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Characterization of the secretion pathway of the collagen adhesin EmaA of *Aggregatibacter actinomycetemcomitans*

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SUMMARY

The extracellular matrix protein adhesin A (EmaA) surface antennae-like structures of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* are composed of three identical protein monomers. Recently, we have demonstrated that the protein is synthesized with an extended signal peptide of 56 amino acids necessary for membrane targeting and protein translocation. In this study, EmaA secretion was demonstrated to be reliant on a chaperone-dependent secretion pathway. Deletion of *secB* partially reduced but did not abolish the amount of EmaA in the membrane. This observation was attributed to an increase in the synthesis of DnaK in the Δ*secB* strain. Overexpression of a DnaK substitution mutant (A174T), with diminished activity, in the Δ*secB* strain further reduced the amount of EmaA in the membrane. Expression of *dnaK* A174T in the wild-type strain did not affect the amount of EmaA in the membrane when grown under optimal growth conditions at 37°C. However, EmaA was found to be reduced when this strain was grown at heat-shock temperature. A chromosomal deletion of amino acids 16–39 of the EmaA extended signal peptide, transformed with either the wild-type or *dnaK* A174T-expressing plasmid, did not affect the amount of EmaA in the membrane. In addition, the level of EmaA in a Δ*secB*/*emaA*$^-$ double mutant strain expressing EmaA Δ16–39 was unchanged when grown at both temperatures. The data suggest that chaperones are required for the targeting of EmaA to the membrane and a specific region of the signal peptide is necessary for secretion under stress conditions.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a human pathogen associated with both adult and localized aggressive periodontitis (Slots *et al.*, 1980; Zambon, 1985; Christersson, 1993). Multiple extra-oral systemic diseases are also attributed to this gram-negative bacterium including pneumonia, bone disease, soft-tissue abscesses and infective endocarditis (Muhle *et al.*, 1979; Reider & Wheat, 1979; Mauff *et al.*, 1983; Carlile *et al.*, 1984). A mechanism associated with the pathogenesis of this microorganism is the capability of this bacterium to bind to fibrillar collagens (Fives-Taylor *et al.*, 1999; Mintz & Fives-Taylor, 1999). The binding to collagen is mediated by antennae-like surface structures composed of trimers of the extracellular matrix protein adhesin A (EmaA) (Mintz, 2004; Ruiz *et al.*, 2006). The collagen-binding domain has been assigned to the most distal domain

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of the structure, which is composed of amino acids 70–386 of the protein sequence (Yu *et al.*, 2008). The disruption of the *emaA* gene results in the loss of these surface structures and leads to decreased tissue colonization (Ruiz *et al.*, 2006; Tang *et al.*, 2008).

Each EmaA monomer is synthesized as a 202-kDa protein containing an unusually long signal peptide of 56 amino acids required for protein secretion (Jiang *et al.*, 2011). In general, proteins containing signal peptides require interaction with either cytoplasmic proteins or a ribonucleoprotein complex for targeting to the inner membrane and translocation via the SecYEG translocon (Driessen & Nouwen, 2008). The common adaptors include chaperones, e.g. SecB, DnaK, and the signal recognition particle (SRP). These molecules associate with the nascent polypeptide chain to maintain an export-competent state before membrane translocation (Driessen & Nouwen, 2008).

Some periplasmic and outer membrane precursor proteins are SecB-dependent. SecB is a homotetrameric chaperone of 17.3 kDa monomers assembled as a dimer of dimers and binds diverse polypeptides rapidly with high affinity and low specificity (Hardy & Randall, 1991; Randall & Hardy, 1995). SecB has a high affinity for SecA, a peripheral inner membrane protein associated with the YEG translocon (Hartl *et al.*, 1990; Crane *et al.*, 2006). The binding of SecB and the interaction of the signal peptide with SecA results in the passage of the precursor protein through the translocon by hydrolysis of ATP (Randall & Hardy, 1995; Miller *et al.*, 2002).

Independent of the dedicated SecB chaperone pathway, the general cytoplasmic chaperone DnaK and co-chaperones DnaJ and GrpE can participate in translocation of secretory proteins (Wild *et al.*, 1992a, 1996). DnaK belongs to a conserved family of proteins, heat-shock protein 70, whose synthesis is induced by increases in temperature and other forms of stress (Neidhardt *et al.*, 1984). DnaK consists of a substrate-binding domain and a nucleotide-binding domain with weak ATPase activity (Palleros *et al.*, 1993; Schmid *et al.*, 1994; Zhu *et al.*, 1996). Interaction with the co-chaperones DnaJ and GrpE increases the basal rate of ATP hydrolysis (Kamath-Loeb *et al.*, 1995).

Alternatively, some signal peptide bearing proteins are recognized by the SRP and its receptor for membrane translocation through the Sec translocon (Sijbrandi *et al.*, 2003; Froderberg *et al.*, 2004). The SRP is a conserved ribonucleoprotein complex composed of a single 54 kDa protein subunit, Ffh, and a small 4.5S RNA component (Poritz *et al.*, 1990). Ffh, a homolog of the SRP54 protein in mammalian systems, is a GTPase that binds to signal sequences and is also an RNA-binding protein that interacts with the 4.5S RNA (Luirink *et al.*, 1992; Valent *et al.*, 1995). In this pathway, the SRP–nascent protein complex interacts with an inner membrane associated SRP receptor FtsY and transfers the protein to the Sec translocon powered by hydrolysis of GTP (Bernstein *et al.*, 1989). In addition to its role in outer membrane protein secretion, the SRP is also used for some proteins destined for insertion into the bacterial inner membrane (Luirink *et al.*, 2005).

