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ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · JULY 2013

Impact Factor: 1.7 · DOI: 10.1016/j.pep.2013.07.002 · Source: PubMed

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Kinetic characterization of arginine deiminase and carbamate kinase from *Streptococcus pyogenes* M49

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ARTICLE INFO

Article history:
Received 22 May 2013
and in revised form 27 June 2013
Available online 16 July 2013

Keywords:
Arginine deiminase
Lactic acid bacteria
LAB
Streptococcus pyogenes
Carbamate kinase

ABSTRACT

Streptococcus pyogenes (group A *Streptococcus*, GAS) is an important human pathogen causing mild superficial infections of skin and mucous membranes, but also life-threatening systemic diseases. *S. pyogenes* and other prokaryotic organisms use the arginine deiminase system (ADS) for survival in acidic environments. In this study, the arginine deiminase (AD), and carbamate kinase (CK) from *S. pyogenes* M49 strain 591 were heterologously expressed in *Escherichia coli* DH5α, purified, and kinetically characterized. AD and CK from *S. pyogenes* M49 share high amino acid sequence similarity with the respective enzymes from *Lactococcus lactis* subsp. *lactis* IL1403 (45.6% and 53.5% identical amino acids) and *Enterococcus faecalis* V583 (66.8% and 66.8% identical amino acids). We found that the arginine deiminase of *S. pyogenes* is not allosterically regulated by the intermediates and products of the arginine degradation (e.g., ATP, citrulline, carbamoyl phosphate). The K_m and V_{max} values for arginine were 1.13 ± 0.12 mM (mean ± SD) and 1.51 ± 0.07 μmol/min/mg protein. The carbamate kinase is inhibited by ATP but unaffected by arginine and citrulline. The K_m and V_{max} values for ADP were 0.72 ± 0.08 mM and 1.10 ± 0.10 μmol/min/mg protein and the K_m for carbamoyl phosphate was 0.65 ± 0.07 mM. The optimum pH and temperature for both enzymes were 6.5 and 37 °C, respectively.

Introduction

Streptococcus pyogenes belongs to the group A streptococci (GAS) and is an important bacterial pathogen causing purulent respiratory tract and skin infections in humans in more than 720 million patients per year [1,2]. From the metabolic point of view, *S. pyogenes* belongs to the lactic acid bacteria (LAB)[1] generating energy from glucose by substrate chain phosphorylation under homofermentative production of lactic acid [3,4]. Under unfavorable conditions, the metabolism of *S. pyogenes* switches to mixed acid fermentation to generate more ATP. Furthermore, there is evidence that under glucose limited conditions *S. pyogenes* also uses L-arginine to obtain energy [3]. L-arginine is used by a number of microorganisms to generate ATP via the arginine deiminase system (ADS) [5,6] as shown in Fig. 1. Arginine degradation via ADS has been shown to generate sufficient energy to maintain growth of several LAB such as *Enterococcus faecalis* [7–9], *Lactobacillus planatrum* [10] and *Lactococcus lactis* [9,11].

The ADS essentially comprises the three enzymes arginine deiminase, ornithine-carbamoyltransferase, and carbamate kinase. Arginine deiminase (E.C. 3.5.3.6) is a hydrolase that converts L-arginine to ammonia and L-citrulline. The resulting L-citrulline is converted to carbamoyl phosphate and ornithine by ornithine-carbamoyltransferase (E.C. 2.1.3.3). The carbamate kinase (E.C. 2.7.2.2) transfers the phosphate group from carbamoyl phosphate to ADP and thereby generates ATP and ammonia. CK catalyzes not only the last step in the fermentative catabolism of arginine but also of agmatine and allantoin/oxalurate [12–14].

Next to its role in generation of ATP, it has been shown that ADS is the primary defense mechanism against acidic environments in oral streptococci and *S. pyogenes* [15,16]. Ammonium generated by the degradation of L-arginine by the ADS within and outside cells can raise the pH in the environment and in the cytoplasm of *S. pyogenes* [16]. Depending on the environmental niche of bacteria, ADS activity has different consequences. E.g., oral bacteria increase the local pH via ADS, which can neutralize the impacts of acidification from sugar metabolism and thus reduce the cariogenicity of oral biofilms [17]. For *S. pyogenes* it has been shown that AD activity leads to a potent inhibition of the proliferation of human T cells [18]. For *Lactobacillus sakei* it was shown that arginine degradation prolonged the viability of the organism in the stationary phase independent from the external pH [19].

Next to the natural role of ADS in bacteria, arginine deiminases proved to be promising tools in therapy of argininosuccinate

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[1] *Abbreviations used:* LAB, lactic acid bacteria; ADS, arginine deiminase system; ASS, argininosuccinate synthetase; PBS, phosphate buffered saline; KGA, α-ketoglutaric acid; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ORFs, open reading frames; CK, carbamate kinase; HCCs, hepatocellular carcinomas; ArcA, arginine deiminase; AP, alkaline phosphatase.

