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Original article

The arginine deiminase system facilitates environmental adaptability of *Streptococcus equi* ssp. *zooepidemicus* through pH adjustment

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Abstract

The arginine deiminase system (ADS) is a secondary metabolic system found in many different bacterial pathogens and it is often associated with virulence. Here, a systematic study of ADS functions in *Streptococcus equi* subsp. *zooepidemicus* (SEZ) was performed. Transcriptional levels of ADS operon genes were observed to be significantly increased when SEZ was grown under acidic conditions. We constructed *arcA* and *arcD* deletion mutants (SEZ *ΔarcA* and SEZ *ΔarcD*, respectively) and found that SEZ *ΔarcA* was unable to metabolize arginine and synthesize ammonia; however, *arcD* deletion resulted in an initial decrease in arginine consumption and ammonia production, followed by recovery to the levels of wild-type SEZ after 24 h of cultivation. Cell extracts of SEZ *ΔarcA* showed no arginine deiminase (AD) activity, whereas no difference in AD activity between SEZ *ΔarcD* and wild-type SEZ was observed. SEZ survival tests demonstrated a significant decrease in survival for SEZ *ΔarcA*, when compared with wild-type SEZ, under acidic conditions and in epithelial cells. These findings indicate that ADS in SEZ contributes to environmental adaptability via ammonia synthesis to reduce pH stress.

Keywords: *Streptococcus equi* ssp. *zooepidemicus*; Arginine deiminase system; Arginine; Ammonia; Acid tolerance

1. Introduction

Streptococcus equi ssp. *zooepidemicus* (SEZ), one of Lancefield group C beta-hemolytic streptococci (GCBHS), is responsible for septicemia, meningitis, arthritis and several other diseases in various animal species. It is especially prevalent in horses, pigs, dogs and occasionally humans [1–3]. Infections caused by SEZ have led to serious welfare and economic losses in equine and swine industries [4,5]. Humans are occasionally infected with SEZ through close contact with infected domestic animals or ingestion of unpasteurized dairy products, resulting in septicemia or meningitis [6]. Despite the clinical relevance of SEZ, factors that contribute to its virulence are still not well known.

The arginine deiminase system (ADS) is highly conserved in bacteria and it results in the conversion of arginine to ornithine, carbon dioxide and ammonia, with the concomitant release of ATP. Three core enzymes are involved in ADS: arginine deiminase (ArcA) catalyzes the conversion of arginine and water substrates to citrulline and ammonia; ornithine carbamoyltransferase (ArcB) catalyzes the phosphorolysis of citrulline and the manufacture of carbamoyl phosphate and ornithine; and carbamate kinase (ArcC) catalyzes the transfer of phosphate from carbamoyl phosphate to ADP, generating ATP, carbon dioxide and ammonia [7,8]. The recombinant ArcA of SEZ has been demonstrated to inhibit the growth activity of MOLT-3 cells and induce apoptotic cell death [9]. The ADS also contains ArcD and ArcT, which encode a putative arginine/ornithine antiporter and a putative Xaa-His dipeptidase, respectively [10,11]. Transcriptional upregulation of *arcD* was observed during SEZ infection in piglet lungs [12], and the product of *arcD* in *Streptococcus suis* has been

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demonstrated to be important for supplying arginine as a substrate for the ADS [13]. Moreover, as a secondary metabolic pathway, the ADS is regulated by a complex network. For instance, in some other bacteria, the ADS is controlled by the amount of arginine in the substrate via regulators that belong to the ArgR family, including both ArgR and AhrC (but these do not occur in *Streptococcus pneumoniae*) [14–16]; carbon catabolite repression (CCR) via CcpA [8,17]; oxygen, a negative regulatory factor, via the Crp/Fnr family, such as Flp (Fnr like protein) [17,18]; and pH value, in oral streptococcus, via two-component systems (TCSs) CiaRH and ComDE in *Streptococcus gordonii* or TCS VicRK in *Streptococcus mutans* but not in *S. gordonii* [19].