EmaA belongs to a family of autotransporters that contain unusually long signal peptides (Jiang *et al.*, 2011). Although these proteins share structural similarities in the signal peptide region of the protein, different secretion targeting pathways have been described for these proteins (Sijbrandi *et al.*, 2003; Peterson *et al.*, 2006; Desvaux *et al.*, 2007). In this study, we have used genetic approaches to determine the membrane targeting pathway for EmaA in *A. actinomycetemcomitans*. Our results suggest that EmaA is targeted to the membrane via a chaperone-dependent pathway, which may involve multiple chaperones. Evidence is also presented suggesting that DnaK chaperone activity is dependent on a specific sequence of the EmaA extended signal peptide and regulates the secretion of this protein during infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All *A. actinomycetemcomitans* strains were grown statically in 3% (weight/volume) trypticase soy broth supplemented with 0.6% (weight/volume) yeast extract (TSBYE) in a 10% (volume/volume) CO_2 humidified atmosphere at 37°C. Antibiotics, when necessary, were added to a final concentration of 50 μg kanamycin ml^{-1} or 1 μg chloramphenicol ml^{-1}. All *Escherichia coli* strains were grown with agitation in Luria–Bertani broth at 37°C containing either 50 μg kanamycin ml^{-1} or 20 μg chloramphenicol

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Table 1 Strains and plasmids

Strains or plasmids	Relative description	References
Strains		
Aggregatibacter actinomycetemcomitans		
VT1169	Wild-type strain, a derivative of SUNY465	(Mintz & Fives-Taylor, 1999)
emaA	The *emaA* mutant strain	(Mintz, 2004)
ffh S382T	*ffh* gene substitution mutant strain	This study
Δ*secB*	*secB* gene deletion mutant strain	This study
KM333	In-frame deletion of *emaA* gene region corresponding to amino acids 16–39 on the chromosome	(Jiang *et al.*, 2011)
Δ*secB*/*emaA*$^-$	Strain with *secB* gene deleted and *emaA* gene inactivated	This study
Escherichia coli		
DH10B	F$^-$ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ$^-$	(Grant *et al.*, 1990)
DH5α λ *pir*	*endA1 hsdR17* (*r-m +*) *supE44 thi-1 recA1 gyrA1*(*Nalr*)*relA1* *D*(*lac-IZYAargF*)*U169 deo* (*F80 dLacD*(*lacZ*)*M15*) *pir* R6K	
SM10 λ *pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2Tc::Mu I *pir* R6K	(Priefer *et al.*, 1985)
Plasmid		
pKM2	Shuttle plasmid, Cm^r	(Gallant *et al.*, 2008)
pKM9	Full-length *emaA* gene in a shuttle plasmid pKM1	(Ruiz *et al.*, 2006)
pVT1460	Mobilization plasmid, Spec^r	(Mintz *et al.*, 2002)
pVT1642	Mobilization plasmid, Cm^r	(Mintz *et al.*, 2002)
pKM303	*morC* promoter in pKM2	(Gallant *et al.*, 2008)
pKM421	Full-length *ffh* gene in pKM303	This study
pKM422	Full-length *secB* gene in pKM303	This study
pKMΔ16-39	In-frame deletion of bp 46–117 of *emaA* in pKM9	(Jiang *et al.*, 2011)
pDnaK	The *dnaK* gene with the endogenous promoter in pKM2	This study
pDnaK A174T	The amino acid substitution of A174T in dnaK	This study

col ml^{-1}, as appropriate. Bacterial growth was measured at a wavelength of 495 nm for *A. actinomycetemcomitans* or 600 nm for *E. coli* using a Spectronic 20+ (Thermo Scientific, Waltham, MA).

Heat-shock experiments were performed as described previously (Jiang *et al.*, 2011). Briefly, cells were initially grown in TSBYE at 37°C overnight, diluted, grown at 37°C for 30 min, sealed with parafilm, and incubated at 42°C for 3 h before analysis. Bacterial growth at 42°C was comparable to those cells grown at 37°C for the stated period of time.

Allelic replacement mutagenesis

All mutants were generated by conjugation using a modified mobility plasmid for the transfer between *E. coli* and *A. actinomycetemcomitans* as described previously (Mintz *et al.*, 2002; Mintz, 2004).

The *secB* gene was originally identified in the *A. actinomycetemcomitans* genome based on homologous sequences with the *E. coli secB* gene. The *secB* deletion strain was generated by polymerase chain reaction (PCR) amplification of 1 kbp upstream and downstream of the gene (Gene ID AA01506, Oralgen database) using VT1169 genomic DNA as template and primers 5′secBup/3′secBdn (Table 2). The PCR product was cloned into the TOPO TA Cloning vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* strain DH10B. The *SecB* gene was deleted by inverse PCR using primers with engineered unique *Stu*I restriction sites (StuInvsecB3′/secBinv5′). The purified PCR product was treated with *Stu*I and ligated with the spectinomycin adenylyltransferase (*aad9*) (Accession Number M69221) previously treated with *Stu*I. The modified DNA fragment was released by *Eco*RI digestion and cloned into the mobility plasmid pVT1460 for conjugation. The resulting deletion mutant was confirmed by DNA sequencing performed at the Vermont Cancer Center DNA Analysis Facility, University of Vermont.

