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Arcanolysin is a cholesterol-dependent cytolysin of the human pathogen *Arcanobacterium haemolyticum*

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Abstract

Background: *Arcanobacterium haemolyticum* is an emerging human pathogen that causes pharyngitis, wound infections, and a variety of occasional invasive diseases. Since its initial discovery in 1946, this Gram positive organism has been known to have hemolytic activity, yet no hemolysin has been previously reported. *A. haemolyticum* also displays variable hemolytic activity on laboratory blood agar that is dependent upon which species the blood is derived.

Results: Here we describe a cholesterol-dependent cytolysin (CDC) secreted by *A. haemolyticum*, designated arcanolysin (*aln*), which is present in all strains (n = 52) tested by DNA dot hybridization. Among the known CDCs, ALN is most closely related to pyolysin (PLO) from *Trueperella* (formerly *Arcanobacterium*) *pyogenes*. The *aln* probe, however, did not hybridize to DNA from *T. pyogenes*. The *aln* open reading frame has a lower mol %G+C (46.7%) than the rest of the *A. haemolyticum* genome (53.1%) and is flanked by two tRNA genes, consistent with probable acquisition by horizontal transfer. The ALN protein (~ 64 kDa) contains a predicted signal sequence, a putative PEST sequence, and a variant undecapeptide within domain 4, which is typically important for function of the toxins. The gene encoding ALN was cloned and expressed in *Escherichia coli* as a functional recombinant toxin. Recombinant ALN had hemolytic activity on erythrocytes and cytolytic activity on cultured cells from human, rabbit, pig and horse origins but was poorly active on ovine, bovine, murine, and canine cells. ALN was less sensitive to inhibition by free cholesterol than perfringolysin O, consistent with the presence of the variant undecapeptide.

Conclusions: ALN is a newly identified CDC with hemolytic activity and unique properties in the CDC family and may be a virulence determinant for *A. haemolyticum*.

Background

Arcanobacterium haemolyticum, a Gram positive, pleomorphic rod, causes wound infections and pharyngitis and can occasionally cause more severe invasive diseases such as endocarditis, meningitis, septic arthritis, pneumonia and osteomyelitis in humans [1]. There is strong epidemiologic evidence for *A. haemolyticum* being the only or primary isolate from throat specimens of some humans with pharyngitis [1-4] and these data suggest that the number of cases per year of *A. haemolyticum*-mediated pharyngitis is ~240,000-480,000 with 0.5-1 million lost work days in the United States. The organism, previously in the *Corynebacterium* genus, was classified as the first member of the genus *Arcanobacterium* [5]. The other members of the genus are uncommonly isolated and remain largely uncharacterized, with the exception of *Trueperella* (*Arcanobacterium*) *pyogenes*, which is an important opportunistic livestock pathogen [6].

Little is known about *A. haemolyticum* virulence factors with the exception of a phospholipase D (PLD) [7], which causes dermonecrosis [8]. We recently described the ability of PLD to reorganize host membrane lipid rafts, leading to enhanced bacterial adhesion [9]. Furthermore, *A. haemolyticum* was able to invade HeLa cells and once intracellular, PLD was able to kill host

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cells via direct necrosis [9]. These effects could potentially lead to bacterial dissemination to deeper tissues.

It is thought that clinical microbiology laboratories often miss *A. haemolyticum* in clinical specimens due to the organism's weak hemolytic activity on the commonly-used sheep blood agar, and therefore it may be misinterpreted as commensal diphtheroids and the isolate discarded. However, this organism displays more pronounced hemolysis on human and rabbit blood [10,11]. The organism has been known to have hemolytic activity since its initial discovery in 1946 [12], yet no *bona fide* hemolysin has been previously reported. PLD itself is not directly hemolytic, but causes synergistic hemolysis with bacteria that express cholesterol oxidase [13], prompting a search for the *A. haemolyticum* hemolysin. Possible clues to the identity of the *A. haemolyticum* hemolysin come from studies on the hemolytic bacterium *T. pyogenes*, which is closely related to *A. haemolyticum*. *T. pyogenes* expresses PLO, a member of the cholesterol-dependent cytolysin (CDC) toxin family, as its primary virulence factor and this molecule is a hemolysin [14]. Thus, we hypothesized that the hemolytic activity expressed by *A. haemolyticum* was due to the presence of an uncharacterized CDC.

Here we report the identification and characterization of a CDC from *A. haemolyticum*, designated arcanolysin (ALN). We show that ALN has several distinct structural features among the CDC family and demonstrate that ALN is cholesterol-dependent and provide evidence that ALN has variable hemolytic and cytotoxic activity against mammalian cells from different species. We propose ALN is the long, sought-after hemolysin.

Methods
Bacteria and growth conditions
ATCC 9345 is the *A haemolyticum* type strain. The other *A. haemolyticum* strains used in this study were archival isolates obtained from diverse human clinical cases (Table 1). *A. haemolyticum* and *Escherichia coli* strains were grown as previously described [9].

DNA techniques
E. coli DH5αMCR plasmid DNA extraction, transformation, DNA restriction, ligation and agarose gel electrophoresis were by standard methods [15]. DNA hybridization was performed using the DIG DNA Labeling and Detection Kit (Roche). PCR DNA amplification was performed using Vent DNA polymerase (NEB) for 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min/kb at 72°C, with a final extension step of 72°C for 7 min.

Nucleotide sequence determination and analysis
Prior to the recent GenBank deposit of the 1.986 MB genome from strain ATCC9345 (= DSM20595 = 11018)

[16], we sequenced the same strain to > 20× coverage (454 Life Sciences), with ~1.945 MB of unique sequence (> 98% complete) with essentially identical sequence data. A translated ORF with amino acid similarity to CDCs, Arch_1062, was identified within this sequence. Oligonucleotide primers flanking this ORF were used to amplify the region by PCR. The nucleotide sequence was confirmed by automated DNA sequencing of both strands. The *aln* sequence data and flanking regions were submitted to the GenBank/EMBL/DDBJ databases under accession number FJ785427.

