Zinc chloride inhibits lysine decarboxylase production from *Eikenella corrodens in vitro* and its therapeutic implications

Martin Levine [a,*], Lindsay M Collins [a], Zsolt Lohinai [b]

[a] *Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States*
[b] *Department of Conservative Dentistry, Semmelweis University, Budapest, Hungary*

ARTICLE INFO

ABSTRACT

Objectives: Dentifrices containing zinc reduce gingival inflammation and bleeding better than control dentifrices (no zinc). How zinc might work is not understood. We have shown that lysine decarboxylase (LdcE), an enzyme from *Eikenella corrodens*, converts lysine to cadaverine in dental biofilms. The lack of lysine impairs the dentally attached cell barrier to biofilm, causing biofilm products to leak into junctional epithelium and stimulate inflammation. In year-old beagle dogs, immunization with LdcE, induces antibodies that inhibit LdcE activity and retard gingivitis development. We therefore examined whether a zinc-mediated loss of LdcE activity could explain the beneficial effect of zinc dentifrices.

Methods: We grew *E. corrodens* in modified tryptic soy broth with or without zinc chloride, and extracted LdcE from the cell surface using a Potter Elvehjem homogenizer.

Results: Up to 0.96 mM zinc chloride in the bacterial growth medium did not change cell yield, but reduced the extracted protein content by 41% ($R^2 = 0.27$, $p < 0.05$) and LdcE activity/mg extracted protein by 85% ($R^2 = 0.90$, $p < 0.001$). In extracts from cells grown without zinc, 78 times this zinc chloride concentration (73 mM) was required to reduce LdcE activity by 75%.

Conclusions: Zinc ions inhibit the production of protein with LdcE activity at *E. corrodens* cell surfaces. The zinc ions may attach to cysteine residues that are unique to the N-terminal region of LdcE by interfering with the non-covalent polypeptide assembly that produces enzyme activity.

Clinical significance: Zinc ion-mediated inhibition of LdcE assembly may provide a rationale for the improved control of gingival inflammation by zinc dentifrices.

1. Introduction

Periodontal disease is an important worldwide health problem [1], and a cause of systemic infections that induce or exacerbate chronic diseases such as: diabetes [2], atherosclerosis [3], rheumatoid arthritis [4], some cancers [5], adverse pregnancy outcomes [6] and Alzheimer's disease [7]. Periodontal disease begins as gingival inflammation in which the gingiva (gums) become red, swollen and bleed on brushing. Persistent gingival inflammation (gingivitis) may develop into periodontitis in which the epithelial attachment to the tooth recedes apically from the enamel-cemental junction. Gingivitis and periodontitis are earlier and later stages of the same disease [8].

Experimental gingivitis is a procedure in which oral hygiene is restricted for 3 weeks causing gingivitis to develop as the colonization of dentally adherent bacteria from saliva increases on teeth surfaces [9]. Within a week the adherent bacteria extend into shallow crevices where the teeth and gingiva meet in the oral cavity and an inflammatory exudate from the crevice, gingival crevicular fluid (GCF), appears at gingival margins. Compared to saliva, GCF is derived from serum and much richer in amino acids and proteins than saliva [10], causing the ecological environment of the crevice, and therefore the crevice biofilm's microbiological composition to be very different [11]. Over the following 2–3 weeks, GCF exudation increases and gingivitis appears [12]. If gingivitis persists beyond that, GCF increases further and periodontal pathogens such as *Porphorymonas gingivalis* appear and increase in what is a periodontal disease-causing (dysbiotic) biofilm [13].

Although regular oral hygiene and professional tooth cleaning controls gingivitis and chronic periodontitis [14], the high prevalence of

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* Corresponding author: Dept of Biochemistry & Molecular Biology, University of Oklahoma HSC, BMSB Rm 802, 940 S.L. Young Blvd, Oklahoma City, OK 73104. United States.