The *ffh* gene (Gene ID AA01117, Oralgen database) was identified in the *A. actinomycetemcomitans* genome by sequence homology with the *E. coli* gene. The gene and 1 kbp of surrounding sequence was

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Table 2 Oligonucleotides used in this study

Oligonucleotides	Sequence (5′ → 3′)
5′secBup	CACTTGATAAATTTGTTCGG
3′secBdn	GATTACGACGTAAAATATTAG
StuInvsecB3′	GCAGGCCTCGAGTCCTTATTTTATTTTTTAAC
secBinv5′	GCAGGCCTTAGTCTTGAAAAGTGGG
SecB*Xho*IFor	CGCTCGAGATGACCGAAGAAAACAAA GAAGTGAC
SecB*Xba*IRev	GTCTAGAATTTACTGTTGGTTCGCTTTGCTCG
Ffh5′	CTCACCGCACTTTTCTCTCATCCTTTG
Ffh3′	CCCTGAGCATTATCAAAGCGTATC
Ffh Serine382F	GAAGCCATCATCAATACCATGACCTTAA AGAA
Ffh Serine382R	TTCTTTAAGGTCATGGTATTGATGATGGCTTC
FfhInv*Stu*I3′	AGGCCTCCATCACCCTCAACCATC
FfhInv*Stu*I5′	AGGCCTAATATTGCCTGTGGTTAA
Ffh2	ACGTCTGAACATTCCGCCTAACCC
Ffh3	CATGTGAAAAATCAAGTGGATGAC
Ffh*Xho*IFor	CTCGAGATGTTTGAAAATTTATCCGATGG
Ffh*Xba*IRev	TCTAGAACGTCTGAACATTCCGCCTAAC
CBP1-738F	GCTGATTGAGGCGATGTATAAC
CBP1259Rev	GGAGAGAAATAATAACGGGGC
CBP1-5′up	ACATGCATGCAACAAATCGCCGTCATCGCC
CBP1-7	TTGACGCATCATCGCAAG
SecB*Xho*IFor	CGCTCGAGATGACCGAAGAAAACAAAGA AGTGAC
SecB*Xba*IRev	GTCTAGAATTTACTGTTGGTTCGCTTTGCTCG
DnaK*Xho*IFor	CCGCTCGAGGAAAACGAACAGGAAATCG
DnaK*Xba*IRev	CTGCTCTAGACACGAAAAGGGCGTAATAA TAC
DnaKA174TFor	CATCAACGAACCGACCACCGCGGCATTA GCTTATG
DnaKA174TRev	CATAAGCTAATGCCGCGGTGGTCGGTTC GTTGATG

amplified by PCR using high-fidelity *Taq* polymerase (Roche, Basel, Switzerland), VT1169 genomic DNA and primers Ffh5′/Ffh3′ (Table 2). The *aad9* selectable marker was introduced into the *ffh* sequence adjacent to the transcriptional stop codon by inverse PCR, using primers with engineered unique *Stu*I restriction sites (FfhInv*Stu*I3′/FfhInv*Stu*I5′, Table 2), followed by ligation with the marker. The *ffh* gene expressing a protein with reduced activity was generated by substitution mutagenesis of Serine 382 to Threonine (S382T) in the coding sequence using primers Ffh Serine382F/Ffh Serine382R (Table 2) and QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) based on the *E. coli ffh* gene (Tian & Beckwith, 2002). The resulting substitution mutant was confirmed by PCR (primers Ffh2/Ffh3, Table 2) and sequencing.

The *emaA* gene in the Δ*secB* mutant strain was disrupted by insertional mutagenesis described previously, except the gene coding for chloramphenicol acetyl transferase (*cat*) (Accession number AAC64937) was substituted for *aad9* (Dennis & Zylstra, 1998). A DNA fragment corresponding to approximately 300 bp before the start codon of *emaA* and the first approximately 100 bp of the *emaA* coding sequence were amplified using VT1169 genomic DNA and primer set CBP1-738F/CBP1259Rev. The PCR product was cloned into mobility plasmid pVT1660 via unique *Eco*RI site for conjugation described previously (Mintz *et al.*, 2002). The transconjugants were selected on TSBYE plates containing 100 μg rifampin ml^{-1}, 50 μg nalidixic acid ml^{-1} and 1 μg chloramphenicol ml^{-1}. Mutants were confirmed by PCR using a primer set (CBP1-5′up/CBP1-7, Table 2), which corresponds to the sequences outside the target sequence.

Complementation of *secB* and *ffh*

The full-length *secB* and *ffh* gene sequence was amplified with *Taq* polymerase using primers with engineered restriction sites for directional cloning into the shuttle plasmid pKM2 – SecB*Xho*IFor/SecB*Xba*IRev and Ffh2/Ffh3 (Table 2), for *secB* and *ffh*, respectively. The PCR products were purified, treated with enzyme and ligated with pKM303 containing the *morC* promoter (Gallant *et al.*, 2008). Plasmids were transformed into the mutant strains by electroporation (Sreenivasan *et al.*, 1991).

Generation of *dnaK* substitution mutants

The *A. actinomycetemcomitans dnaK* (Accession number YP_003255533) was identified based on homology with the *E. coli* gene. The full-length *dnaK* gene with the endogenous promoter was amplified with engineered *Xho*I and *Xba*I restriction (DnaK*Xho*IFor and DnaK*Xba*IRev). The PCR product was purified using Qiagen Gel Extraction Kit, restricted with *Xho*I and *Xba*I and ligated with plasmid pKM2 previously treated with the same restriction enzymes (Gallant *et al.*, 2008). The ligation mixture was transformed and selected by plating on Luria–Bertani plates containing 20 μg chloramphenicol ml^{-1}. The DnaK mutant was generated by substitution mutagenesis using and the QuikChange XL site-

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directed mutagenesis kit (Stratagene) based on the *E. coli dnaK* sequence (Kamath-Loeb *et al.*, 1995). The active site mutant A174T was developed using the primers DnaKA174TFor/DnaKA174TRev in Table 2. The full-length *A. actinomycetemcomitans dnaK* gene with the endogenous promoter was ligated with pKM2 for transformation to be used as a control.