Database searches were performed using the BlastX and BlastP algorithms [17]. tRNA sequences were identified using the tRNAscan-SE program [18]. Signal sequence prediction was performed using SignalP [19]. Transcriptional terminators were identified using mfold [20]. Multiple sequence alignments were performed using CLUSTAL W [21], and tree construction was with the neighbor-joining algorithm and midpoint rooting, carried out in MacVector version 12.0.3 (MacVector, Inc.). PEST sequence prediction used the pestfind algorithm http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind.

Cloning and purification of a recombinant, 6xHis tagged-ALN (His-ALN)
The *aln* gene, without the signal sequence, was amplified from *A. haemolyticum* ATCC9345 genomic DNA by PCR with His-ALNF (5'-CCCGGCGTTGCGGATCCAGTTGACGC-3') and ALN5 (5'-GGACCTTCTCGAGTATGTATCACTC-3') encoding *Bam*HI and *Xho*I sites (underlined in the primer sequence), respectively. These primers amplified a 1,669 bp product. The PCR fragment was digested with *Bam*HI-*Xho*I and cloned into pTrcHisB (Invitrogen), to generate pBJ51, which encoded the 63.7 kDa His-ALN. The final His-ALN translational fusion protein thus has the MWVGSQKHYFFYQDRGKIMTRRFLATVAGTALLAGAFAPGVAFG signal sequence removed and replaced with the sequence from the vector that leads to MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDP (6 His underlined). No other ALN native amino acids were removed. Cultures for purification of His-ALN were grown and lysed as described [9]. His-ALN was purified from the soluble cell fraction using TALON Metal Affinity Resin, as described (Clontech). His-ALN was eluted from the resin with 50 mM imidazole, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0 (elution buffer). Total protein concentration was determined using Bradford Protein Assay Reagent (Bio-Rad).

For some experiments ALN was amplified from ATCC 9345 DNA using the primers ALN26-F (GCCGCCGCTAGCGTTGACGCTTCAACACAAACCGATCC) and ALN-R (GCCGCCCTCGAGTCACTCG

Table 1 *Arcanobacterium* strains used in this study.

Strain (all *A. haemolyticum* except as noted)	Relevant characteristics	Source
AhS1	Biotype S*; wound infection; 73 year old male; 1991	Petteri Carlson
AhS2	Biotype S; paronychia; 16 year old male; 1991	Petteri Carlson
AhS3	Biotype S; wound infection; 11 year old male; 1991	Petteri Carlson
AhS4	Biotype S; infected leg ulcer; 47 year old male; 1991	Petteri Carlson
AhS5	Biotype S; wound infection; 64 year old male; 1991	Petteri Carlson
AhS6	Biotype S; wound infection; 43 year old male; 1991	Petteri Carlson
AhS7	Biotype S; infected leg ulcer; 68 year old female; 1991	Petteri Carlson
AhS8	Biotype S; wound infection; 62 year old male; 1991	Petteri Carlson
AhS9	Biotype S; wound infection; 38 year old male; 1991	Petteri Carlson
AhS10	Biotype S; paronychia; 21 year old male; 1991	Petteri Carlson
AhS11	Biotype S; pharyngitis; 3 year old male; 1991	Petteri Carlson
AhS12	Biotype S; pharyngitis; 23 year old female; 1992	Petteri Carlson
AhS13	Biotype S; pharyngitis; 28 year old female; 1992	Petteri Carlson
AhS14	Biotype S; pharyngitis; 23 year old female; 1992	Petteri Carlson
AhS15	Biotype S; pharyngitis; 20 year old male; 1992	Petteri Carlson
AhS16	Biotype S; sinusitis; 41 year old male; 1990	Petteri Carlson
AhS17	Biotype S; sinusitis; 65 year old female; 1991	Petteri Carlson
AhS18	Biotype S; pharyngitis; 12 year old male; 1992	Petteri Carlson
AhS19	Biotype S; pharyngitis; 20 year old female; 1992	Petteri Carlson
AhS20	Biotype S; pharyngitis; 34 year old male; 1992	Petteri Carlson
AhS21	Biotype S; peritonsillar abscess; 15 year old male; 1996	Petteri Carlson
AhS22	Biotype S; pharyngitis, pneumonia; 42 year old male; 1996	Petteri Carlson
AhS23	Biotype S; diabetic foot gangrene; 45 year old male; 1997	Petteri Carlson
AhS24	Biotype S; tonsillitis; 16 year old female; 1998	Petteri Carlson
AhS25	Biotype S; metatarsal osteitis; 37 year old male; 1998	Petteri Carlson
AhR26	Biotype R; wound infection; 43 year old male; 1991	Petteri Carlson
AhR27	Biotype R; wound infection; 53 year old male; 1991	Petteri Carlson
AhR28	Biotype R; pharyngitis; 13 year old female; 1991	Petteri Carlson
AhR29	Biotype R (uncertain); peritonsillar abscess; 18 year old male; 1991	Petteri Carlson
AhR30	Biotype R; sinusitis; 14 year old male; 1992	Petteri Carlson
AhR31	Biotype R; peritonsillar abscess; 21 year old male; 1986	Petteri Carlson
AhR32	Biotype R; peritonsillar abscess; 15 year old female; 1992	Petteri Carlson
AhR33	Biotype R; pharyngitis; 26 year old male; 1992	Petteri Carlson
AhR34	Biotype R; pharyngitis; 15 year old male; 1992	Petteri Carlson
AhR35	Biotype R; pharyngitis; 18 year old male; 1992	Petteri Carlson
AhR36	Biotype R; pharyngitis; 21 year old male; 1992	Petteri Carlson
AhR37	Biotype R; peritonsillar abscess; 15 year old female; 1992	Petteri Carlson
AhR38	Biotype R; wound infection; 21 year old female; 1992	Petteri Carlson
AhR39	Biotype R; pharyngitis; 18 year old female; 1992	Petteri Carlson
AhR40	Biotype R; pharyngitis; 17 year old male; 1992	Petteri Carlson
AhR41	Biotype R; pharyngitis; 24 year old male; 1992	Petteri Carlson
AhR42	Biotype R; pharyngitis; 16 year old female; 1992	Petteri Carlson
AhR43	Biotype R; pharyngitis; 12 year old male; 1992	Petteri Carlson
AhR44	Biotype R; pharyngitis; 18 year old male; 1992	Petteri Carlson
AhR45	Biotype R; pharyngitis; 16 year old male; 1992	Petteri Carlson