E-mail address: martin-levine@ouhsc.edu (M. Levine).

periodontal disease [1,15] suggests that oral hygiene is often inefficient. To overcome this problem, antiseptics such as uncharged phenolic oils [16], triclosan [17], or stannous ions [18] were added to dentifrices with the aim of supplementing biofilm control by oral hygiene. More recently, a dentifrice containing enzymes and proteins designed to supplement the natural control of oral bacteria by saliva was at least as effective as antiseptics and potentially less disruptive to the salivary microbiota over 6 months of use [19].

We recently reported a new target for biofilm control [20], lysine decarboxylase, an enzyme from *Eikenella corrodens* (LdcE) that converts lysine to cadaverine and carbon dioxide. Antibodies that inhibit LdcE activity were induced in beagle dogs and retarded the rate of gingivitis development when the dogs were given a water-softened diet [21]. In humans, *E. corrodens* is present in the saliva microbiome where it grows by oxidizing key amino acids to the corresponding ketoacids, ammonia and electrons [22]. Nitrate is absorbed from saliva or GCF, and reduced to nitrite which is excreted [23]. *E. corrodens* is a core component of biofilms in both health and periodontitis [24].

The gingiva is a site of masticatory stress, which damages the host microbial barrier mediated by dentally attached cells of junctional epithelium. Damage is evidenced by the persistence of leukocytes (T17 helper cells) and activated interleukin 6 (IL-6) beneath the gingival crevice [25]. The leukocytes leak from the crevice with traces of GCF, which provides the dentally attached cells with adequate amounts of lysine, an essential amino acid inadequately provided by saliva [26]. Once experimental gingivitis begins, *E. corrodens* in the salivary microbiome is an early colonizer of teeth surfaces [27], and its LdcE converts the lysine in GCF to cadaverine [20,26]. The lack of lysine causes the coronal layer of dentally attached cells to stop dividing and lose their attachment [28].

The impaired epithelium in this region allows microbial-associated molecular patterns from the adjacent dentally adherent biofilm to enter the junctional epithelium where pattern recognition receptors recognize them as foreign. This recognition causes the epithelium to secrete and activate interleukin-1α (IL-1α), an alarmin that mediates intrinsic microbial immunity [29]. IL-1α appears after a week of inducing experimental gingivitis [30] where it attracts and stimulates leukocytes and many other cytokines to activate a broad range of hydrolytic enzymes in an effort to remove the infection and epithelial impairment if oral hygiene is not re-established [31].

Other studies indicate that mouthwashes or dentifrices containing 2% (w/v) zinc chloride (ZC) or zinc citrate reduce gingivitis and the precipitation of dental calculus from calcium phosphate in GCF [32–37]. Unfortunately, these dentifrices leave a persistent taste that made them unsuitable for regular use. Recently, a group of investigators found that an arginine stabilized 0.96% w/v zinc oxide/zinc citrate suspension improved the acceptability of zinc ions in dentifrices [38]. Because our previous studies led us to propose that LdcE could be a new therapeutic target [20], we investigated whether a zinc-mediated loss of LdcE activity could explain the beneficial effect of zinc dentifrices.

2. Materials and methods

2.1. E. corrodens growth and extract preparation

Cultures of the *E. corrodens* type strain (ATCC 23834) were maintained on tryptic soy agar containing 5% sheep blood (Wards Science, Rochester NY), and grown in tryptic soy broth (30 g/L; Becton Dickinson, Franklin Lakes, NJ) containing 2 g/L of potassium nitrate (Mallinckrodt Baker Inc., Phillipsburg NJ). Aliquots (200 mL) were autoclaved for 45 min and cooled. Solutions of hemin (Sigma Chemical Co. St Louis, MO) and sodium bicarbonate were filter-sterilized (0.45 μ) and added to the autoclaved nitrate/tryptic soy broth to a final concentration of 0.07% and 0.45% w/v respectively [20].