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http://dx.doi.org/10.1016/j.pep.2013.07.002

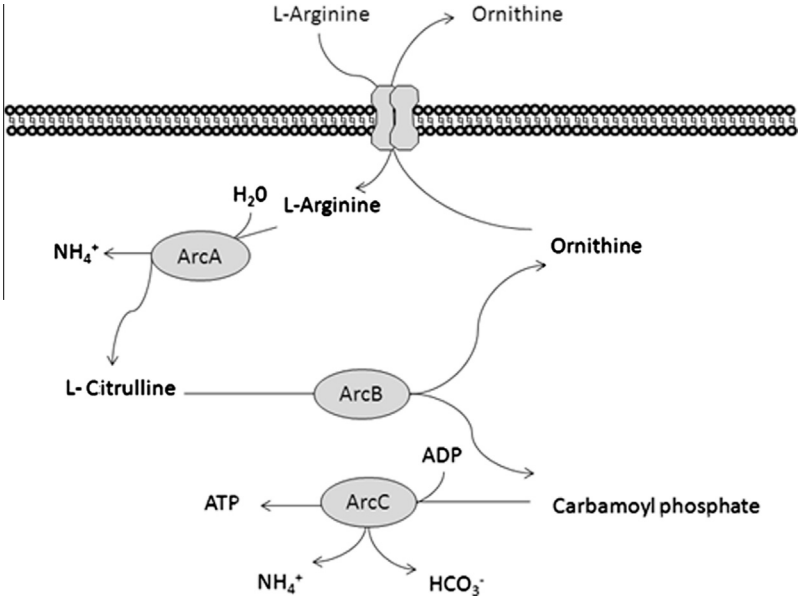

Fig. 1. Overview of the arginine deiminase system. Arginine is transported into the cell via an arginine ornithine antiporter. The arginine deiminase (ArcA) hydrolyzed arginine to ammonia and citrulline. Resulting citrulline is converted to ornithine and carbamoyl phosphate by the ornithine carbamoyltransferase (ArcB). The energetic reaction is the transfer of phosphate from carbamoyl phosphate to ADP. The phosphorylation is catalyzed by the carbamate kinase (ArcC).

synthetase (ASS) negative tumors [20,21]. These tumors are not able to synthesize arginine, they face nutrient starvation when arginine degrading enzymes such as AD or arginase are present [20–25]. E.g., beneficial effects of application of AD have been shown in patients with hepatocellular carcinoma and metastasized malignant melanoma [26,27]. Consequently, it is worthwhile investigating kinetic parameters of ADS enzymes for the understanding of bacterial amino acid metabolism on the one hand and for finding efficient arginine degrading enzymes for therapy of ASS negative tumors on the other hand.

In this study we heterologously expressed and characterized the arginine deiminase and carbamate kinase of *S. pyogenes*.

Methods

Strains and culture conditions

S. pyogenes M49 strain 591 was grown in Todd-Hewitt broth supplemented with 0.5% (wt/vol) yeast extract (THY medium) as standing cultures at 37 °C in a 5% CO_2/20% O_2 atmosphere. Unless otherwise indicated, *Escherichia coli* DH5α strains were grown in lysogeny broth (LB−) medium at 37 °C under vigorous shaking in ambient air conditions. For cultivation of recombinant *E. coli* DH5α strains carrying plasmid pASK-IBA2 or derivatives, culture medium was supplemented with ampicillin (100 mg/L).

Construction of expression plasmids

Chromosomal DNA of *S. pyogenes* M49 591 was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and used as template for PCR amplification with the Phusion™ High Fidelity DNA Polymerase and the primers listed in Table 1. All PCR products were purified using the QIAquick PCR purification kit (Qiagen). The resulting PCR fragments of *arcA* and *arcC* genes were digested with BamHI and SalI and ligated into the equally treated pASK-IBA2 vector (IBA GmbH, Göttingen, Germany) via T4 DNA ligase (Qiagen). The recombinant vectors were transformed in *E. coli* DH5α cells.

Expression and purification

Expression and purification of recombinant enzymes were carried out as described previously [28]. In short, recombinant *E. coli* strains were grown to an optical density at 600 nm (OD_{600}) of

Table 1
Oligonucleotide primers used in this study. Restriction sites are underlined.