Table 1 *Arcanobacterium* strains used in this study. *(Continued)*

AhR46	Biotype R; pharyngitis; 14 year old female; 1992	Petteri Carlson
AhR47	Biotype R; pharyngitis; 13 year old female; 1991	Petteri Carlson
AhR48	Biotype R; pharyngitis; 15 year old male; 1992	Petteri Carlson
AhR49	Biotype R; pharyngitis; 20 year old male; 1991	Petteri Carlson
AhR50	Biotype R; pharyngitis; 19 year old female; 1992	Petteri Carlson
CCUG39796	Biotype unknown; infected leg ulcer; 52 year old male; 1998	Petteri Carlson
ATCC9345; DSM 20595	Biotype unknown, 1946	American Type Culture Collection; [12]
Trueperella (Arcanobacterium) pyogenes BBR1	*plo nanH nanP cbpA fimAB tet*(W); Isolated from a bovine abscess	[14]

* S, Smooth type; R, rough type.

CTATGAACGATGTTCTTG), cloned into expression vector pET28a (Novagen) using *Nhe*I and *Xho*I sites (underlined), and confirmed by sequencing. The *plo* gene encoding PLO was amplified from *T. pyogenes* ATCC 49698 DNA using the primers PYO28-F (GCCGCCCATATGGCCGGATTGGGAAACAGTTCG) and PYO-R (GCCGCCCTCGAGCTAGGATTTGA-CATTTTCCTC), cloned into pET28a using *Nde*I and *Xho*I sites (underlined), and confirmed by sequencing. The *ily* gene encoding ILY was amplified from *Streptococcus intermedius* and cloned into pET28a as described [22]. Purification of the His-tagged CDCs was as previously described [22,23].

SDS-PAGE and Western blotting
Proteins were separated by electrophoresis in 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose [15]. Western blots were immunostained using rabbit anti-His-ALN (prepared by immunization of a rabbit with His-ALN, Antibodies Inc., Davis, CA) and rabbit anti-goat IgG(H+L)-peroxidase conjugate (KPL), as the primary and secondary antibodies, respectively. Rabbit antiserum against PFO was kindly provided by Rodney K. Tweten, University of Oklahoma Health Sciences Center, OK.

Hemolytic assays
The hemolytic titers of His-ALN preparations were determined by incubation of two-fold serial dilutions of protein with an equal volume of 0.5% blood (Cleveland Scientific, Bath, OH) at 37°C for 1 h [14]. The hemolytic titer was the reciprocal of the highest dilution which resulted in 50% cell lysis, expressed as hemolytic units (HU) [14]. The specific activity of purified His-ALN was determined as HU/μg protein. Thiol activation was assessed by incubation of 5 HU His-ALN with 2% β-mercaptoethanol for 10 min at room temperature, prior to performing a hemolytic assay with human blood (Cleveland Scientific). Cholesterol inhibition was assessed by

incubation of 5 HU His-ALN with 0.01-1 μM cholesterol for 30 min at room temperature with shaking, prior to performing a hemolytic assay with human blood. Cholesterol was diluted in absolute ethanol and an equal volume of ethanol was used as the cholesterol-free control. His-tagged perfringolysin O (PFO) [24] and His-tagged PLO [14] were used as controls in the various hemolytic assays. For some experiments hemolysis assays were performed as described [22,23].

Epithelial cell cytotoxicity
The epithelial cell cytotoxicity of His-ALN was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). A549 (human lung, CCL-185), CHO (hamster ovary, CCL-61), HCT-8 (human colon, CCL-244), J774A.1 (mouse macrophage, TIB-67), MDBK (bovine kidney, CCL-22), MDCK (canine kidney, CCL-34) and RK-13 (rabbit kidney, CCL-37) cells were cultured in Iscove's Modified Dulbecco's Medium or RMPI 1640 with 10% fetal bovine serum and 10 μg/ml gentamicin in a humidified, 5% CO_2 atmosphere at 37°C. Cells were seeded into 96-well plates at 2×10^4 cells/well and incubated for 18 h to achieve 80% confluence. Triplicate wells were incubated with doubling dilutions of His-ALN (0-2000 ng) and incubated for 2 h, prior to addition of substrate for 3 h. Determination of cell viability was performed using the appropriate control values (Promega).