To obtain similar amounts of bacterial cells, a 2 mL aliquot of broth was mixed with 4–6 bacterial colonies and 0.2 mL of the suspension was

added to each of 4 × 200 mL aliquots of broth. *E. corrodens* is slow-growing [39], and 48 h incubation at 37 °C was necessary before harvesting sufficient bacteria by centrifugation (10 min at 4000 rpm in 250 mL centrifuge tubes). After centrifugation, pellets were washed twice with physiological phosphate buffered saline, and each pellet's wet weight was recorded. A 4-fold excess by weight of 65 mM NaCl was added and the pellet was homogenized using 20 up-and-down strokes of a rapidly rotating Potter-Elvehjem pestle. Following centrifugation at 10,000 rpm [40], the supernatant fraction was sterilized by Millipore filtration to give an LdcE-active cell extract solution containing only cell surface proteins [40].

ZC powder (Mallinckrodt Baker Inc., Phillipsburg NJ) was dissolved in water to 200 mM or 24.2 mM. For assay of LdcE activity, *E. corrodens* pellets from cultures without ZC were extracted and Millipore filter-sterilized dilutions of 200 mM ZC (tests) or distilled water controls were added as indicated in Table 1 and section 2.2 below. For assay of LdcE production, a commercial mouthwash (0.33%, 24.2 mM) was made benzoate-free to permit bacterial growth for this study (Triumph Pharmaceuticals Oral Health Laboratories, St Louis, MO). Varying amounts of the Triumph or a 24.2 mM Mallinckrodt ZC solution (test), or sterile water (control) were added as indicated likewise in Table 1 and section 2.2.

2.2. Assay of LdcE

Extracts of *E. corrodens* cells grown without ZC were incubated at 37 °C for 20 min in 96-well plates with the reagents and amounts shown in Table 1. After cooling the plates on ice for another 20 min, the reaction was stopped by adding 10 μL of 1.0 M sodium hydroxide. The solution from each well was added to polypropylene tubes containing 0.8 mL of ice-cold buffer pH 7.0 (0.15 M NaCl in 60 mM Na phosphate). Potassium carbonate (1.0 mL of 1.0 M) was added, followed by 1.0 mL of a 10% (w/v) solution of 2,4,6 trinitrobenzenesulfonic acid (TNBS). After incubating the tubes together in a water bath at 43 °C for exactly 5 min, and cooling to room temperature, toluene (2.0 mL) was added. The tubes were capped and vortexed strongly in a fume cupboard for exactly 30 s each, and centrifuged at 2000 rpm for 10 min. The toluene soluble TNBS-cadaverine product (upper layer) was transferred to a 96-well plate and the samples from tests, controls and standards (legend to Table 1) were read at 340 nm [41]. The cadaverine produced in 20 min in the presence of ZC (test) was expressed as a percentage of cadaverine produced by the controls (no ZC).

Using similar procedures, the effect of ZC on *E. corrodens* growth and LdcE production were determined. Washed cell pellets were extracted, and the cadaverine produced from 3.0 and 3.5 μmol lysine were averaged from extracts grown with and without ZC. The pellet weight, the

Table 1

Assays for LdcE cadaverine production.

Agent	Volume μL
NaCl (450 mM) in Na phosphate pH 7.0 (60 mM)	67
Pyridoxal phosphate cofactor (80 mM)	3
LdcE extract made to 1.5 μg protein in 7.5 mM NaCl[a,b]	10
ZC solutions (0–73 mM[c], or 0 – 0.96 mM[d])	100
Lysine (0–175 mM)[e]	20
Total volume of assay	200

[a] Tested without or with zinc chloride (ZC) in the growth medium.

[b] To standardize cadaverine content, LdcE extract was replaced with cadaverine in 7.5 mM NaCl to give final concentrations of 0.00, 0.160, 0.32, 0.64 and 0.80 mM. The absorbance from the cadaverine standards (controls) was used to determine how much cadaverine was produced in the test assays.

[c] For the LdcE activity inhibition assay; final concentrations added to extracts of cells grown without ZC.

[d] For the LdcE production inhibition assay; final concentrations of ZC added in the growth medium.

[e] Added last to start LdcE catalysis.

concentration of protein in the pellet extract (mg protein extracted/g pellet), and the mean μmol cadaverine produced/mg protein were expressed as a percentage of these amounts in the respective controls (no ZC in the growth medium).