Primer	Sequence (5′-3′)	Restriction sites	PCR product (bp)
arcA_for	GTAATTGGATCCACTGCTCAAACACC	BamHI	*arcA* gene (1236)
arcA_rev	CATAGCGTCGACAATATCTTCACGTT	SalI	
arcC_for	GAGTAGGATCCACGAAACAAAAAATCGTAG	BamHI	*arcC* gene (951)
arcC_rev	CCTCTGTCGACTCCTGCGATAATTTGTG	SalI	

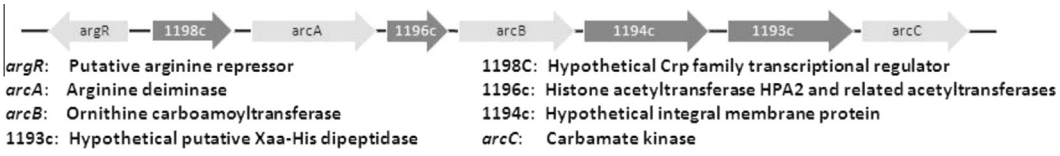

Fig. 2. Gene locus of *S. pyogenes* containing *arc* operon.

about 0.4 before heterologous expression was induced by addition of anhydrotetracycline (0.2 mg/L). Cells were harvested after overnight shaking at 22 °C and pellets were stored at −80 °C. For purification of the Strep-tagged proteins, cell pellets were suspended in buffer W (100 mM Tris–HCl pH 8.0, 1 mM EDTA, 150 mM NaCl) and cell disruption was achieved by the FastPrep method with acid-washed glass beads. Cell debris was removed by centrifugation and clear supernatants were loaded on 5-ml columns contain-

