield was determined by NanoDrop One (Thermo Fisher Scientific, Waltham, USA).

For further analysis and storage at 4 °C, the buffer of the protein sample was changed to 50 mM Tris–HCl (pH 7.5) with a 5000 MWCO polyethersulfone membrane (Vivaspin 2, Vivascience Sartorius group, VS0211). His-tag was removed from the purified protein with thrombin (Sigma-Aldrich, #RECOMT, St. Louis, USA) treatment according to the Sigma-Aldrich CleanCleave™ Kit manual (available on the Sigma-Aldrich website). Prior to use, the thrombin resin (Sigma-Aldrich, #RECOMT, St. Louis, MO, USA) was washed with 50 mM Tris–HCl (pH 7.5) according to the Sigma-Aldrich CleanCleave™ Kit manual. The protein sample was combined with the thrombin resin (1 ml of thrombin resin aliquot/1 mg protein) was incubated with the resin overnight at room temperature in gentle agitation. After incubation, the thrombin resin was packed into a chromatography column provided by the Sigma-Aldrich CleanCleave™ Kit, and the protein sample was collected. Analysis of cleavage reaction was done by SDS-PAGE.

**Kinetic studies**

For assaying of CA-catalyzed CO2 hydration activity an Applied Photophysics, stopped-flow instrument was used (Khalifah 1971). The pH indicator was phenol red (at a concentration of 0.2 mM), working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer and 20 mM Na2SO4 (for maintaining constant ionic strength), following the initial rates of the CA-catalyzed CO2 hydration reaction for a period of 10 − 100 s. For the determination of the kinetic parameters and inhibition constants, CO2 concentrations ranged from 1.7 to 17 mM.

**Inhibitory studies**

**Sulfonamide inhibition**

Inhibition of LrhCA was investigated with a set of designated sulfonamides, involving 15 clinically used drugs acetazolamide through hydrochlorothiazide (AAZ-HCT) and their heterocyclic derivatives (1–24). For the determination of the initial velocity, at least six traces of the initial 5–10% of the reaction were used for each inhibitor. The uncatalyzed rates were determined similarly and subtracted from the total observed rates. Stock solutions of the inhibitor (0.1 mM) were prepared in distilled-deionized water. Subsequent series of dilutions up to 0.01 nM were prepared with distilled-deionized water. Inhibitor (I) and enzyme (E) solutions were preincubated together for 15 min at room temperature prior to the assay to allow formation of the E-I complex. The inhibition constants were obtained by nonlinear least squares methods using PRISM 3. The mean values were calculated from at least three different determinations (data not shown).

**In vitro inhibition assay with acetazolamide**

The effect of AAZ on cell growth was investigated with 96- and 24-well microplates (Nunc™, Thermo Fisher, Waltham, USA) and 15 ml test tubes. The plates and the test tubes were inoculated with (OD595 = 0.45) overnight anaerobic
MRS pre-culture (37 °C, 170 rpm) of *L. rhamnosus* GG. The AAZ solution was prepared by diluting AAZ powder (Diamox by Mercury Pharmaceuticals Ltd., Finlab, Tampere, Finland) with sterile water. Subsequently, the studied concentrations were done by serial dilution in MRS. In case of microplates, 4–6 separate samples were prepared with half of the well volume being cells and the other half of the studied AAZ dilution (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM, and 10 mM). Control samples without inhibitor (cells only) were prepared as well. The microplates and the test tubes were cultured anaerobically/ aerobically in MRS at 37 °C and 230–400 rpm (PST-60HL Plate-Shaker-Thermostat, Biosan, Riga, Latvia/MaxQ™ 6000 Incubated Shaker, Thermo Fisher, Waltham, USA). The total culturing time was set for 96 h, with OD595 measurements in time points 0, 24, 48, 72, and 96 h. The cultures were analyzed for OD595 with UV/VIS Envision Multimode Plate Reader (PerkinElmer Oy, Finland). Each condition was studied three times, from which the mean was calculated to obtain the growth rate.