Membrane binding assay
The membrane binding assay was performed using erythrocytes as previously described [25]. His-ALN was diluted to 12.5 μg ml^{-1} in PBS, 40 μl was added to an equal volume of 50% (v/v) blood and the mixture was incubated on ice for 20 min. Cells were harvested by centrifugation at 14,000 g for 5 min at 4°C, resuspended in SDS-PAGE sample buffer and subjected to SDS-PAGE and Western blotting with antiserum against His-ALN.
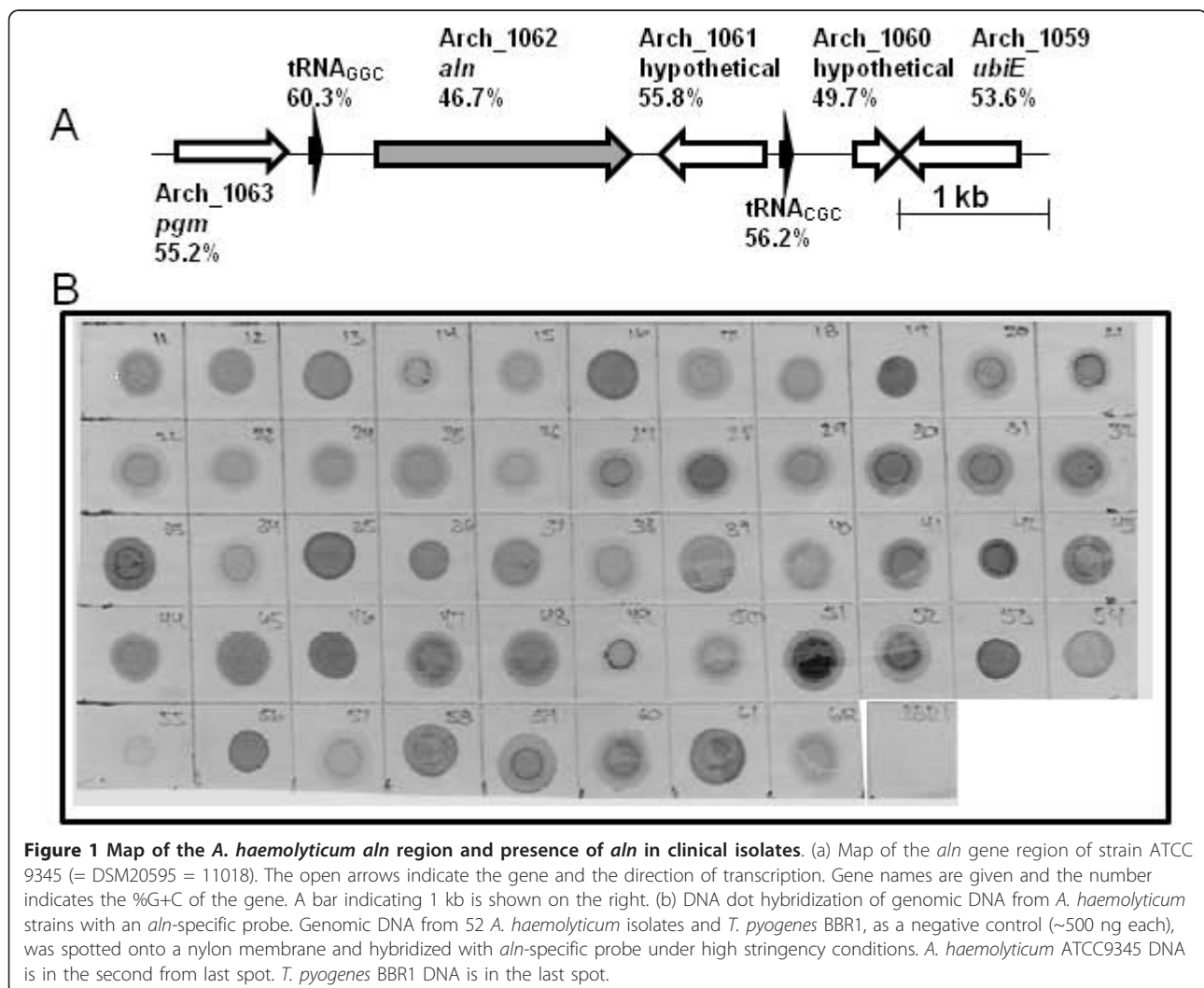
Results

Cloning and nucleotide sequence determination of *aln*

A draft genome sequence of *A. haemolyticum* ATCC 9345 was determined and consists of 46 contigs that encompass ~1.945 Mb in size (D. J. McGee, S. J. Billington, and B. J. Jost, unpublished). 1,639 ORFs were preliminarily identified using the Rapid Annotation using Subsystem Technology (RAST) Server [26]. Within this sequence, we identified ORF Arch_1062, the translation of which displayed similarity to other CDCs. The 1,710 bp gene was designated *aln*, for arcanolysin (ALN). Upstream of *aln* are a phosphoglycerate mutase gene (*pgm*; Arch_1063) (EC 5.4.2.1) and an alanine tRNA$_{GGC}$ (Figure 1). In the 426 bp intergenic region are regulatory signals predicted to be involved in *aln* transcription, including a putative σ^{70} promoter and 3 direct repeats (ATTTT(G/C)(G/T)T) which are similar to those found immediately upstream of *plo*, encoding PLO, the CDC of *T. pyogenes* [27]. 6 bp downstream of *aln* is a transcriptional terminator with a ΔG = -18.05 kcal/mol. Downstream of *aln* and divergently transcribed is Arch_1061. The Arch_1061 protein displays amino acid similarity to hypothetical proteins from a number of genome sequences, including *Corynebacterium jeikeium* (GenBank YP_249820.1), and features a signal sequence. Further downstream is an additional alanine tRNA$_{CGC}$, which is 91% identical at the nucleotide level to the alanine tRNA$_{GGC}$ upstream of *aln*. Further downstream of the 2nd alanine tRNA is Arch_1060, a gene that is predicted to encode a conserved hypothetical protein related to *Corynebacterium diphtheriae* (DIP0761), and a gene, Arch_1059 (*ubiE*), with similarity to type II or SAM-dependent methyltransferases (EC 2.1.1.-).