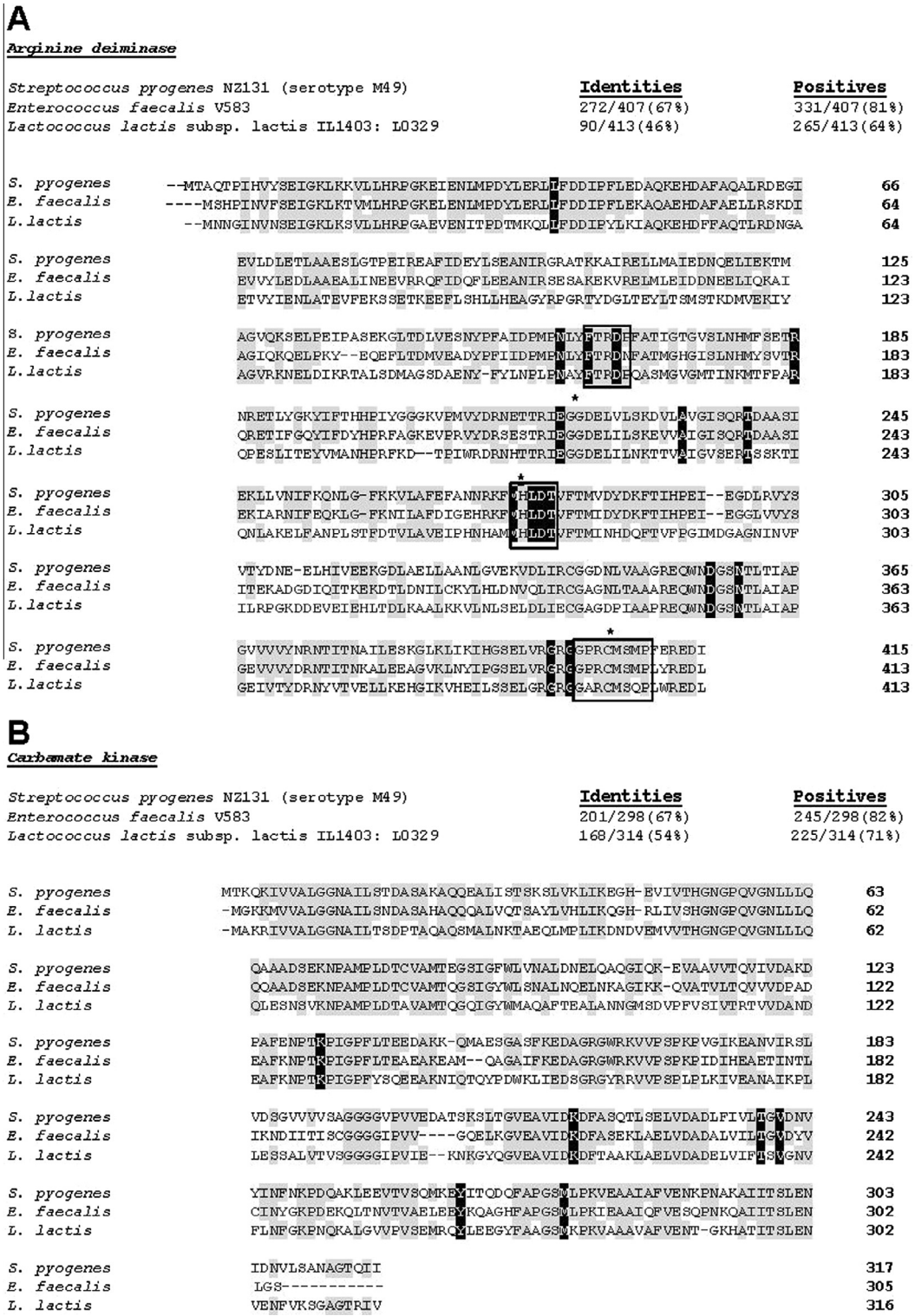

Fig. 3. Alignment of the amino acid sequences of ArcA and ArcC between *S. pyogenes* M49 NZ131, *L. lactis* subsp. *lactis* IL1403 and *E. faecalis* V583. (A) Functionally identical amino acids (= "Positives") are marked in grey, key amino acids involved in binding of arginine and water molecules are marked in black, amino acids involved in catalysis are indicated by an asterisk. The motifs in guanidino-group-modifying enzymes are boxed. (B) The analogies in the amino acids sequences of the different CKs are marked in grey. The key amino acids, which are involved in binding of ADP and carbamoyl phosphate are marked in black. For the analysis the SIM – Alignment Tool for protein sequences was used [59].

ing 1 ml StrepTactin Sepharose (volume) prewashed with buffer W (3 times column volume). Unbound proteins were washed from the column with buffer W (5–7 times column volume). The protein carrying a StrepTag was eluted from the column in fractions of 0.5 ml each in buffer E (buffer W with 2.5 mM desthiobiotin). Elution fractions were analyzed for purified protein with SDS-PAGE and Western Blot.

SDS–PAGE and Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [29]. Proteins were visualized by staining with Coomassie Brilliant Blue. Electrophoretic transfer was done by the semi-dry method [30]. After blotting (30 min, 0.12 A) the membrane was blocked overnight with 20 mM Tris–HCl (pH 7.5) containing 0.15 M NaCl and 3% bovine serum albumin. The blot was washed three times with phosphate buffered saline (PBS). For chromogenic detection of Strep-tag® proteins on Western blots the Strep-Tactin® alkaline phosphatase (AP) conjugate was used. For that purpose blots were incubated for 1 h with Strep-Tactin® AP conjugate under vigorous shaking, and washed with PBS. Subsequently, bound AP conjugate was detected with nitro blue tetrazolium (NBT, 50 g/L) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 g/L).

Protein concentration measurements

Protein concentrations were determined with the Bradford method using the Pierce Coomassie Plus (Bradford) Protein Assay from Thermo Scientific (Germany). The standard curve was generated using bovine serum albumin in concentrations ranging from 0–1 g/L.

Table 2
Expression and purification of *S. pyogenes* M49 AD and CK in *E. coli* DH5α.

Purification step	Total protein (mg)	Specific activity (μmol/min/mg)
Arginine deiminase		
Crude extract[a]	309.5	n/d[b]
Last washing fraction	0.02	0.0
Elution fractions	3.54	1.5
Carbamate kinase		
Crude extract[a]	296.8	n/d[b]
Last washing fraction	0.04	0.0
Elution fractions	2.42	1.1

[a] From 500 ml of induced *E. coli* culture.
[b] "Not definable" due to the high background activity.

Enzymatic assays

Arginine deiminase assay

L-arginine deiminase activity was assayed by measuring the rate of ammonia production during the conversion of L-arginine to L-citrulline. Briefly, the reaction mixture (final volume 250 μl) contained the purified enzyme, 5 mM $MgCl_2$, and 100 mM MES/KOH (pH 6.5) buffer. The reaction was started by addition of L-arginine. L-arginine was added in the range from 0 to 20 mM for the kinetic measurements. After incubation for 30 min at 37 °C the amount of ammonia produced was determined. For that purpose 100 μl of the reaction mixture were analyzed with the Ammonia Assay Kit (Sigma). Here, L-glutamate dehydrogenase catalyzes the reaction of ammonia, α-ketoglutaric acid (KGA), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$). The decrease in absorbance at 340 nm, due to the oxidation of NADPH, is proportional to the ammonia concentration.

Carbamate kinase assay

Carbamate kinase activity was determined by measuring the production of ammonia during the conversion of carbamoyl phosphate and ADP. The reaction mixture included carbamoyl phosphate, 5 mM $MgCl_2$, ADP and 100 mM MES/KOH (pH 6.5) buffer. The concentration of carbamoyl phosphate and ADP were varied in range from 0 to 10 mM. After 30 min at 37 °C the ammonia concentration in the reaction mixture was measured with the Ammonia Assay Kit as described above.