Isolation of bacterial membranes

Bacterial membranes were prepared as described previously (Mintz, 2004). The protein concentration was determined spectrophotometrically at a wavelength of 280 nm following addition of sodium dodecyl sulfate (SDS) to a final concentration of 2% (weight/volume).

Immunoblot analysis

EmaA membrane protein tends to aggregate after solubilization of the membrane and the monomers do not consistently resolve in the separating gel (Yu *et al.*, 2008) so the amount of EmaA synthesized by the different bacterial strains was quantified by immunodot blot analysis following the procedure described previously using a monoclonal antibody specific for the stalk region of denatured EmaA (Yu *et al.*, 2008). Equal protein concentrations, measured as described above, or equal cell numbers were applied to the membrane in all experiments. Before immunodetection, membranes were stained with Ponceau S to verify equivalent protein loads. The nitrocellulose membranes were exposed to Kodak X-OMAT LS film (Carestream Health, Rochester, NY) or imaged using a Bio-Rad Molecular Imager Gel Doc XR+ System (Bio-Rad Laboratories, Hercules, CA). The intensity of the dots was quantified using the Bio-Rad Quantity One software as described previously (Jiang *et al.*, 2011). Experimental samples were investigated in duplicate and repeated a minimum of three times each.

Secreted leukotoxin was concentrated from stationary-phase cell growth medium using Amicon Ultra-15 10K (Millipore Corporation, Billerica, MA) and detected using a polyclonal rabbit anti-leukotoxin antibody (provided by E.T. Lally, University of Pennsylvania) as described previously (Gallant *et al.*, 2008).

The presence of DnaK in bacterial cell lysates of equal cell numbers were determined by immunoblot of SDS–polyacrylamide gels transferred onto polyvinylidene fluoride membrane (Whatman Inc., Florham Park, NJ). DnaK was detected using a mouse monoclonal antibody developed to *E. coli* DnaK (Abcam Inc., Cambridge, MA). The immune complexes were recognized by horseradish peroxidase-conjugated goat anti-rabbit antibodies and exposed to Kodak X-OMAT LS film (Carestream Health) or imaged using a Bio-Rad Molecular Imager Gel Doc XR+ System (Bio-Rad Laboratories). The intensity of the bands was quantified using the Bio-Rad Quantity One software as described previously (Jiang *et al.*, 2011). Data were collected from two individual experiments, and statistically analysed using Student's *t*-test for significance ($P < 0.05$).

Collagen-binding assay

Collagen-binding activity was determined as described previously (Yu *et al.*, 2008). Data were collected from three individual experiments in triplicate, and statistically analysed for significance using Student's *t*-test ($P < 0.05$) or two-way analysis of variance where appropriate.

Transmission electron microscopy

Bacterial samples were prepared as described by Ruiz *et al.* (2006) using Nano-W (Nanoprobes, Yaphank, NY) as the staining agent. Data collection was carried out using a Tecnai 12 electron microscope (FEI, Hillsboro, OR) equipped with a LaB_6 cathode, a 14-μm 2048 × 2048-pixel charge-coupled device (CCD) camera (TVIPS, Gauting, Germany) and a dual-axis tilt tomography holder (Fischione, Export, PA), operating at 100 kV. Micrographs were recorded in the CCD camera at a 52,000× nominal magnification, which corresponds to a 0.25-nm pixel size on the specimen scale.

RESULTS

Secretion of EmaA is mediated by a chaperone-dependent pathway

The protein secretion machinery required for EmaA inner membrane targeting was determined in isogenic mutant strains with either a deletion of *secB* or expressing a mutant Ffh protein, a cofactor of the SRP,

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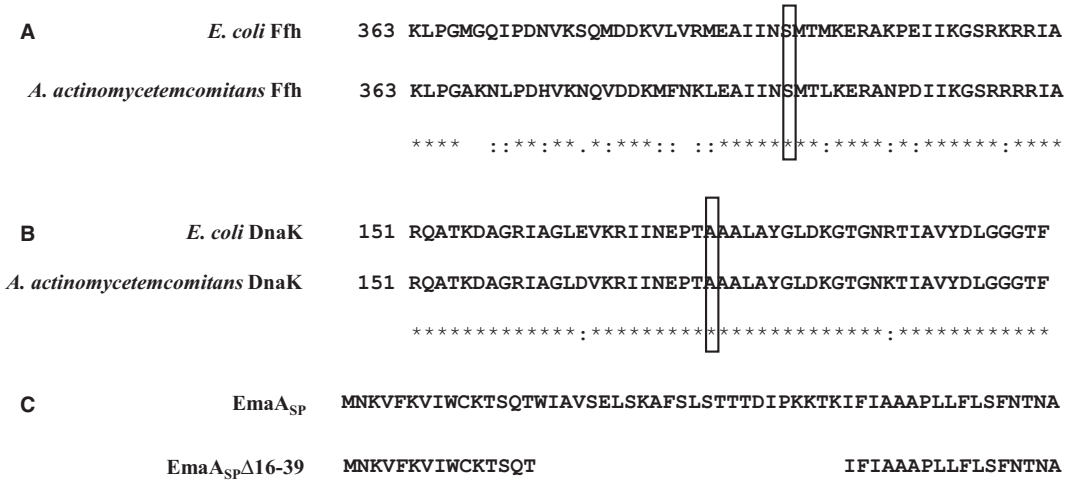