2.3. Statistical analysis

ANOVA within the statistics program Xlstat (Addinsoft, Paris, France) were used to regress the cadaverine produced in 20 min against concentration of ZC in the assay, as well as the weight of cells, amount of protein extracted and amount of cadaverine produced/mg protein against the concentration of ZC in the growth medium.

3. Results

3.1. Effect of ZC on LdcE in extracts of E. corrodens cells grown without ZC in growth medium

As ZC increased to 73 mM, cadaverine produced from 3.0 μmol lysine in the extract reduced the amount of cadaverine by 75%, indicating that ZC inhibited LdcE activity directly but weakly (Fig. 1).

3.2. Effect of ZC on LdcE in extracts of E. corrodens cells grown with ZC in growth medium

Adding zinc to the *E. corrodens* growth medium also reduced cadaverine production, the greatest decrease being observed at 3.0 and 3.5 μmol lysine in the assay. Cell surface extracts from cultures grown in 0.8 mM ZC inhibited cadaverine production more strongly than extracts from cultures grown in 0.4 mM ZC (Fig. 2a and b).

The yield of *E. corrodens* cells was unrelated to the ZC concentration (Fig. 3a), but the amount of extracted protein per g of cells decreased significantly by about 41% despite the large variation (Fig. 3b). Cadaverine produced/mg extracted protein fell more significantly, indicating that LdcE accounted for much of the protein lost from extracts of *E. corrodens* cells when grown in the presence of ZC. The maximal ZC concentration tested, 0.96 mM, reduced the amount of cadaverine/mg protein by 85% (Fig. 3c).

4. Discussion

4.1. Zinc inhibition of LdcE and dentifrice control of gingivitis

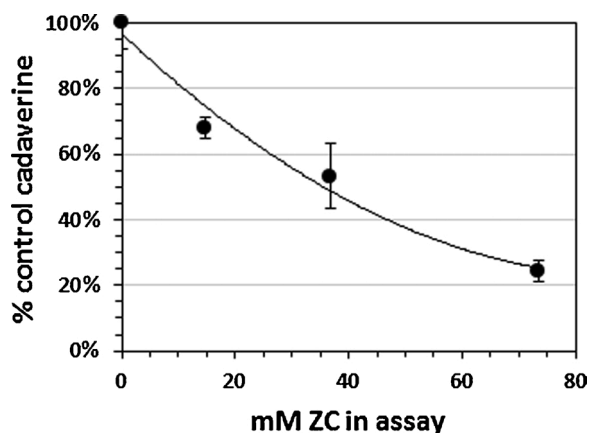Our results strongly suggest that growing *E. corrodens* in medium

Fig. 1. Inhibition of LdcE activity by zinc chloride (ZC). Mean and range of duplicate assays are shown. Linear regression. Percent control = 0.912–0.010*mM ZC, R^2 = 0.94, p < 0.04. A slightly stronger model was provided by the second-degree polynomial model shown in the Fig., R^2 = 0.98. Percent control = 0.968–0.016*ZC +0.00008*ZC^2.

containing almost 1 mM zinc inactivates most of the LdcE obtained from cell surface extracts, whereas 73 mM zinc was required to inhibit the LdcE from similar extracts of *E. corrodens* cells grown without ZC. Thus, zinc inhibits LdcE production, not its activity. The molar concentration of 2% zinc citrate dentifrices is 147 mM [33,34], and that of the arginine-stabilized 0.96% zinc citrate/zinc oxide dentifrice is 74 mM [38]. The former dentifrice therefore contains 150-times more zinc ions than required to inhibit LdcE by 85% and the latter 75-times more. After six months of use, reductions for mean plaque (biofilm) and gingival indices were 25% and 20% respectively, compared with zinc-free dentifrices. Stronger protection occurred at sites that had bled spontaneously or on probing, a mean reduction of 67.6% and 56.6% respectively [33,38].