The %G+C for *aln* is 46.7% (Figure 1) compared with 49.7-60.3% for the surrounding genes and 53.1% for the entire genome. Given the lower %G+C of the *aln* gene and the presence of flanking tRNA genes, which can act as sites of foreign gene insertion [28], it is possible that

Figure 1 Map of the *A. haemolyticum* *aln* region and presence of *aln* in clinical isolates. (a) Map of the *aln* gene region of strain ATCC 9345 (= DSM20595 = 11018). The open arrows indicate the gene and the direction of transcription. Gene names are given and the number indicates the %G+C of the gene. A bar indicating 1 kb is shown on the right. (b) DNA dot hybridization of genomic DNA from *A. haemolyticum* strains with an *aln*-specific probe. Genomic DNA from 52 *A. haemolyticum* isolates and *T. pyogenes* BBR1, as a negative control (~500 ng each), was spotted onto a nylon membrane and hybridized with *aln*-specific probe under high stringency conditions. *A. haemolyticum* ATCC9345 DNA is in the second from last spot. *T. pyogenes* BBR1 DNA is in the last spot.

the *A. haemolyticum aln* gene was acquired by horizontal gene transfer.

aln is widely distributed in *A. haemolyticum* isolates

The prevalence of the *aln* gene was determined by DNA hybridization. A DIG-labeled probe spanning bases 492-1,052 of the *aln* ORF was hybridized to genomic DNA from *A. haemolyticum* ATCC9345, 51 *A. haemolyticum* clinical isolates (Table 1) and *T. pyogenes* BBR1, as a negative control. The *aln* probe hybridized at high stringency to all *A. haemolyticum* isolates (n = 52), but not *T. pyogenes* genomic DNA (Figure 1b), indicating that this gene appears to be highly prevalent in *A. haemolyticum*. The region of *aln* from which the probe was derived has 62.8% identity to the corresponding nucleotide region in *plo* of *T. pyogenes*. Under high stringency hybridization conditions, DNA sequences which are less than 70% identical do not hybridize.

Analysis of the primary structure of ALN

The predicted ALN protein is 569 amino acids in length, including a 26 amino acid signal sequence predicted by SignalP. The mature protein lacking the signal sequence has a predicted molecular mass of 60.1 kDa. ALN is most similar to PLO with 59.4% and 71.5% amino acid identity and similarity (Figure 2) and has ~50% similarity to other CDC family members. Within

the ALN N-terminus, the pestfind algorithm identified a putative PEST sequence not present in PLO or most other CDC sequences (Figure 3a). Listeriolysin O (LLO), which contains a *bona fide* PEST sequence [29], returned a pestfind score of 4.71, while ALN had a score of 7.58, indicating a higher probability of containing a functional PEST sequence. Given that *A. haemolyticum* invades host cells [9], it is possible that the PEST sequence allows for a similar compartmentalization of ALN activity within the host cell. Like PLO, the predicted amino acid sequence of ALN has a variant undecapeptide in domain 4 and both lack the conserved cysteine residue (Figure 3b). The tryptophan spacing of ALN and PLO (WxxWW) also differs from the consensus sequence (WxWW) (Figure 3b).