### Results

#### Attempts of endogenous protein purification

The sequence data of the genome of *L. rhamnosus* (strain ATCC 53,103/GG) was obtained from UniProt and used to identify LrhCA gene (UniProt ID C8URX6) (Uniprot Consortium 2002-2017). The presence of the LrhCA gene in *L. rhamnosus* bacteria was confirmed using colony- and cDNA-polymerase chain reaction (PCR) (data not shown). To obtain the cDNA-PCR result, mRNA was isolated from *L. rhamnosus* GG cells and transcribed to cDNA to see the expression level of the α-CA. Primers used in the studies are presented in Table 1.

Since *L. rhamnosus* could be obtained from commercial probiotic products (Gefilus Basic capsules containing *L. rhamnosus* GG strain, Yliopiston Apteekki, Helsinki, Finland), we first planned experiments to isolate CA protein from these bacteria. Attempts to purify LrhCA from the bacteria were designed according to the inhibitor affinity chromatography and procedures that have been previously used to isolate human endogenous CAs (Falkbring et al. 1972; Johansen 1976; Maupin et al. 2009; Wistrand and Knuttila 1989). *L. rhamnosus* was successfully cultured on Petri dishes as well as in broth media (MRS, whey). After the inhibitor affinity chromatography, only a faint band equal to the theoretical size of LrhCA (Mw = 24.3 kDa) could be detected on SDS-PAGE. However, the identification of the protein by mass spectrometry (MS/MS) failed to confirm the product as LrhCA (data not shown). The purification procedure was repeated multiple times by culturing the cells in the presence or absence of O2 and by altering temperatures, pH, culturing time, medium broth (MRS-glucose, MRS-galactose, whey), the amount of resin, incubation time with the resin, and washing and elution procedures. In addition to *L. rhamnosus* GG, the cultured lactobacilli strains finally included *L. rhamnosus* DSMZ, *L. helveticus* FMB Lh5, and *L. paracasei* LPC-S01. After several unsuccessful attempts, we initiated recombinant production instead of isolating the endogenous CA protein.

#### Recombinant protein production

Recombinant LrhCA was successfully expressed in *E. coli* and purified by affinity chromatography. The yield of the protein was approximately 0.1 mg of purified protein per L of culture. Removal of the 6xHis-tag was carried out by treatment with thrombin (Sigma-Aldrich, St. Louis, MO, USA) followed by Ni2+.-NTA purification and analysis via SDS-PAGE. In Fig. 2, the produced recombinant LrhCA is seen as a two-band polypeptide (lane 2, without His-tag). The size of the band visualized on the gel is in line with the theoretical mass of LrhCA, Mw = 24.3 kDa. All polypeptide bands on both lanes were identified as LrhCA by tandem mass spectrometry (MS/MS), the 48 kDa band therefore representing a dimer.

#### Kinetic studies

The kinetic characteristics of LrhCA are shown in Table 2, alongside human α-CA isoforms I, II and III, for comparison. The kinetic results show that the enzymatic activity of LrhCA is between the values reported for human CA I and II. Notably LrhCA exhibited almost five times higher kcat value compared to hCA I but 3.5 times lower kcat/Km.

#### Inhibitory studies

**Sulfonamide inhibition**

Table 3 shows the inhibition results of designated sulfonamide derivatives 1–24 and the clinically used drugs AAZ-HCT. The molecular structures of AAZ-HCT and the derivatives 1–24 are shown in Fig. 3.
From the inhibition data presented in Table 3, the following observations could be made:

(i) The three most efficient sulfonamide inhibitors for LrhCA were 5-((4-aminophenyl)sulfonamido)-1,3,4-thiadiazole-2-sulfonamide (compound 20), 4-(2-hydroxymethyl-4-nitrophenyl-sulfonamidoethyl)-benzenesulfonamide (compound 21), and benzolamide (BZA) with $K_i$ values of 319 nM, 378 nM, and 387 nM, respectively.