The ability to apply zinc to biofilm-infected gingival surfaces [33,34, 38] resembles its use for healing infected or wounded skin [42]. Moreover, individuals with low serum zinc levels are more predisposed to chronic inflammatory diseases, such as bacterial infections, delayed wound healing, atherosclerosis, and type-2 diabetes [42,43]. About 90% of the zinc in resting whole saliva (spit) is likely from GCF, which is derived from serum [10], not from salivary gland secretions [44]. The normal (reference) range of zinc in serum from adults of all ages was recently reported to be 9.3–30.8 μM (61–201 μg/dL) [45] with a mean of 16.9 ± 5.5 μM (110 ± 5.5 μg/dL), at least an order of magnitude too small to alter LdcE action in dental biofilms.

LdcE is a typical pyridoxal-5-phosphate (PLP) dependent lysine decarboxylase whose sequence is 65% identical to two lysine decarboxylases in *Escherichia coli*: one that is acid-induced (LdcI) and one that is constitutive (LdcC). *In vitro*, LdcI is inactive at physiologic pH, but active in acid with a sharp peak at pH 5.5 [46]. LdcC is inactive at pH 5.5, but fully active at physiological pH [47]. *In vivo*, *E. coli* and other intestinal bacteria traverse the stomach where lysine is a product of pepsin proteolysis. The acidic environment induces LdcI expression to convert the lysine to cadaverine, which locally alkalinizes the medium, and decreases porin permeability so that protons cannot penetrate the cell interior and retard *E. coli* growth.

Urinary tract infections are acidic, frequently colonized by *E. coli*, and rich in bactericidal reactive nitrogen species derived from the host. The acidic environment induces expression of *E. coli* LdcI, whose cadaverine decreases porin permeability to both acid and reactive nitrogen species, thereby enhancing *E. coli* growth [48,49]. If the gene for LdcI is mutated, the exogenous addition of cadaverine (or other polyamines) rescues the growth of these mutants under reactive nitrogen species stress [49]. In periodontitis, the environment is alkaline [50] but the tissues contain reactive nitrogen species [51] as in urinary tract infections. Biofilm bacteria mix with GCF and both bacteria and host neutrophils express proteolytic enzymes that release lysine and other free amino acids from proteins in GCF and the surrounding tissues [10, 52]. In clinically inflamed periodontal pockets, lysine degradation is overrepresented and its synthesis underrepresented [53].

LdcE production of cadaverine from lysine may protect bacteria from reactive nitrogen stress in alkaline environments, like LdcI in acidic environments. If so, ZC inhibition of LdcE would reduce cadaverine production, and allow reactive nitrogen species to penetrate and kill *E. corrodens*, and probably other biofilm bacteria. Standard therapy for periodontitis consists of scaling and root planing. Antibiotics provide minimal additional protection and promote bacterial resistance [54]. Our study indicates that zinc ion-mediated inhibition of LdcE not only maintains an intact dental epithelial attachment (see Introduction), but may also promote bacterial killing by reactive nitrogen species. The stronger protection of zinc dentifrices at sites that are inflamed or bleed on probing after therapy (see Introduction) gives credence to these mechanisms, and could improve post-periodontal maintenance better than antibiotics [55].
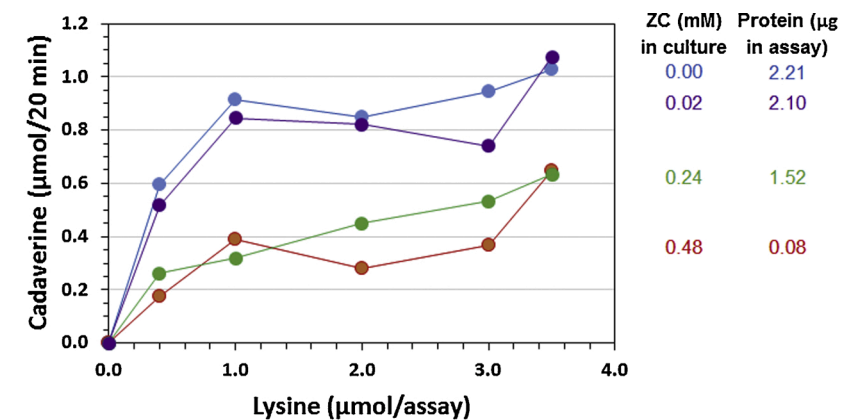
ZC (mM) in culture	Protein (µg in assay)
0.00	2.21
0.02	2.10
0.24	1.52
0.48	0.08