Results and discussion

The arc operon of S. pyogenes

The genes encoding the ADS enzymes of *S. pyogenes* are located in the *arc* operon as shown in Fig. 2. Next to the enzymes encoded in *arcA* (arginine deiminase), *arcB* (ornithine carbamoyltransferase), and *arcC* (carbamate kinase), the *S. pyogenes arc* operon com-

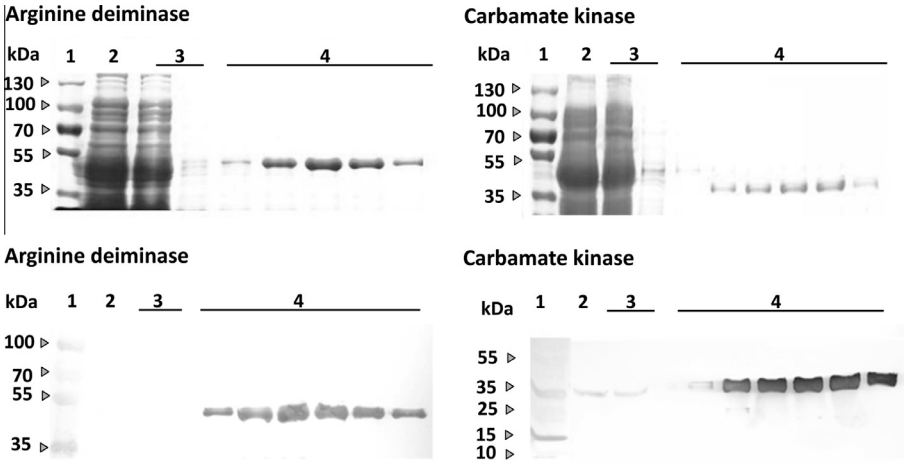

Fig. 4. Purification of arginine deiminase and carbamate kinase. SDS–PAGE gels were loaded with PageRuler Prestained Protein Ladder (1), flow through fraction (2), washing fractions (3), and elution fractions (4) of the StrepTactin affinity chromatography. For chromogenic detection of proteins carrying a Strep-tag®, a Strep-Tactin® alkaline phosphatase (AP) conjugate and NBT/BCIP were used. The upper panel shows Coomassie-stained SDS–PAGE gels, the lower panel shows Western blot membranes.

prises at least four additional open reading frames (ORFs) encoding for hypothetical proteins (Fig. 2). Also in numerous other organisms the *arc* clusters include genes for transport proteins, aminotransferases, or regulators of the ArgR or Crp/Fnr family [31]. The gene arrangement and regulation as well as the biological role of the ADS differ among different bacterial species [5,15,32–35]. In *Pseudomonas aeruginosa* and *Bacillus licheniformis*, the expression of the ADS genes is controlled by regulators of the Crp/Fnr family [35,36]. The induction by Crp/Fnr can be further enhanced in presence of arginine by the transcriptional regulator (ArgR) [36]. In LAB [5] and oral streptococci [11], the expression of the ADS is under the control of CCR and is inducible by arginine and anaerobiosis. The regulation and the structure of the ADS in *S. pyogenes* have not been analyzed in detail yet.

Sequence similarity analysis

Direct amino acid sequence comparison revealed 45.6% identical amino acids between the AD of *S. pyogenes* M49 NZ131 and the AD of *L. lactis* subsp. *lactis* IL1403 and 66.8% with the AD of *E. faecalis* V583 (Fig. 3).

Arginine deiminase belongs to a superfamily of guanidine group modifying enzymes [37,38]. Consequently, the four conserved motifs (FTRD, EGGDV, MHLDT, and CMSxP) and the catalytic triad (C-H-[g,D]) characteristic for enzymes of this superfamily [38,39] can also be found in the *S. pyogenes* AD (Fig. 3). As deduced from *L. lactis* AD data, the amino acid residues Leu40, Met275, Phe163, Arg233 and Gly399 are most likely responsible for binding of arginine, while the residues Thr279 and Asp356 mediate the binding of water molecules [37].