(ii) In 14 of the studied sulfonamides, the result was a $K_i < 1 \mu M$, 11 of which had a $K_i$ of $< 720 \text{nM}$ and could therefore be regarded as having a moderate inhibitory effect on LrhCA. These inhibitors were, in addition to those already mentioned in part (i), 4-(2-hydroxymethyl-4-nitrophenyl-sulfonamido)ethylbenzenesulfonamide (compound 24), brinzolamide (BRZ), dorzolamide (DRZ), 4-(4-sulfaminy1-aminomethyl)-benzenesulfonamide (compound 23), topiramate (TPM), sulfanylated sulfonamide (compound 22), 4-hydroxyethyl-benzenesulfonamide (compound 17), and 4-methyl-5-imino-1,3,4-thiadiazole-2-sulfonamide (compound 14). The compounds with a $K_i$ in the range of 733–902 nM and therefore considered to be medium-weak inhibitors were AAZ, methazolamide (MZA), 5-amino-1,3,4-thiadizole-2-sulfonamide (compound 13), and ethoxzolamide (EZA).

(iii) Out of the studied sulfonamides, 24 had a $K_i$ value in the range of 1–10 $\mu M$, indicating little or no inhibition of LrhCA. These ineffective inhibitors were the simple derivatives 4-aminomethyl-benzenesulfonamides (compounds 5 and 6), 3-fluoro-4-aminobenzenesulfonamide (compound 7), 3-bromo-4-aminobenzenesulfonamide (compound 9), and 4-amino-/4-hydroxymethyl-benzenesulfonamide (compound 15) as well as the clinically used drugs sulpiride.

Table 2 Kinetic properties of LrhCA, and human isoforms CA I, CA II, and CA III, for comparison

| Enzyme          | $k_{cat}$ (s$^{-1}$) | $K_M$ (nM) | $k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$) | $K_i$ (AAZ$^1$, nM) | Ref                                 |
|-----------------|---------------------|------------|----------------------------------|---------------------|------------------------------------|
| LrhCA$^5$      | $9.9 \times 10^5$   | 7.0        | 1.4 $\times 10^7$               | 733                 | Current study                      |
| hCA I$^2$      | $2.0 \times 10^5$   | 4.0        | 5.0 $\times 10^7$               | 250                 | (Supuran 2008; Supuran et al. 2003) |
| hCA II$^3$     | $1.4 \times 10^6$   | 9.3        | 1.5 $\times 10^8$               | 12                  | (Supuran 2008; Supuran et al. 2003) |
| hCA III$^4$    | $1.0 \times 10^8$   | 33.3       | 3.0 $\times 10^5$               | > 10,000            | (Supuran 2008; Supuran et al. 2003) |

$^1$AAZ acetazolamide, $^2$hCA I human carbonic anhydrase isozyme I, $^3$hCA II human carbonic anhydrase isozyme II, $^4$hCA III human carbonic anhydrase isozyme III, $^5$LrhCA α-carbonic anhydrase of Lactobacillus rhamnosus GG
(iv) The clinically used drug AAZ showed moderate/weak inhibition of LrhCA with a $K_i$ of 733 nM. The corresponding values with hCA I and hCA II are 250 nM and 12 nM, respectively.

**Table 3** Sulfonamide inhibition data of the α-carbonic anhydrase of *L. rhamnosus* (LrhCA) and human isoforms CA I and CA II, for comparison. All the CAs studied here belong to the enzyme class α.