Adaptability of bacteria to acidic conditions found in the host is extremely important for survival, colonization and infection of locations such as the skin surface and some mucous membranes, areas of tissue inflammation and acidic lysosomes in cells [20–23]. After years of research, a number of different acid-resistant mechanisms operated by some bacteria have been studied, including proton pumps such as the F_1-F_0-ATPase [24], a series of protein and DNA repair mechanisms [25], antioxidant enzymes such as superoxide dismutase and hydroperoxidase [26], glutamate decarboxylase [27], regulators such as TCSs [19] and production of alkali by the ADS [28,29] or urease system [30,31]. However, related research has not yet entirely elucidated the mechanisms of acid tolerance on SEZ. In this study, we focused on the potential function of the main *arc* operon genes and the influence of the ADS in SEZ for tolerating acidic environments.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1.

SEZ ATCC35246 and its derivative mutants were cultured in Todd-Hewitt (TH, BD) broth or agar at 37 °C [32]. For cell tests, the strains were grown in tryptic soy broth (TSB, BD). For studies related to arginine metabolism, the strains were grown in a tryptone-yeast-based (TY) medium supplemented with galactose and, if indicated, with arginine [33,34] and were adjusted to 2.5×10^6 CFU/ml at the start of cultivation. Growth was monitored every hour using the SmartSpec Plus spectrophotometer (Bio-Rad). Experiments were performed at least three times. *Escherichia coli* DH5α hosting plasmid pSET4S [35] and pSET2 [36] were cultured at 37 °C in Luria-Bertani (Sigma) medium. For construction of the recombinant plasmid and selection of the mutants, spectinomycin was added to the medium at a concentration of 100 μg/ml for SEZ and 50 μg/ml for *E. coli*.

2.2. Construction of gene-specific knockout mutants and their complementation strains

In this study, we separately constructed *arcA* and *arcD* deletion mutants utilizing a methodology of homologous recombination [35], and named them SEZ *ΔarcA* and SEZ *ΔarcD*, respectively. The primers used in these experiments are listed in Table S1. First, the upstream and downstream flanking sequences of the gene to be knocked out were amplified and integrated using PCR. The product was digested with restriction endonucleases (*Sal* I and *Bam*H I, TaKaTa) and ligated into pSET4s with T4 DNA Ligase (TaKaRa). The recombinant plasmid was introduced into SEZ by using electroporation. Transformants were grown at 37 °C on TH agar plates containing spectinomycin. A single colony was subjected to serial passages at 28 °C in TH broth without spectinomycin. Then, the inoculum was diluted and plated on TH agar. Next, colonies were picked and plated on TH agar plates with or without spectinomycin. Spectinomycin-sensitive colonies were verified for specific gene deletion by PCR. In particular, we amplified the locus of the gene to be deleted, including its upstream and downstream flanking sequences, and sequenced the product; we made sure that just the whole ORF of the specific gene had been knocked out. After allelic

Table 1
Bacterial strains and plasmids used in the study.

Bacterial strain or plasmid	Notable characteristic (s)[a]	Source or reference
Bacterial strains		
E. coli DH5α	Plasmid cloning host and Gram-negative replicon	Purchased from Invitrogen
SEZ ATCC35246	Virulent strain of SEZ isolated from infected pig	Purchased from ATCC™
SEZ ATCC35246 *ΔarcA*	*arcA* allelic replacement mutant of SEZ ATCC35246	This study
SEZ ATCC35246 *ΔarcD*	*arcD* allelic replacement mutant of SEZ ATCC35246	This study
SEZ ATCC35246 C*ΔarcA*	Complemented strain of SEZ *ΔarcA*, Spc^r	This study
SEZ ATCC35246 C*ΔarcD*	Complemented strain of SEZ *ΔarcD*, Spc^r	This study
Plasmid		
pSET4s	*Streptococcus* thermosensitive suicide vector, Spc^r	[35]
pSET2	*E. coli* – *Streptococcus* shuttle cloning vector, Spc^r	[36]
pSET4s:: *arcA* UD[b]	*arcA* allelic replacement vector, Spc^r	This study
pSET4s:: *arcD* UD[b]	*arcD* allelic replacement vector, Spc^r	This study
pSET2:: *arcA*	pSET2 containing *arcA* in Sal I-BamH I restriction sites, Spc^r	This study
pSET2:: *arcD*	pSET2 containing *arcD* in Sal I-BamH I restriction sites, Spc^r	This study

[a] Spc, spectinomycin.
[b] UD, upstream and downstream flanking sequence of a gene.