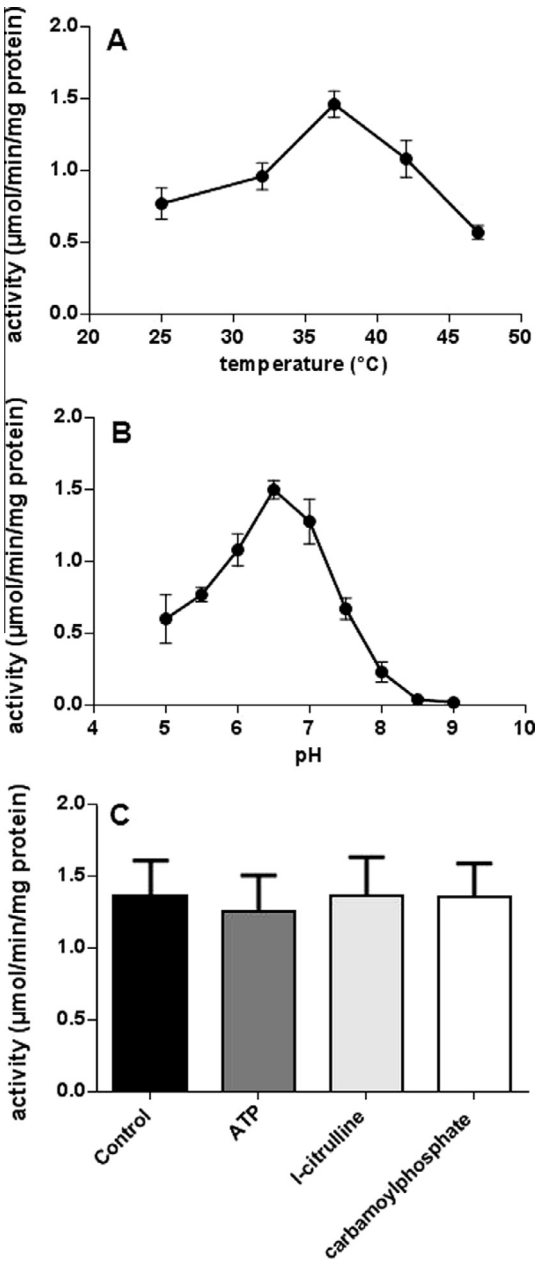

Fig. 5. Enzymatic properties of purified AD from *S. pyogenes* M49 591. Impact of (A) temperature, (B) pH, and (C) potential allosteric regulators (10 mM) on activity of purified AD were analyzed. Activities are shown in µmol/min/mg and results are mean values from four experiments.

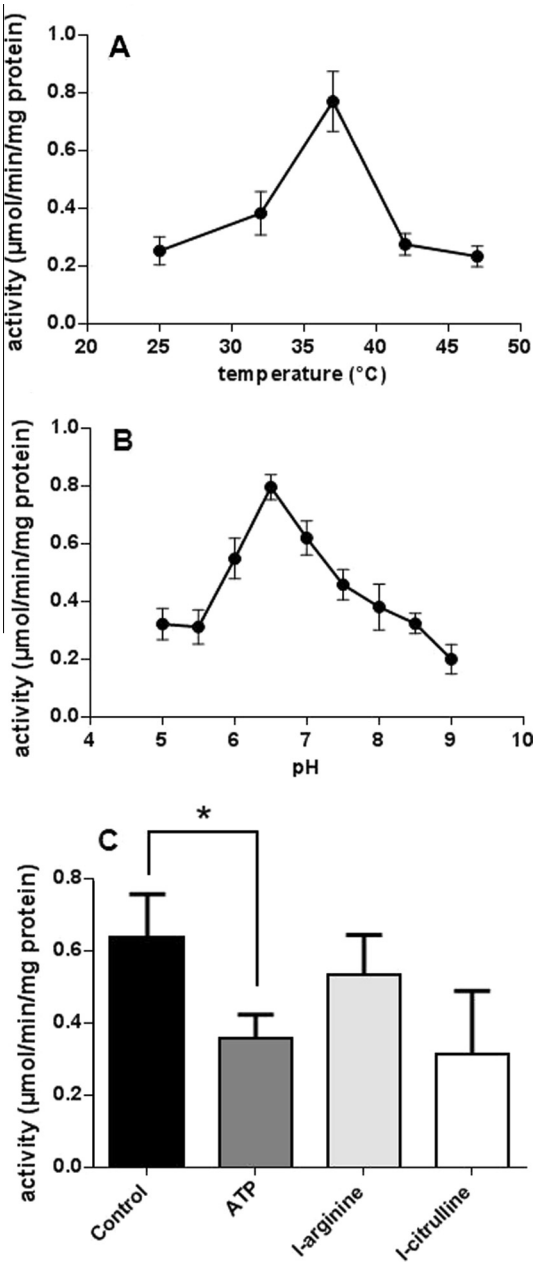

Fig. 6. Enzymatic properties of purified CK from *S. pyogenes* M49 591. Impact of (A) temperature, (B) pH, and (C) potential allosteric regulators (10 mM) on activity of purified CK were analyzed. Activities are shown in µmol/min/mg and results are mean values from four experiments. Asterisks indicate significant differences ($p \leqslant 0.05$, Mann–Whitney *U*-test).

In spite of the amino acid sequence similarities the number of subunits, and hence the structure of AD, differs between different bacterial species. In most cases the AD is a homodimer, e.g., in *Pseudomonas putida* [40] or *Mycoplasma arginini* [39]. However the enzyme in *L. lactis* is organized as homotrimer [37] and in *P. aeruginosa* as homotretramer [41,42]. The AD from *S. pyogenes* consists of two identical subunits [18].