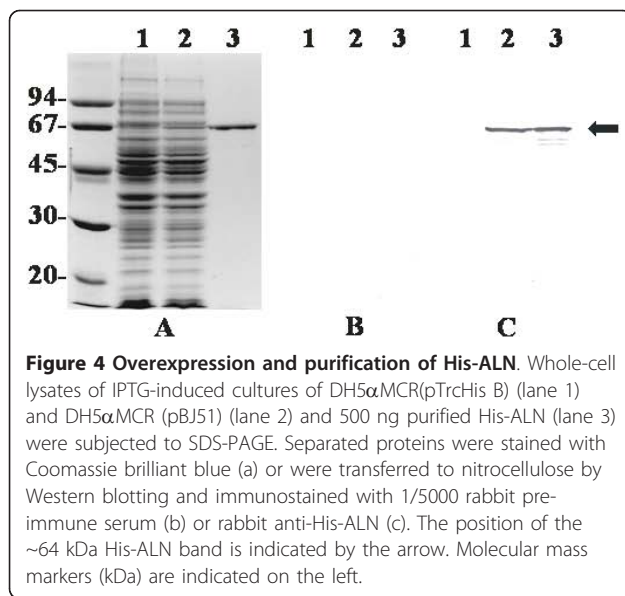### Cloning and expression of His-ALN

SDS-PAGE and Coomassie Brilliant Blue staining of IPTG-induced cultures of pBJ51-containing *E. coli* indicated the presence of an over-expressed protein of ~64 kDa (Figure 4a). His-ALN was purified to > 95% homogeneity using TALON resin (Figure 4a), and the size of this protein (~64 kDa) corresponded to its predicted molecular mass. Antiserum against ALN, but not pre-immune antiserum, reacted specifically with His-ALN and some possible HIS-ALN degradation products (Figure 4b and 4c).
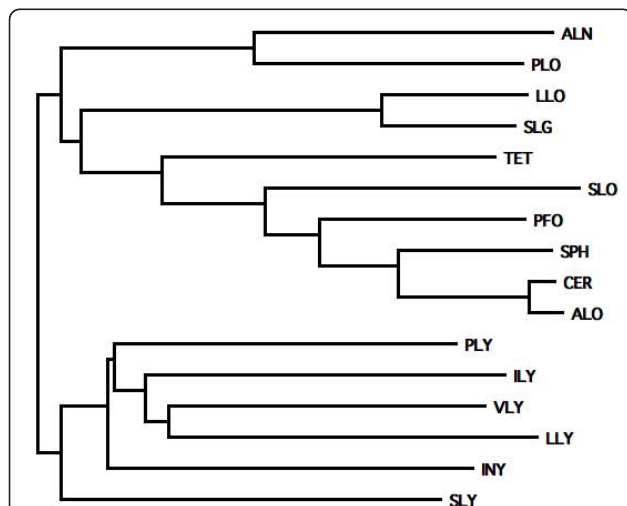
Figure 2 Neighbor joining tree of amino acid sequences showing the relationship of ALN to other selected CDC family members. Abbreviations and gi ascession numbers from the NCBI protein database: ALN, arcanolysin (259156857); PLO, pyolysin (2252800); LLO, listeriolysin O (16802248); SLG seeligeriolysin (40889013); TET, tetanolysin (28211522); SLO, streptolysin O (15674372); PFO, perfringolysin O (18309145); SPH, sphaericolysin (146455206); CER, cereolysin (62550724); ALO, anthrolysin O (49186114); PLY, pneumolysin (15901747); ILY, intermedilysin (6729344); VLY, vaginolysin (187940699); LLY, lectinolysin (190576835); INY, inerolysin (259167149); SLY, suilysin (253752120).
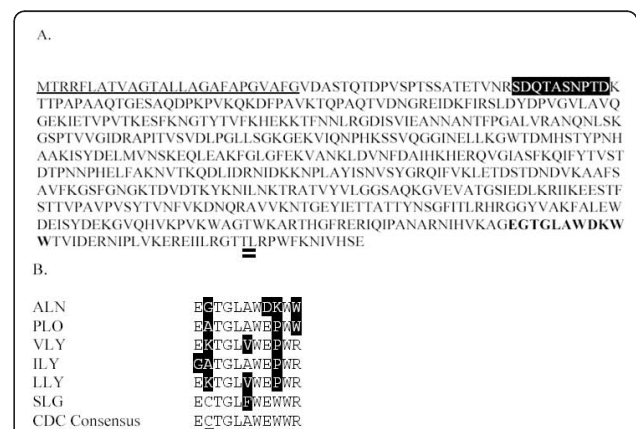
Figure 3 Salient features of the ALN predicted amino acid sequence. (a) ALN sequence with predicted signal sequence (underlined), putative PEST motif (inverse), undecapeptide (bold), and cholesterol-interacting TL motif (double underlined). (b) Undecapeptide sequences of ALN, other CDC undecapeptides known to differ from consensus, and the consensus CDC undecapeptide. The cysteine conserved in thiol-activated CDCs (but absent from ALN) is underlined in the consensus sequence. Differences from consensus depicted as inverse letters. Abbreviations as in Figure 2.

Figure 4 Overexpression and purification of His-ALN. Whole-cell lysates of IPTG-induced cultures of DH5αMCR(pTrcHis B) (lane 1) and DH5αMCR (pBJ51) (lane 2) and 500 ng purified His-ALN (lane 3) were subjected to SDS-PAGE. Separated proteins were stained with Coomassie brilliant blue (a) or were transferred to nitrocellulose by Western blotting and immunostained with 1/5000 rabbit pre-immune serum (b) or rabbit anti-His-ALN (c). The position of the ~64 kDa His-ALN band is indicated by the arrow. Molecular mass markers (kDa) are indicated on the left.

Recombinant ALN has cytotoxic activity

A. haemolyticum is not strongly hemolytic when grown on ovine (sheep) blood agar [10]. Likewise, the *E. coli* strain expressing His-ALN did not display hemolysis when grown on bovine blood agar (data not shown). Similarly, His-ALN displays low hemolysis with bovine or ovine erythrocytes (Figure 5a). In contrast, His-ALN had ~4- and 10-fold increased hemolytic activity on rabbit and human erythrocytes, respectively (Figure 5a). This is in contrast to PFO or PLO, which show little difference in specific activity on erythrocytes from different hosts. Consistent with these findings, hemolysis assays demonstrated that ALN has a preference for horse or human cells over porcine cells but lyses all of these at high toxin concentrations (Figure 5b). This is in contrast to intermedilysin (ILY) from *Streptococcus intermedius*, which retains human-specific tropism over a wide concentration range, and PLO, which is less selective than ALN (Figure 5b).

The highly-conserved Cys residue in the undecapeptide of CDCs is responsible for Thiol activation of this group of toxins [30]. ALN lacks the Cys residue in the undecapeptide (Figure 3a), and like PLO [14], its activity was unaffected by treatment with β-mercaptoethanol (data not shown).

We also determined the effect of recombinant ALN on cultured mammalian cells. His-ALN was applied to human, bovine, canine, hamster, mouse and rabbit cell lines and was highly active on human and rabbit cells (Figure 5c), with low activity on bovine, mouse and canine cells. This toxin had intermediate activity on hamster cells (Figure 5c). This finding mirrors the activity of ALN on blood from different host species (Figure 5a), and is less species-specific than intermedilysin (ILY)

or vaginolysin (VLY) [23,31]. ILY, VLY, and lectinolysin (LLY) use human CD59 (hCD59) as a membrane receptor [23,32,33], leading to host-specificity. Unlike these other CDC toxins ALN hemolysis was not blocked with a monoclonal antibody against hCD59 (data not shown). Consistent with this finding, the predicted ALN amino acid sequence lacks the Tyr-X-Tyr-X_{14}-Ser-Arg signature motif common to all known hCD59-dependent CDCs [33].

### The activity of ALN is less sensitive to cholesterol inhibition than PFO

Given the more restrictive host species preference of ALN over that of PFO, along with the variant undecapeptide sequence in ALN, we hypothesized that ALN might be less sensitive to inhibition by free cholesterol. As expected, PFO activity was almost completely inhibited by exogenous 0.5 μM cholesterol (7.6%; Figure 6). In contrast, PLO and ALN retained 52.5% and 41.4% activity, respectively, when incubated with 0.5 μM cholesterol and retained ~20% of hemolytic activity at 1 μM cholesterol (Figure 6). These data indicate that ALN and PLO have intermediate sensitivity to cholesterol compared to a CDC (PFO) with the conserved undecapeptide sequence.