| Inhibitor | hCA I | hCA II | LrhCA |
|-----------|-------|--------|--------|
| 1         | 28,000| 300    | 7611   |
| 2         | 25,000| 240    | 7583   |
| 3         | 79    | 8      | 3019   |
| 4         | 78,500| 320    | 5603   |
| 5         | 25,000| 170    | >10,000|
| 6         | 21,000| 160    | >10,000|
| 7         | 8300  | 60     | >10,000|
| 8         | 9800  | 110    | 8983   |
| 9         | 6500  | 40     | >10,000|
| 10        | 7300  | 54     | 5029   |
| 11        | 5800  | 63     | 7556   |
| 12        | 8400  | 75     | 7927   |
| 13        | 8600  | 60     | 866    |
| 14        | 9300  | 19     | 691    |
| 15        | 5500  | 80     | >10,000|
| 16        | 9500  | 94     | 6006   |
| 17        | 21,000| 125    | 681    |
| 18        | 164   | 46     | 2707   |
| 19        | 109   | 33     | 4407   |
| 20        | 6     | 2      | 319    |
| 21        | 69    | 11     | 378    |
| 22        | 164   | 46     | 559    |
| 23        | 109   | 33     | 487    |
| 24        | 95    | 30     | 403    |
| AAZ       | 250   | 12     | 733    |
| MZA       | 50    | 14     | 837    |
| EZA       | 25    | 8      | 902    |
| DZA       | 50,000| 9      | 474    |
| BRZ       | 45,000| 3      | 433    |
| BZA       | 15    | 9      | 387    |
| TPM       | 250   | 10     | 508    |
| ZNS       | 56    | 35     | 8946   |
| SLP       | 1200  | 40     | >10,000|
| IND       | 31    | 15     | >10,000|
| VLX       | 54,000| 43     | >10,000|
| CLX       | 50,000| 21     | >10,000|
| SLT       | 374   | 9      | 9314   |
| SAC       | 18,540| 5959   | >10,000|
| HCT       | 328   | 290    | >10,000|

*Mean from three different assays, measured by stopped-flow CO$_2$ hydrase assay method (Khalifah 1971). Errors are in the range of 5–10% of the reported data. *Human recombinant isozymes, data published previously (Supuran 2008)
these groups of CAs are absent in humans and lactobacilli. Many of the sulfonamides studied in this report are in use as clinical drugs making inhibition data important information when designing and using novel CAIs with the least adverse side/off-target effects towards human body and its probiotic organisms. Therefore, commensal and probiotic bacteria \textit{L. rhamnosus} GG, which carries only an α-CA in its genome, is a fascinating study subject to learn if the use of CAIs targeting β- or γ-CAs is tolerated.

The α-CA of \textit{L. rhamnosus} GG was successfully produced as a recombinant protein, resulting in a pure protein sample after metal-affinity chromatography. SDS-PAGE revealed a band corresponding to the theoretical $M_w$ of 24.3 kDa and a trace of dimeric protein. A very similar result was reported previously by Li et al. (2015), whose production of α-CA from \textit{L. delbrueckii} resulted in a double-band polypeptide on SDS-PAGE with the $M_w$ of 23.8 kDa (Li et al. 2015). Prior to the recombinant protein production, we failed to isolate the endogenously produced CA from \textit{L. rhamnosus} GG and from other \textit{Lactobacillus} species tried. Inhibitory studies revealed that LrhCA has low affinity towards several sulfonamide CA inhibitors. Likely, this is the reason for the inhibitor affinity chromatography based on p-aminomethylbenzene sulfonamide not being successful. In a study by Mendes et al., another plausible explanation emerged; they observed no CA expression in \textit{Lactobacillus plantarum} or in \textit{L. delbrueckii} subsp. \textit{bulgaricus} cultures supplied with CO$_2$, even though the presence of the CA gene was confirmed (Mendes et al. 2013). It has also been discovered that CA expression is present only during non-exponential growth of bacteria, i.e.,

![Molecular structures of sulfonamide derivatives](image-url)
in stationary phases (16–31 h). This seems to occur when they shift from glucose fermentation to galactose and from homolactic to mixed acid fermentation (Laakso et al. 2011). Hence, we also attempted the isolation from lactobacilli cultured in the presence of both MRS-galactose instead of MRS-glucose and the same whey medium as used in the previous experiments. These experiments again showed no success. Although we consider the weak affinity of LrhCA towards the sulfonamide-coupled resin the most plausible explanation for the failure in isolation of the endogenous CA, the expression of α-CA in lactobacilli may also require very specific conditions, which warrant further studies.