As shown in Fig. 3B, also the amino acid sequence of CK from *S. pyogenes* is highly similar to the corresponding enzymes from *L. lactis* subsp. *lactis* IL1403 (53.5%) and *E. faecalis* V583 (66.8%). The CK belongs to the amino acids kinase enzyme family [43]. In contrast to AD, CK consists of two identical subunits in all organisms investigated so far, e.g., *E. faecalis*, *Enterococcus faecium*, or *P. aeruginosa* [44,45]. Consequently, it is likely that the CK of *S. pyogenes* also is a homodimer. The amino acid residues responsible for binding of the substrates carbamoyl phosphate and ADP are conserved among CK of bacteria and are shown in Fig. 3B. As described for *E. faecalis*, the positive charges of Lys131 and Lys217 interact with the negative charges of the phosphate group of carbamoyl phosphate and the binding site for ADP contains Thr238, Val240 and Met277 [46].

Protein expression and purification

We heterologously expressed *arcA* and *arcC* of *S. pyogenes* M49 591 in *E. coli* DH5α using the expression system pASK-IBA2. For that purpose both open reading frames were ligated into the BamHI and SalI sites of pASK-IBA2 without their intrinsic Start- and Stop-codons. Recombinant proteins were produced in *E. coli* DH5α and purified from cell lysates via affinity chromatography on StrepTactin Sepharose. The elution fractions of AD contained about 3.5 mg protein, accounting for about 1% of the total protein in the *E. coli* crude extracts (Table 2). For CK the total amount of protein in the elution fractions with 2.4 mg accounted for about 0.8% of the proteins in the crude extract. The calculated molecular weights of the recombinant enzymes including the Strep-Tag were 47.2 kDa for AD and 34.2 kDa for CK of *S. pyogenes*. The success of the purification was confirmed by SDS–PAGE and by Western blot using StrepTactin-alkaline phosphatase conjugates (Fig. 4). The purified recombinant proteins were used to determine the temperature and pH optima, Michaelis constants (K_m), and allosteric regulators of the enzymes.

Impact of temperature and pH on AD and CK activity

The optimal temperature for both, arginine deiminase and carbamate kinase, was determined by measuring the activity of both enzymes in a temperature range between 25 and 47 °C (Figs. 5 and 6A). Both enzymes had highest activities at 37 °C. This is in accordance with the temperature in the human body – the natural habitat of *S. pyogenes*. Apparently the AD temperature optimum is not necessarily correlating with the natural environment of the organisms it originates from. The optimal temperature for the AD of *P. putida* and *Lactobacillus buchneri* was determined to be 50 °C [40,47] although for instance the natural habitats of *P. putida* are moist soil and water and it grows optimally at 25–30 °C [48]. Also for *L. lactis*, the optimum growth temperature is 30 °C but the AD optimum was measured at 60 °C [37]. In contrast to that the AD of *Bacillus cereus* has the highest activity at 25 °C [49] and *B. cereus* is growing optimally at temperatures between 20 and 40 °C [50]. Hence, a wide range of temperature optima can be found among AD from different microorganisms and they do not correlate with the temperatures at the natural habitats or the optimal growth temperatures of the respective bacteria. The fact that AD from *S. pyogenes* obviously works best at 37 °C makes this enzyme a promising candidate for the application in treatment of ASS-negative tumors. Immunogenicity of bacterial proteins such as AD in the human body can be circumvented by formulation with polyethylene glycol without loss of enzymatic activity [51].

Table 3
Comparison of kinetic parameters of known arginine deiminases and carbamate kinases.

Strain	K_m L-arginine (mM)	Optimum pH	Optimum temp. (°C)	References
Arginine deiminase				
Streptococcus pyogenes M49 591	1.33	6.5	37	This work
Lactococcus lactis ssp. *lactis* ATCC 7962	8.67	7.2	60	[37]
Lactobacillus buchneri NCDO110	0.83	6.0	50	[52]
Pseudomonas putida	0.20	6.0	50	[40]
Pseudomonas aeruginosa	0.14	5.6	25	[49]
Bacillus cereus	0.06	7.0	25	[49]
Escherichia coli	0.32	6.0	25	[49]

Strain	K_m ADP (mM)	K_m CAP* (mM)	Optimum pH	Optimum temp. (°C)	References
Carbamate kinase					
Streptococcus pyogenes M49 591	0.72	0.65	6.5	37	This work
Lactobacillus buchneri NCDO110	0.71	1.53	5.4	30	[56]
Enterococcus faecalis ATCC 8043	0.04	1.40	–	–	[44]

* Carbamoyl phosphate.

The influence of pH on activity of AD and CK has been determined in a range between pH 5 and pH 9. The optimum for both enzymes was pH 6.5 (Figs. 5 and 6B). In contrast to the variable temperature preferences of different AD, the pH range in which AD and CK from different bacteria have their highest activity is relatively narrow. As can be seen in Table 3, the optimal pH for most enzymes described ranges from neutral to slightly acidic.