ALN binds differentially to host cell membranes

Hemolytic assays measure the full spectrum of CDC binding, oligomerization and pore formation leading to cell lysis. However, initial toxin binding to membranes can be determined by incubation of CDCs with host cells at 4°C, which prevents subsequent oligomerization and pore formation [34]. Using this approach, His-ALN bound to human and rabbit erythrocytes as determined by Western blotting (Figure 7). Probable ALN degradation products were also detected. His-ALN did not exhibit detectable binding to bovine or ovine erythrocyte membranes under these conditions. As a control, His-PFO was incubated with human, bovine, ovine or rabbit erythrocytes, and bound toxin was detected with anti-PFO antiserum. His-PFO bound to all cell types at approximately equivalent amounts (data not shown). These data suggest that ALN host preference may occur at the initial contact of the toxin with the host cell membrane.

Discussion

The CDCs are a family of bacterial toxins produced by diverse Gram-positive bacteria and are generally important in pathogenesis [35-37]. CDCs have a four-domain structure and a conserved C-terminal undecapeptide sequence in domain 4 that is important for toxin function. Soluble CDC monomers bind to host membrane targets, oligomerize into a large homomeric structure
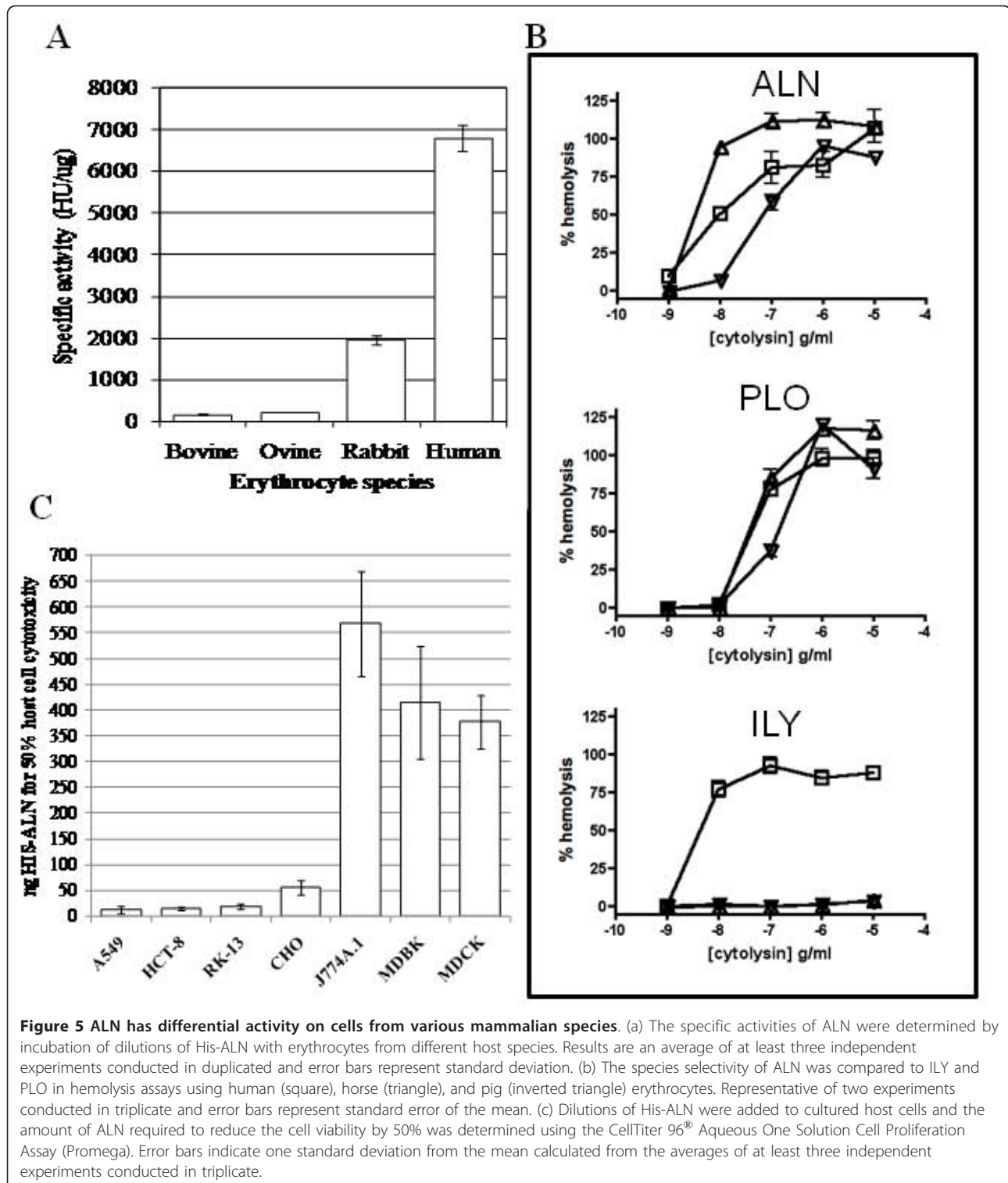
Figure 5 ALN has differential activity on cells from various mammalian species. (a) The specific activities of ALN were determined by incubation of dilutions of His-ALN with erythrocytes from different host species. Results are an average of at least three independent experiments conducted in duplicated and error bars represent standard deviation. (b) The species selectivity of ALN was compared to ILY and PLO in hemolysis assays using human (square), horse (triangle), and pig (inverted triangle) erythrocytes. Representative of two experiments conducted in triplicate and error bars represent standard error of the mean. (c) Dilutions of His-ALN were added to cultured host cells and the amount of ALN required to reduce the cell viability by 50% was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). Error bars indicate one standard deviation from the mean calculated from the averages of at least three independent experiments conducted in triplicate.

known as the prepore complex, and transition to a true pore, leading to cytolysis of target cells [38]. CDCs interact with membrane cholesterol through a conserved threonine-leucine pair in domain 4, and this interaction is crucial to the formation of functional pores [39].