A total of 11 of the 39 studied sulfonamides resulted in moderate inhibition; the rest had little or no inhibitory effect towards LrhCA. Many of these inhibitors possess a bulky scaffold with one or more substituents attached, which could interfere with effective binding within the active cavity of LrhCA. The most effective inhibitors were 5-((4-aminophenyl)sulfonamido)-1,3,4-thiadiazole-2-sulfonamide (compound 20), 4-(2-hydroxymethyl-4-nitrophenyl-sulfonamidoethyl)-benzenesulfonamide (compound 21), and BZA with $K_i$ values of 319 nM, 378 nM, and 387 nM, respectively. Our inhibition study provides valuable information for the development of potent and more specific molecules against β- and γ-CAs which could be designed against various pathogenic infections. As it is important to avoid CAIs that have affinity towards the human α-CAs, it is also important to prevent similar effects on the α-CAs of commensal bacteria, such as lactobacilli. To gain more information on this topic, we also performed an in vitro study to see the effect of the clinically used drug, AAZ, on lactobacilli cell growth. AAZ showed no significant effect on the growth of *L. rhamnosus* GG. The relatively weak affinity of AAZ ($K_i$ of 733 nM) towards LrhCA may be the main factor explaining this finding. It could also be possible that some of these bacteria express other CAs which compensate the potential loss of one enzyme. Of the known lactobacilli, *L. reuteri* has been identified with both an α-CA and additional γ-CA (Uniprot Consortium). However, we found no hits of a γ-CA or other CAs in the *L. rhamnosus* GG genome. The fact that AAZ did not inhibit the *L. rhamnosus* GG cell growth is a positive result considering the current usage of AAZ in treatment of glaucoma, epilepsy, etc. (Masini et al. 2013; Thiry et al. 2007). It is also encouraging for the future design of novel antimicrobial drugs resembling AAZ, since they may not impair the viability of the probiotic lactobacilli present in the human body.

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**Author contribution** All authors designed and conceived the experiments. LJU, MK, SB, and AA performed the experiments. All authors analyzed the data. LJU wrote the first draft of the manuscript, and all authors read and commented on previous versions of the manuscript. All authors have given approval to the final version of the manuscript. ‡Equal contribution as senior authors.

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**Data availability** The authors confirm that the data supporting the findings of this study are available within the article.
D’Ambrosio K, Supuran CT, De Simone G (2018) Are carbonic anhydrases suitable targets to fight protozoan parasitic diseases? Curr Med Chem 25(39):5266–5278. https://doi.org/10.2174/092967325666180326160121

De Vita D, Angeli A, Pandolfi F, Bortolami M, Costi R, Di Santo R, Suffredini E, Ceruso M, Del Prete S, Capasso C, Scipione L, Supuran CT (2017) Inhibition of the alpha-carbonic anhydrase from Vibrio cholerae with amides and sulfonamides incorporating imidazole moieties. J Enzyme Inhib Med Chem 32(1):798–804. https://doi.org/10.1080/14756366.2017.1327522

Doron S, Snyderman DR, Gorbach SL (2005) Lactobacillus GG: bacteriology and clinical applications. Gastroenterology clinics of North America 34(3):483–98, ix. https://doi.org/10.1016/j.gtc.2005.05.011

Falagas M, Betsi GI, Athanasiou S (2007) Probiotics for the treatment of women with bacterial vaginoses. Clin Microbiol Infect 13(7):657–664

Falkbring SO, Gothe PO, Nyman PO, Sundberg L, Porath J (1972) Affinity chromatography of carbonic anhydrase. FEBS Lett 24(2):229–235. https://doi.org/10.1016/0014-5793(72)80773-7

French GL (2010) The continuing crisis in antibiotic resistance. Int J Antimicrob Agents 36(Suppl 3):S3-7. https://doi.org/10.1016/S0924-8579(10)00030-0

Frost SC, McKenna R (2013) Carbonic anhydrase: mechanism, regulation, links to disease, and industrial applications. Springer 1st Edn. Subcell Biochem. https://doi.org/10.1007/978-94-007-3759-2