Figure 1 Partial amino acid sequence alignment of Ffh and DnaK of *Escherichia coli* and *Aggregatibacter actinomycetemcomitans* and EmaA signal peptide. (A) Sequence alignment of the amino acids surrounding and composing the active site of the *E. coli* and *A. actinomycetemcomitans* Ffh protein. (B) Sequence alignment of the amino acids surrounding and composing the active site of DnaK of *E. coli* and *A. actinomycetemcomitans*. Identical amino acids (*), highly conserved amino acids (:), and weakly conserved amino acids (.) are indicated below the sequence alignment. The boxed amino acid indicates the amino acid substituted to generate a protein with reduced activity. (C) Sequence of the full-length and the in-frame deletion [amino acid 16–39 ($EmaA_{SP}$Δ16–39)] signal peptide.

which decreases SRP activity. Although the sequences of Ffh from *A. actinomycetemcomitans* and *E. coli* share 81% identity in the whole sequence, the amino acids necessary for the interaction of Ffh with the 4.5S RNA and surrounding amino acids are identical in both species (Tian & Beckwith, 2002) (Fig. 1A). Substitution of *E. coli* amino acid serine 382 to threonine results in an Ffh protein with reduced SRP activity (Tian & Beckwith, 2002). An *A. actinomycetemcomitans* S382T substitution mutant strain was developed and the *ffh* S382T strain was demonstrated to have no growth defects (data not shown). In the S382T substitution mutant strain the amount of EmaA in the membrane fraction was unchanged when compared with the parent strain (Fig. 2A,B). In addition, the S382T substitution mutant strain displayed surface structures, and collagen-binding activity similar to the wild-type strain (Figs 2C, 3). The data suggest that EmaA is not dependent on the SRP pathway for membrane localization.

To confirm that the *ffh* S382T strain was defective in SRP activity, the secretion of leukotoxin was determined using anti-leukotoxin polyclonal antibodies, assuming the inner membrane transporter proteins are SRP-dependent. Immunoblot analysis of the secreted proteins from the *ffh* S382T strain demonstrated little immunoreactive material present in the spent culture medium. In the complemented strain, which was generated by transformation of a plasmid expressing the full-length *ffh* gene, leukotoxin was detected and quantification of the immunoreactivity suggested that a similar amount of the toxin was detected in the spent medium of the complemented strain when compared with the wild-type strain (Fig. 2D).

The lack of an impact on EmaA secretion in the *ffh* mutant strain suggested that secretion may be mediated by the chaperone-dependent pathway. SecB is a well-characterized chaperone associated with targeting secreted proteins to the Sec translocon for inner membrane translocation (Watanabe & Blobel, 1989; Baars *et al.*, 2006). The *A. actinomycetemcomitans* Δ*secB* strain was found to contain 40% less EmaA in the membrane fraction when compared with the parent strain (Fig. 4A). No obvious difference in the membrane protein profile or concentration of membrane proteins was observed in the ΔsecB strain (data not shown). Furthermore, an increase in the amount of EmaA in the cytoplasm of the Δ*secB* strain was not observed within the detection limit of the technique.

The reduction of EmaA in the membrane of the Δ*secB* mutant strain correlated with a significant decrease in the collagen-binding activity (Fig. 4C).

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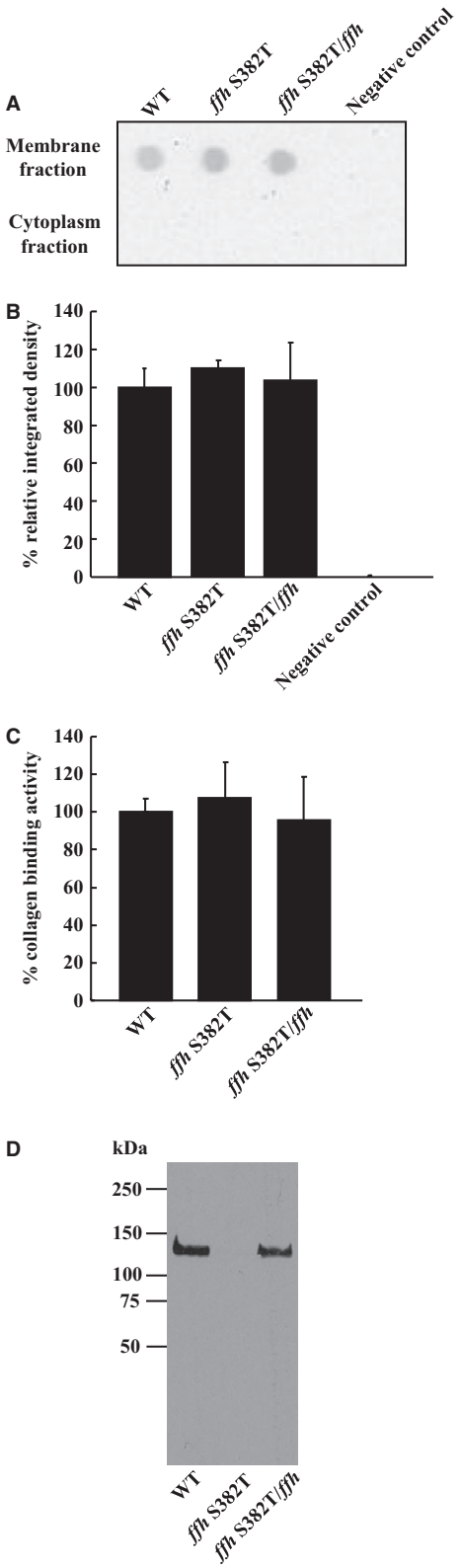