Some CDCs, including ILY, VLY, and LLY, require the presence of hCD59 as a membrane receptor, conferring human-specific activity [23,33,40]. Among the CDCs, PLO is unusual, as it contains a variant of the highly conserved domain 4 undecapeptide, and this divergent
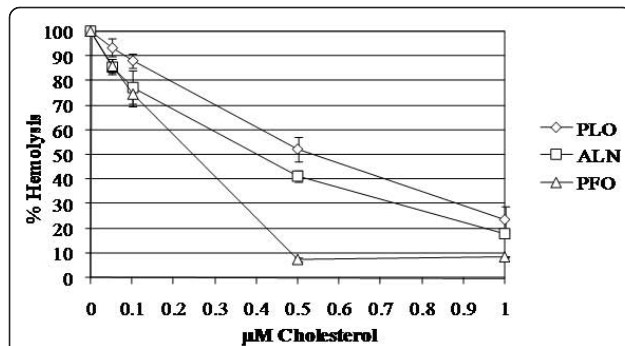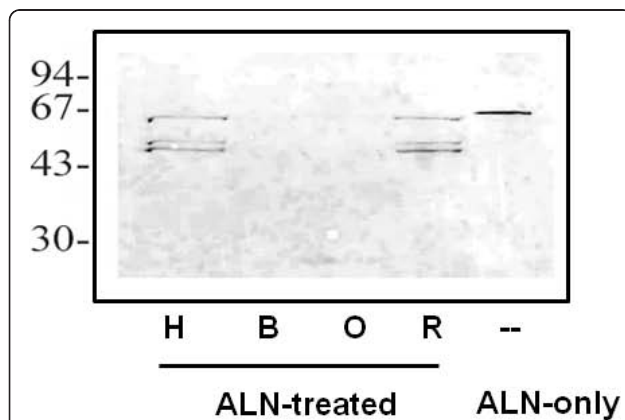
Figure 6 ALN, a cholesterol-dependent cytolysin, has hemolytic activity that is less sensitive to cholesterol inhibition than PFO. His-tagged CDCs were preincubated with dilutions of cholesterol for 30 min at room temperature prior to hemolytic assay. Abbreviations as in Figure 2. Error bars indicate one standard deviation from the mean calculated from the averages of three independent experiments conducted in triplicate.

sequence is essential for full PLO activity [41]. The ALN undecapeptide is most similar to that of PLO (Figure 3B), in that it retains the three tryptophan residues of the consensus undecapeptide but employs an alternate spacing (i.e. WxxWW rather than WxWW). The tryptophan residues of the undecapeptide are known to be important for insertion of domain 4 into host cell membranes [42]. Like the human-specific CDCs (VLY, ILY, and LLY), ALN contains a proline in its undecapeptide sequence. However, the hemolytic activity of ALN was

Figure 7 ALN has a differential ability to bind to erythrocyte cell membranes from different host species. His-ALN (500 ng) or buffer (negative control) was added to erythrocytes, and the mixture was incubated on ice for 20 min. Untreated (no reactivity, data not shown) or ALN-treated erythrocyte membrane fractions from human (H), bovine (B), ovine (O) or rabbit (R) blood were separated by SDS-PAGE, transferred to nitrocellulose, and immunostained with 1/1000 rabbit anti-His-ALN. His-Aln (500 ng) in absence of erythrocyte membrane fractions (ALN) serves as the positive control. Molecular mass markers (kDa) are indicated on the left.

not blocked by antibodies to human CD59, which acts as a receptor for the human-specific CDCs [23,32,33], suggesting that ALN may interact with a distinct membrane receptor, perhaps in addition to cholesterol. The nature of the ALN receptor is currently unknown and is under investigation. Although the cysteine residue in the consensus undecapeptide confers the property of thiol activation to CDCs, the cysteine is not essential for streptolysin O and pneumolysin toxin function [43,44]. The human-specific CDCs (VLY, ILY, LLY), PLO, and ALN all lack this conserved cysteine residue, but the contribution of this sequence variation to toxin function is not yet known for these toxins.

Some CDCs have a number of functions beyond simple pore formation. *Streptococcus pyogenes* uses streptolysin O to introduce a bacterial effector into host cells via a novel mechanism termed cytolysin-mediated translocation (CMT) [45]. At sublytic concentrations, CDCs may act as ligands for toll-like receptors [46,47] and may induce a cycle of p38 mitogen-activated protein kinase (MAPK) phosphorylation and dephosphorylation [48,49]. LLO allows *Listeria monocytogenes* to escape from the vacuole into the cytoplasm where the organism can rapidly multiply [50]. The site-specific nature of LLO is controlled by cytosolic down-regulation of LLO function due to an N-terminal PEST-like sequence, which usually targets eukaryotic proteins for cytosolic degradation. The PEST sequence results in a substantially reduced half-life of LLO in the cytoplasm of the host cell [29].

Conclusions

ALN has several unique features among the CDC family. ALN has a variant undecapeptide and possesses an unusual N-terminal extension, with a putative PEST sequence. Moreover, ALN lacks the conserved cysteine of thiol-activated CDCs, explaining why β-mercaptothanol had no effect on ALN function. The unique sequences and predicted structural features of ALN will make it an interesting toxin to conduct future structure-function analyses to identify additional unique properties of this toxin. ALN displays an unusual pattern of target cell species selectivity, with high activity against human, horse, and rabbit cells and lesser activity against cells derived from other species. This selectivity appears to function at the level of membrane binding and may contribute to the host range of *A. haemolyticum*. Further work will focus on understanding the role of ALN in *A. haemolyticum* pathogenesis.

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Authors' contributions
BHJ, EAL and AJR designed and conducted the experiments and analyzed data, BHJ drafted the manuscript, AJR, SJB and DJM revised the manuscript and figures. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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