Heikilä M (2018) Bakteerit hoitavat koko kehoa. Tiede 10. https://www.tiide.fi/artikkelit/tilaajille/bakteerit-hoitavat-koko-kehoa. Accessed 24 May 2021

Hilvo M, Baranauskiene L, Salzano AM, Scaloni A, Matulis D, Incenzi A, Scozzafava A, Monti SM, Di Fiore A, De Simone G, Lindfors M, Janis J, Valjakka J, Pastorekova S, Pastorek J, Kulo- maa MS, Nordlund HR, Supuran CT, Parkkila S (2008) Biochemical characterization of CA IX, one of the most active carbonic anhydrase isozymes. J Biol Chem 283(41):27791–27809. https://doi.org/10.1074/jbc.M800938200

Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Das U, Daugherty L, Duqueinne L, Finn RD, Gough J, Haft D, Hulo N, Kahn D, Kelly E, Lauraud A, Letunic I, Lonsdale D, Lopez R, Madera M, Maslen J, McAnulla C, McDowell J, Mistry J, Mitchell A, Mulder N, Natale D, Orengo C, Quinn AF, Selengut JD, Sgrist JIA, Thimma M, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2009) InterPro: the integrative protein signature database. Nucleic acids research 37(Database issue):D211-D215. https://doi.org/10.1093/nar/gkn785

Jensen EL, Clement R, Kosta A, Maberly SC, Gonterro B (2019) A new widespread subclass of carbonic anhydrase in marine phytoplankton. ISME J 13(8):2094–2106. https://doi.org/10.1038/s41396-019-0426-8

Johansen JT (1976) Isolation of human carbonic anhydrase B and C and apocarbonic anhydrase by affinity chromatography. Carlsberg Res Commun 41(2):73–80. https://doi.org/10.1016/BF02906418

Kaur J, Cao X, Abutaleb NS, Elkashif A, Graboski AL, Krabill AD, AbdelKhalek AH, An W, Bhardwaj A, Seleem MN, Flaherty DP (2020) Optimization of acetazolamide-based scaffold as potent inhibitors of vancomycin-resistant Enterococcus. J Med Chem 63(17):9540–9562. https://doi.org/10.1021/acs.jmedchem.0c00734

Khalifah RG (1971) The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human iso- enzymes B and C. J Biol Chem 246(8):2561–73

Kikutani S, Nakajima K, Nagasato C, Tsuji Y, Miyatake A, Matsuda Y (2016) Thylakoid luminal theta-carbonic anhydrase critical for growth and photosynthesis in the marine diatom Phaeodactylum tricornutum. Proc Natl Acad Sci USA 113(35):9828–9833. https://doi.org/10.1073/pnas.1603112113

References

Alterio V, Pan P, Parkkila S, Buonanno M, Supuran CT, Monti SM, De Simone G (2014) The structural comparison between membrane-associated human carbonic anhydrases provides insights into drug design of selective inhibitors. Biopolymers 101(7):769–778. https://doi.org/10.1002/bip.22456

Aspatwar A, Hammaren M, Parikka M, Parkkila S, Carta F, Bozdag M, Vullo D, Supuran CT (2020) In vitro inhibition of Mycobacterium tuberculosis beta-carbonic anhydrase 3 with mono- and dithiocarbamates and evaluation of their toxicity using zebrafish developing embryos. J Enzyme Inhib Med Chem 35(1):65–71. https://doi.org/10.1080/14756366.2019.1683007

Aspatwar A, Kairy T, Rala S, Parikka M, Bozdag M, Carta F, Supuran CT, Parkkila S (2019) Mycobacterium tuberculosis beta-carbonic anhydrases: novel targets for developing antituberculosis drugs. Int J Mol Sci 20(20). https://doi.org/10.3390/ijms20205153

Avlami A, Kordossis T, Vrizidis N, Sipsas NV (2001) Lactobacillus rhamnosus endocarditis complicating colonoscopy. J Infect 42(4):283–285. https://doi.org/10.1053/jinf.2001.0793