Fig. 2. LdcE activity and protein content of cell surface *E. corrodens* extracts after growth in medium with and without zinc chloride (ZC). (**a**) and (**b**) Cadaverine produced from lysine in two separate extracts compared with the protein content of two respective extracts after growth without ZC (control, blue), and at different ZC concentrations (purple, green and red). Cadaverine contents were single measurements. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).
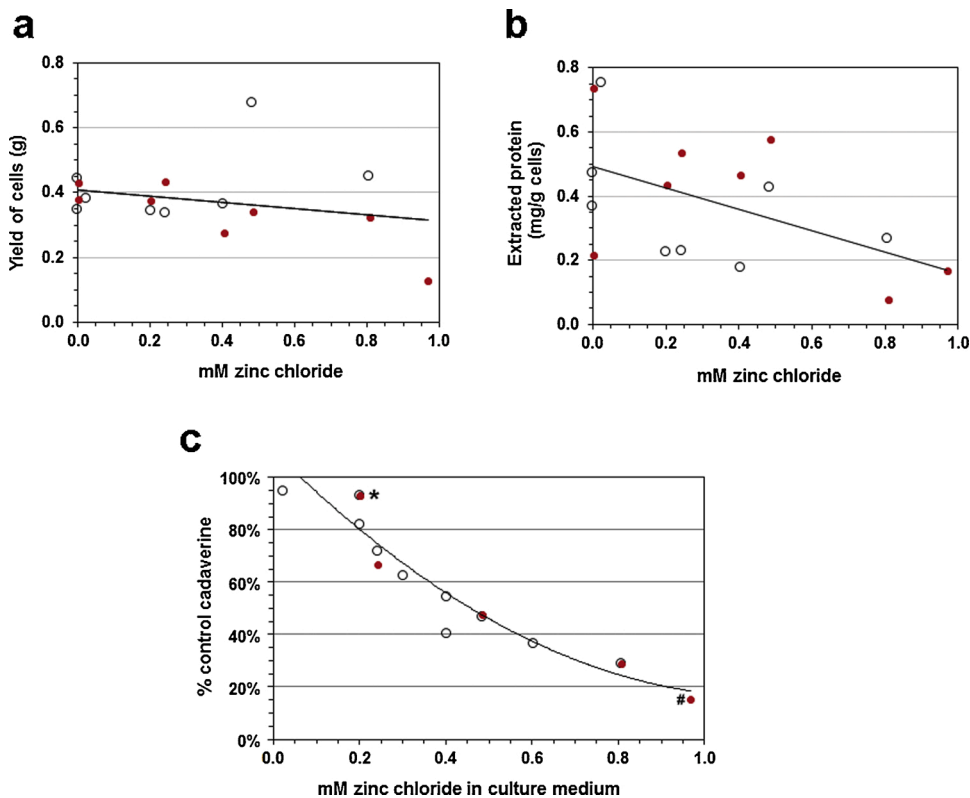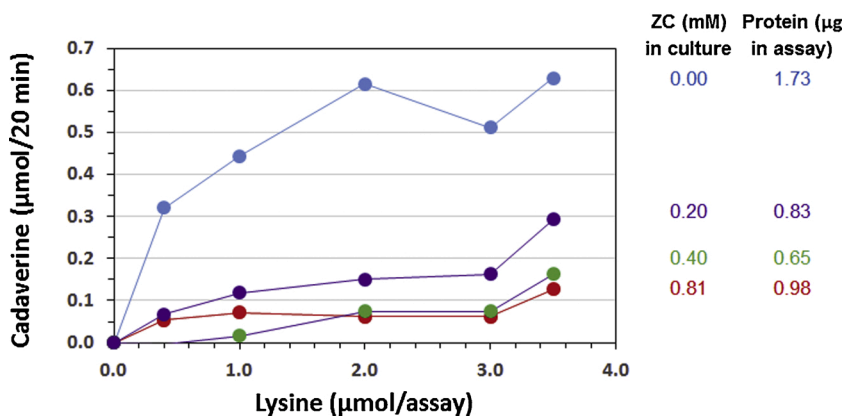