Figure 2 Immunodot blot analysis and collagen-binding activity of *ffh* mutant and wild-type strains. (A) Analysis of EmaA associated with the membrane and cytoplasm fractions from *Aggregatibacter actinomycetemcomitans ffh* mutant and wild-type strains. Membrane and cytoplasm fractions were generated by differential centrifugation; equal protein concentrations were applied to the membrane and probed with an anti-EmaA monoclonal antibody. WT, wild-type strain; *ffh* S382T, strain expressing amino acid serine 382 substituted with amino acid threonine of the Ffh sequence; *ffh* S382T/*ffh*, the *ffh* mutant strain transformed with a plasmid expressing the wild-type *ffh* gene; negative control, a strain containing a disrupted chromosomal copy of *emaA*. (B) Relative integrated densities of the immunodot blot of EmaA expressed by the strains containing the constructs described above for (A). (C) Collagen-binding activities of the strains in (A) and (B) as measured by enzyme-linked immunosorbent assay. (D) Analysis of leukotoxin in the spent medium samples from *A. actinomycetemcomitans* strains using a polyclonal antibody against leukotoxin. Spent medium from equivalent number of cells, as determined by optical density of the cell culture at a wavelength of 495 nm, were concentrated, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed with a polyclonal anti-LtxA antibody. WT, wild-type strain; *ffh* S382T, strain expressing amino acid serine 382 substituted with amino acid threonine of the Ffh sequence; *ffh* S382T/*ffh*, the *ffh* mutant strain transformed with a plasmid expressing the wild-type *ffh* gene.

The number of structures associated with the surface of the bacterium was also reduced, as demonstrated in the electron micrograph (Fig. 3). Complementation of the Δ*secB* strain by transformation with a plasmid containing the full-length *secB* gene restored the amount of EmaA in the membrane fraction, as well as the collagen-binding activity of the strain, when compared with the parent strain (Fig. 4B,C). Transcriptional activity of the *emaA* gene in the Δ*secB* strain was similar to that in the wild-type strain, suggesting that the defect was not the result of changes in the transcription of the *emaA* gene (data not shown). The partial reduction in the amount of EmaA found associated with the membrane suggests that other protein(s) may stabilize or mediate inner membrane targeting of EmaA.

DnaK mediates EmaA secretion

In other bacterial systems, the expression of *dnaK* is increased in Δ*secB* mutant strains (Baars *et al.*, 2006). The *A. actinomycetemcomitans* DnaK sequence shares a high degree of similarity (90%) with the *E. coli* protein (Fig. 1B). Based on this sequence conservation, whole cell lysates of the wild-type and Δ*secB*

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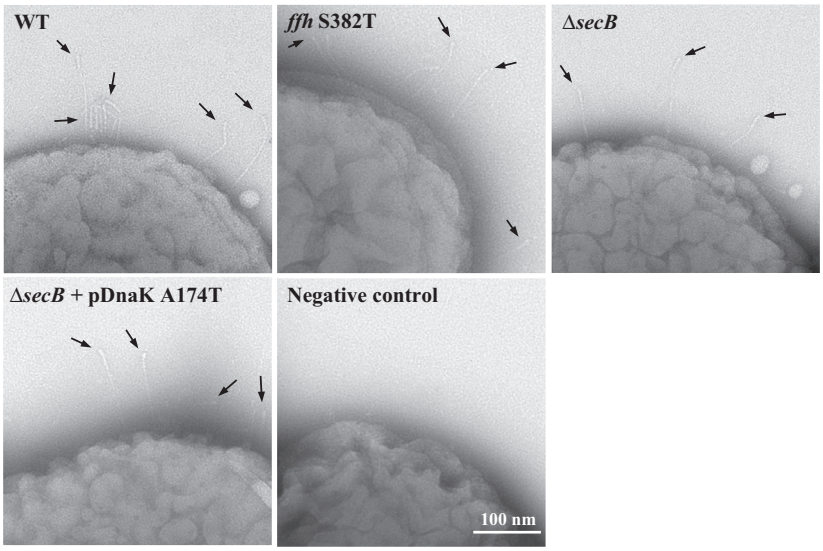

Figure 3 Transmission electron micrographs of whole mount preparations of *Aggregatibacter actinomycetemcomitans* strains negatively stained with Nano-W. WT, wild-type strain; *ffh* S382T, strain expressing amino acid serine 382 substituted with amino acid threonine of the Ffh sequence; Δ*secB*, strain with secB deleted; Δ*secB*/pDnaK A174T, the *secB* deletion strain transformed with a plasmid expressing the alanine to threonine DnaK substitution mutant (A174T) under the control of the endogenous promoter; Negative control, strain with disrupted *emaA*. Black arrows indicate EmaA structures on the bacterial surface. Scale bar: 100 nm.

mutant strains were tested for cross-reactivity with an *E. coli* DnaK antibody. An immunostaining band at the expected molecular weight of DnaK was apparent in both strains (Fig. 5A). However, there was a two-fold increase in the staining intensity of the DnaK band in the Δ*secB* mutant strain when compared with the wild-type strain (Fig. 5B).

The increase in the amount of DnaK suggested the possibility that this protein may compensate for the absence of SecB for EmaA secretion in the Δ*secB* mutant strain. The hypothesis was tested using strains transformed with plasmids expressing either the wild-type *dnaK* or a substitution mutant of *dnaK* with reduced activity, under the control of the endogenous promoter. Substitution of amino acid 174 alanine to threonine (A174T) of the *E. coli* DnaK sequence reduces the ATPase activity of the protein (Wild *et al.*, 1992b; Kamath-Loeb *et al.*, 1995). This identical amino acid substitution was generated in the *A. actinomycetemcomitans* sequence and characterized. Both the wild-type and the Δ*secB* mutant strains transformed with the *dnaK* A174T construct displayed growth characteristics similar to those of the wild-type strain transformed with the empty plasmid, when grown at 37°C (Fig. 5C). An additional *dnaK* substitution mutant (E171K) was also generated, but was found to have growth defects at all temperatures tested (data not shown) and was not used in this study. All of the strains containing the *dnaK* plasmids used in this study were found to express elevated levels of DnaK, compared with strains transformed with the empty plasmid (Fig. 5A,B).