Barrons R, Tassone D (2008) Use of Lactobacillus probiotics for bacterial genitourinary infections in women: a review. Clin Ther 30(3):453–468. https://doi.org/10.1016/j.clinthera.2008.03.013

Bratcher DF (2018) Other Gram-Positive Bacilli. Principles and Practice of Pediatric Infectious Diseases. 5th edn. Elsevier 133:786–790.e4. https://doi.org/10.1016/B978-0-323-40181-4.00133-X

Ceapa C, Davids M, Ritari J, Lambert J, Wels M, Douillard FP, Smokvina T, de Vos WM, Knol J, Kleerebezem M (2016) The variable regions of Lactobacillus rhamnosus genomes reveal the dynamic evolution of metabolic and host-adaptation repertoires. Genome Biol Evol 8(6):1889–1905. https://doi.org/10.1093/gbe/evw123

Cully M (2019) Antibiotics alter the gut microbiome and host health. Nat Milestones:519. Publisher. https://www.nature.com/articles/d42859-019-00019-x. Accessed 24 May 2021
Kim JK, Lee C, Lim SW, Adhikari A, Andring JT, McKenna R, Ghim C-M, Kim CU (2020) Elucidating the role of metal ions in carbonic anhydrase catalysis. Nat Commun 11(1):4557. https://doi.org/10.1038/s41467-020-18422-5

Laakso K, Koskenniemi K, Koponen J, Kankainen M, Surakka A, Salusjärvi T, Auvinen P, Savijoki K, Nyman TA, Kalkkinen N, Tynkkynen S, Varmanen P (2011) Growth phase-associated changes in the proteome and transcriptome of Lactobacillus rhamnosus in industrial-type whey medium. Microbiot Biotechnol 4(6):746–766. https://doi.org/10.1038/111751i-7915.2011.00275.x

Laitinen OH, Airenne KJ, Hytonen VP, Peltonia E, Mahonen AJ, Wirth T, Lind MM, Makela KA, Toivanen PI, Schenkwein D, Heikura T, Nordlund HR, Kulomaa MS, Yla-Herttuala S (2005) A multipurpose vector system for the screening of libraries in bacteria, insect and mammalian cells and expression in vivo. Nucleic Acids Res 33(4):e42. https://doi.org/10.1093/nar/gni042

Li C-X, Jiang X-C, Qiu Y-J, Xu J-H (2015) Identification of a new thermotable and alkaline-tolerant α-carbonic anhydrase from Lactobacillus delbrueckii as a biocatalyst for CO2 biomineralization. Bioresour Bioproc 2(1):44. https://doi.org/10.1186/s40643-015-0074-4

Masini E, Carta F, Scozzafava A, Supuran CT (2013) Antiglaucoma carbonic anhydrase inhibitors: a patent review. Expert Opin Ther Patents 23(6):705–716. https://doi.org/10.1517/15543776.2013.974788

Maupin CM, Zheng J, Tu C, McKenna R, Silverman DN, Voth GA (2009) Effect of active-site mutation at Asn67 on the proton transfer mechanism of human carbonic anhydrase II. Biochemistry 48(33):7996–8005. https://doi.org/10.1021/bi901037u

Mendes F, Sieuwerts S, de Hulster E, Almering MJ, Luttik MA, Pronk JT, Smid EJ,Bron PA, Daran-Lapujade P (2013) Transcriptome-based characterization of interactions between Saccharomyces cerevisiae and Lactobacillus delbrueckii subsp bulgaricus in lactose-grown chemostat cocultures. Appl Environ Microbiol 79(19):5949–61. https://doi.org/10.1128/aem.01115-13

Modak JK, Revitt-Mills SA, Roujeinikova A (2013) Cloning, purification and preliminary crystallographic analysis of the complex of Helicobacter pylori alpha-carbonic anhydrase with acetazolamide. Acta Crystallogr Sect F Struct Biol Cryst Commun 69(Pt 11):1252–1255. https://doi.org/10.1107/S1744309113026146

NHS (2019) Antibiotics - side-effects. Publisher. https://www.nhs.uk/conditions/antibiotics/side-effects/ Accessed 15.11.2021