Kinetic constants and allosteric regulators of AD and CK

As shown in Fig. 5C, AD of *S. pyogenes* M49 591 is not allosterically regulated by the intermediates and products of arginine degradation. No significant changes in AD activity were observed in the presence of 10 mM ATP, citrulline, and carbamoyl phosphate, respectively. While citrulline and carbamoyl phosphate had no effect on the activity of AD of other bacteria either, for *L. buchneri* an inhibitory effect of ATP on AD activity has been shown [52].

The K_m value of the *S. pyogenes* AD for L-arginine was 1.33 ± 0.12 mM (Fig. 7). As shown in Table 3, the *L. lactis* AD has a more than 8 times higher K_m value for arginine. These different affinities might reflect the different availability of arginine in the natural environments of these two species, since the amount of arginine in human plasma with approximately 0.075 mM is relatively low [53] compared to arginine concentration in raw milk which is about 6 mM [54]. Consequently high concentrations of

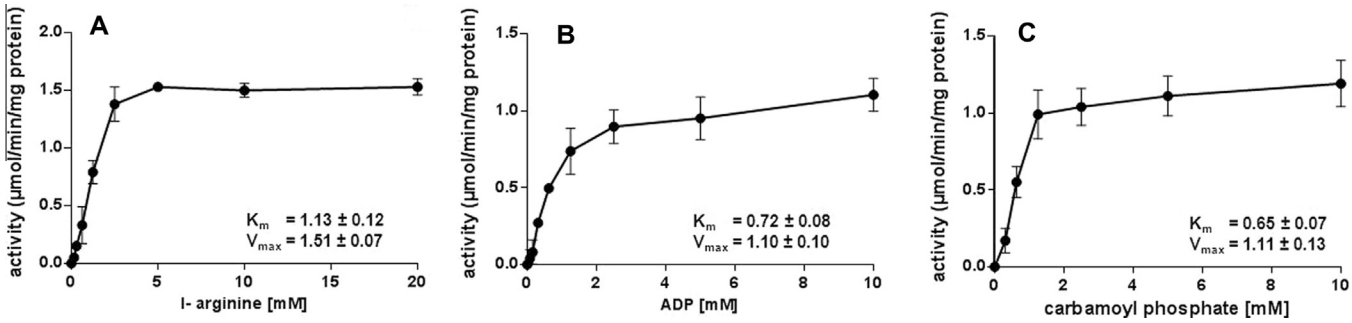

Fig. 7. Michaelis–Menten Plots. Kinetic parameters K_m and V_{max} were determined for purified arginine deiminase (A) and carbamate kinase (B and C) from *S. pyogenes* M49 591.

arginine in the natural environment may be associated with higher K_m values.

The effects of potential allosteric regulators on carbamate kinase (CK) activity are shown in Fig. 6C. In the presence of 10 mM ATP a significant inhibitory effect on enzyme activity was observed. L-arginine and L-citrulline had no effects on CK activity. The CK reaction is reversible, but CK shows a higher affinity for ADP than for ATP [55]. The K_m values were 0.72 ± 0.08 mM for ADP and 0.65 ± 0.07 mM for carbamoyl phosphate (Fig. 7). In Table 3 a comparison of the kinetic parameters for the CK from *L. buchneri*, *E. faecalis* and *S. pyogenes* is shown [52,56]. *S. pyogenes* CK has a lower K_m value for carbamoyl phosphate and thus a higher affinity to carbamoyl phosphate than CK of the other species. In *S. pyogenes* carbamoyl phosphate is a byproduct of arginine and glutamine degradation and an important precursor of pyrimidine biosynthesis. In bacteria not auxotrophic for arginine, carbamoyl phosphate also serves as a precursor for arginine biosynthesis. However, the interdependency of intracellular carbamoyl phosphate pools, carbon dioxide requirements and growth physiology of lactic acid bacteria is not fully understood to date [57].

Conclusions

S. pyogenes uses the arginine deiminase system for ammonia production against acidic stress at low pH [16] and also for ATP production. The arginine deiminase and carbamate kinase have the highest activity at pH 6.5, presumably to produce ammonia for survival in acidifying environments and to maintain ATP production. In comparison to other lactic acid bacteria, the affinity of AD and CK to their substrates is relatively high. This might be explained by the low arginine concentrations in the natural environment of *S. pyogenes* and the importance of generating carbamoyl phosphate. Furthermore, the temperature optimum at 37 °C and the high affinity to arginine make the heterologously produced and purified AD of *S. pyogenes* a candidate for application in the treatment of arginine-auxotrophic tumors such as hepatocellular carcinomas (HCCs) and melanomas [58].

Acknowledgment

This work was conducted for the SYSMO-LAB2 project funded by the German Federal Ministry of Education and Research (BMBF).

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