ZC (mM) in culture	Protein (µg in assay)
0.00	1.73
0.20	0.83
0.40	0.65
0.81	0.98

Fig. 3. Effect of zinc chloride (ZC) on *E. corrodens* yield, protein content of cell surface extracts, and LdcE activity/mg extracted protein. Small solid red circles indicate the Triumph ZC source (See Methods). (**a**) Cell pellet yield (g) against mM ZC ($R^2 = 0.08$, not significant). (**b**) Protein content of cell surface extracts (mg/g cells) against mM ZC, $R^2 = 0.27$, $p < 0.05$; total protein/g cells = 0.49–0.33*ZC. (**c**) Cadaverine produced from 3.0 and 3.5 µmol lysine (LdcE activity) was averaged, expressed per mg protein and graphed as a percentage of controls (no ZC), $R^2 = 0.90$, $p < 0.001$; percent control activity/mg protein (second-degree polynomial model) = $0.74*ZC^2 - 1.69*ZC + 1.13$. Asterisk (*) indicates three identical results at 0.2 mM ZC two from Triumph ZC and one from Mallinckrodt ZC. Hash-tag (#) indicates 85% inhibition of activity at 0.96 mM ZC. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

4.2. Suggested mode of action of zinc ions on LdcE

LdcE is constitutively present on the outer surface of *E. corrodens* cells from which it can be removed without disrupting the cells *in vitro* (Methods, section 2.1). The amino acid sequence of LdcE is 64% identical to that of LdcI, and 58% identical to that of LdcC (Supplement, Fig. 1). LdcE belongs to a class of lysine decarboxylases composed of three domains, of which the largest is a central PLP-binding core domain (residues 130–563) related to the PLP-binding core of aspartate amino acid transferase, AAT (I. Gutschke, Personal communication to ML). The core AAT-fold is common in the lysine decarboxylases of many intestinal bacteria [56], but *E. corrodens* predominates only in the oral cavity [24]. In this class of AAT fold lysine decarboxylases, the N-terminal 'wing' domains in two molecules first form a dimer that causes their core domains to interact (Supplement, Fig. 2). Five such dimers then combine to form a double pentamer [57]. Both the dimer and the double pentamer forms of LdcE may mediate cadaverine production from lysine *in vivo* [58].

Cysteine residues perform essential functions in many proteins and often display high affinity toward zinc ions, which can create or destroy complex supramolecular assemblies [59]. In LdcE, the N-terminal 'wing' domain possesses four cysteine residues, at amino acids 8, 55, 85 and 103, whereas those of LdcI, and LdcC have only two, at amino acids 53 and 67 (Supplementary Fig. 1). Because the N-terminal domain is synthesized first, we propose that zinc in the cell cytosol during growth can interact with cysteine residue 8 until folding and dimer formation can begin. We further note that a cysteine residue within the first 25 residues of the N-terminus is absent from the lysine decarboxylases of intestinal bacterial.

5. Conclusion

Zinc ions inhibit the production of *E. corrodens* lysine decarboxylase (LdcE), preventing its conversion of lysine to cadaverine and carbon dioxide. Lysine is essential for dentally attached epithelial cell turnover, and its depletion initiates gingival inflammation. In addition, the restriction of cadaverine production may enhance bacterial killing by reactive nitrogen species in GCF from inflamed gingiva. The results of this study therefore provide a rationale for the superior control of gingival inflammation by zinc dentifrices.

Funding

CRediT authorship contribution statement

Martin Levine: Conceptualization, Methodology, Supervision, Writing - review & editing. **Lindsay M Collins:** Investigation, Data curation, Writing - original draft. **Zsolt Lohinai:** Writing - review & editing.

Declaration of Competing Interest

None of the authors have a conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdent.2020.103533.

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