Pal DS, Mondal DK, Datta R (2015) Identification of metal dithiocarbamates as a novel class of antileishmanial agents. Antimicrob Agents Chemother 59(4):2144–2152. https://doi.org/10.1128/AAC.00514-16

Pal DS, Abbasi M, Mondal DK, Varghese BA, Paul R, Singh S, Datta R (2017) Interplay between a cytosolic and a cell surface carbonic anhydrase in pH homeostasis and acid tolerance of Leishmania. J Cell Sci 130(4):754–766. https://doi.org/10.1242/jcs.199422

Piao S, Xu Y, Ha NC (2008) Crystallization and preliminary X-ray crystallographic analysis of MacA from Actinobacillus actinomycetemcomitans. Acta Crystallogr Sect F Struct Biol Cryst Commun 64(Pt 5):391–393. https://doi.org/10.1107/S1744309108008701

Reid G (2001) Probiotic agents to protect the urogenital tract against infection. Am J Clin Nutr 73(2 Suppl):437S–443S. https://doi.org/10.1093/ajcn/73.2.437s

Salas-Jara MJ, Ibaraca A, Vega M, Garcia A (2016) Biofilm forming Lactobacillus: new challenges for the development of probiotics. Microorganisms 4(3). https://doi.org/10.3390/microorganisms4030035

Sender R, Fuchs S, Milo R (2016) Are we really vastly outnumbered? Revisiting the Ratio of bacterial to host cells in humans. Cell 164(3):337–340. https://doi.org/10.1016/j.cell.2016.01.013

Sender R, Fuchs S, Milo R (2016) Revised estimates for the number of human and bacteria cells in the body. PLoS Biol 14(8):e1002533. https://doi.org/10.1371/journal.pbio.1002533

Silva M, Jacobs SV, Deneke C, Gorbach SL (1987) Antimicrobial substance from a human Lactobacillus strain. Antimicrob Agents Chemother 31(8):1231–1233. https://doi.org/10.1128/aac.31.8.1231

Smith KS, Ferry JG (2000) Prokaryotic carbonic anhydrases. FEBS Microbiol Rev 24(4):335–366

Smith SB, Ravel J (2017) The vaginal microbiota, host defence and reproductive physiology. J Physiol 595(2):451–463. https://doi.org/10.1113/JP271694

Supuran CT (2008) Carbonic anhydrase inhibitors: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 7(2):168–181. https://doi.org/10.1038/nrd2467

Supuran CT (2011) Bacterial carbonic anhydrases as drug targets: toward novel antibiotics? Front Pharmacol 2:34. https://doi.org/10.3389/fphar.2011.00034

Supuran CT, Scozzafava A, Casini A (2003) Carbonic anhydrase inhibitors. Med Res Rev 23(2):146–189. https://doi.org/10.1002/med.10025

Supuran CT, De Simone G (2015) Carbonic anhydrases as biocatalysts: from theory to medical and industrial applications. Elsevier, 3–13. https://doi.org/10.1016/B978-0-444-63258-6.00001-9

Thiry A, Dogne JM, Supuran CT, Masereel B (2007) Carbonic anhydrase inhibitors as anticonvulsant agents. Curr Top Med Chem 7(9):855–864. https://doi.org/10.2174/156802607780636726

Tiesalo P (2015) Antibioticit voivat sekoittaa suoliston vuosiksi. Yle Uutiset. https://yle.fi/uutiset/3-7862878. Accessed 24 May 2021

Uniprot Consortium (2002–2017) UniProt. Publisher. https://www.uniprot.org/. Accessed 01 March 2017

Uniprot Consortium Seach results on Lactobacillus reuteri gamma carbonic anhydrase. Publisher. https://www.uniprot.org/uniprot/A0A6B3Y2U8. Accessed 15 April 2021

Wistrand PJ, Knuuttila KG (1989) Renal membrane-bound carbonic anhydrase catalysis. Nat Commun 11(1):4557. https://doi.org/10.1113/jphysiol.1989.sp.3390

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