Transformation of the wild-type strain with either *dnaK* or *dnaK* A174T did not affect the secretion of EmaA (Fig. 6A,B). Transformation of the Δ*secB* mutant strain with wild-type *dnaK* did not impact EmaA localization to the membrane. In contrast, transformation of the Δ*secB* mutant strain with *dnaK* A174T significantly reduced the amount of EmaA in the membrane (Fig. 6B). The reduction of EmaA in the membrane of this strain correlated with a decrease in collagen-binding activity (Fig. 6C). Surface structures were still observed on the surface of the bacteria (Fig. 3).

DnaK-associated secretion of EmaA is dependent on amino acids 16–39 of the signal peptide

The data presented above suggested a relationship between EmaA secretion and DnaK activity. Previously, we have demonstrated that the deletion of amino acids 16–39 of the EmaA signal peptide (Fig. 1C) impairs membrane localization at elevated temperatures (Jiang *et al.*, 2011), conditions in which

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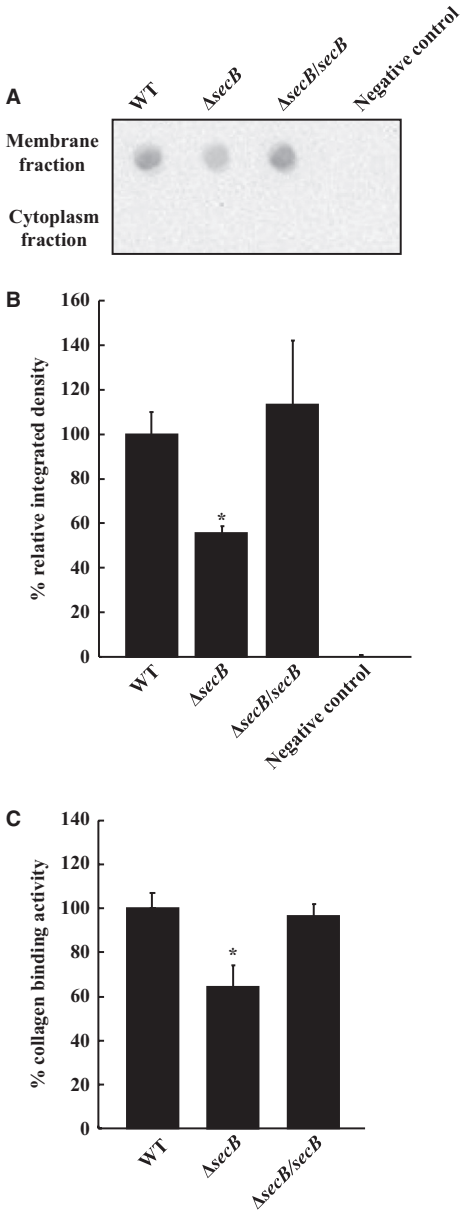

Figure 4 Immunodot blot analysis and collagen-binding activity of *secB* deletion and wild-type strains. (A) Analysis of EmaA associated with the membrane and cytoplasm fractions of *Aggregatibacter actinomycetemcomitans secB* mutant and wild-type strains. Membrane and cytoplasm fractions were generated by differential centrifugation; equal protein concentrations were applied to the membrane and probed with an anti-EmaA monoclonal antibody. WT, wild-type strain; *ΔsecB*, strain with deletion of *secB*; Δ*secB/secB*, the *secB* deletion mutant strain transformed with a plasmid expressing the wild-type *secB* gene; negative control, a strain containing a disrupted chromosomal copy of *emaA*. (B) Relative integrated densities of the immunodot blot of EmaA expressed by the strains containing the constructs described above for (A). (C) Collagen-binding activities of the strains in (A) and (B) as measured by enzyme-linked immunosorbent assay.

dnaK expression is upregulated (Goulhen *et al.*, 2003). The relationship between DnaK and this sequence of the EmaA signal peptide was investigated. Transformation of the wild-type strain with either *dnaK*-expressing or the *dnaK* A174T-expressing plasmids and grown at 37°C resulted in little change in the amount of EmaA associated with the membrane (Fig. 7A,B). However, the identical strains grown at 42°C displayed a difference. A statistically significant reduction in the amount of EmaA and collagen-binding activity was observed in the strain expressing *dnaK* A174T when compared with the *dnaK*-expressing strain (Fig. 7C). In contrast, the expression of either *dnaK* A174T or *dnaK* in the 16–39 amino acid signal peptide deletion strain (KM333) (Jiang *et al.*, 2011) did not change the amount of EmaA in the membrane when grown at either temperature (Fig. 7 A,B). The previous results were confirmed by overexpressing EmaA with deletion of amino acids 16–39 in the Δ*secB/emaA*$^-$ strain, which express higher levels of *dnaK*. Transformation of the Δ*secB/emaA*$^-$ strain with the EmaAΔ16–39-expressing plasmid further reduced the amount of EmaA in the membrane when compared with the Δ*secB/emaA*$^-$ strain expressing the full-length *emaA* gene (Fig. 7D).

DISCUSSION

Protein secretion is a conserved process involving defined pathways (Driessen & Nouwen, 2008). The nascent polypeptide chain, following translation by the ribosome, is targeted by either the chaperone-mediated pathway or the SRP-dependent pathway (Driessen & Nouwen, 2008). Both pathways are comprised of proteins, which share high degrees of sequence similarities and activities across species (van der Sluis & Driessen, 2006). Sorting has been determined to be dependent on the composition of the signal peptide located at the amino-terminus of the nascent protein (Tomilo *et al.*, 1994). Clustering of amino acids of similar charge within the signal peptide, basic (N) or hydrophobic (H) domains, plays an integral role in the association with the components of these secretion pathways (von Heijne, 1990; Hardy & Randall, 1991; Peterson *et al.*, 2003). The hydrophobic domain of the signal peptides bind to the SRP primarily via hydrophobic interactions with the 54 kDa protein (Ffh) subunit (Walter & Johnson, 1994). The formation of salt bridges between the

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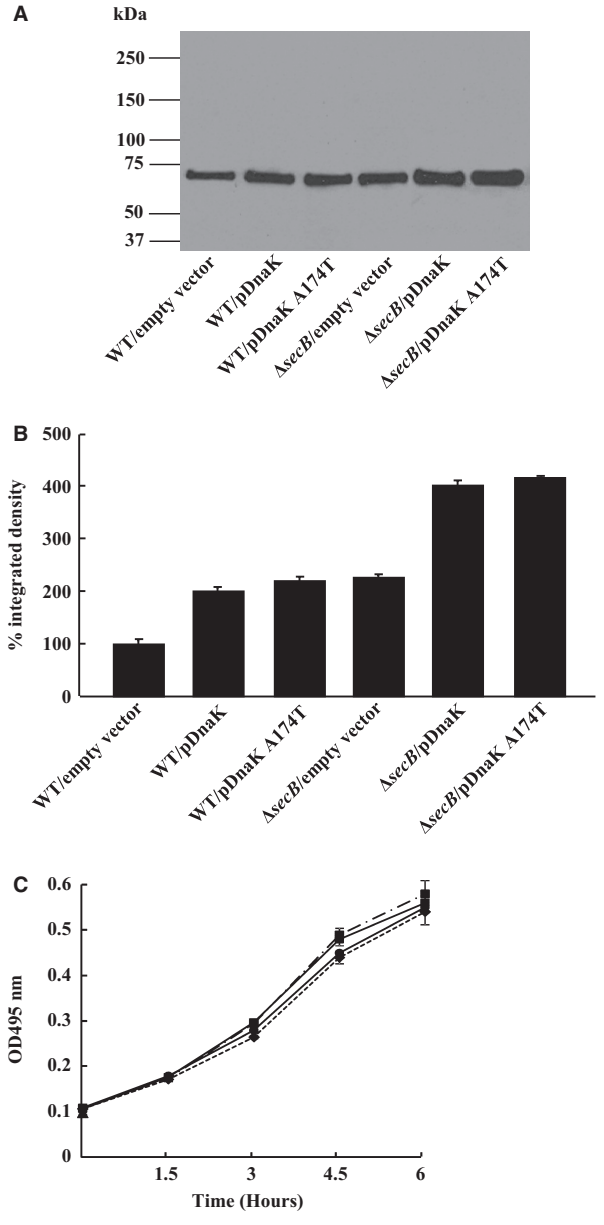

Figure 5 Characterization of *Aggregatibacter actinomycetemcomitans* strains expressing different forms of *dnaK*. (A) Immunoblot analysis of DnaK. Lysates from equal number of bacterial cells were applied to nitrocellulose membranes and probed with an anti-DnaK antibody. WT, the wild-type strain transformed with empty vector pKM2; WT/pDnaK, the wild-type strain transformed with DnaK-expressing plasmid (pDnaK); WT/pDnaK A174T, the wild-type strain transformed with DnaK A174T-expressing plasmid (pDnaK A174T); Δ*secB*/empty vector, the *secB* deletion mutant strain transformed with empty vector pKM2; Δ*secB*/pDnaK, the *secB* deletion mutant strain transformed with DnaK-expressing plasmid (pDnaK); Δ*secB*/pDnaK A174T, the *secB* deletion mutant strain transformed with DnaK A174T-expressing plasmid (pDnaK A174T). (B) Relative integrated densities of the immunodot blot of DnaK expressed by the strains containing the constructs described above for (A). (C) Growth curves of various strains grown at 37°C. WT/pDnaK (dotted line with triangles), the wild-type strain transformed with wild-type *dnaK*-expressing plasmid (pDnaK); WT/pDnaK A174T (solid line with squares), the wild-type strain transformed with a plasmid expressing an alanine to threonine substitution at amino at 174 (A174T) of DnaK; Δ*secB*/pDnaK (dashed line with squares), the *secB* deletion mutant strain transformed with the wild-type DnaK-expressing plasmid (pDnaK); Δ*secB*/pDnaK A174T (solid line with circles), the *secB* deletion mutant strain transformed with the DnaK A174T-expressing plasmid (pDnaK A174T).

SRP RNA and the basic amino acids of the signal sequence of specific peptides may also be involved in the interaction (Batey *et al.*, 2000). In general, it is postulated that the discrimination between different targeting signals by the SRP is the result of slight variations in the hydrophobic nature of the sequence. Furthermore, presecretory proteins that contain moderately hydrophobic signal peptides are bypassed by the SRP and targeted by molecular chaperones by default (Peterson *et al.*, 2003). Some proteins synthesized with unusually long signal peptides, similar to the signal peptide of EmaA, have been demonstrated to be SRP-dependent (Sijbrandi *et al.*, 2003).

The *A. actinomycetemcomitans* SRP mutant generated in this study was based on the >90% sequence similarity with the Ffh of *E. coli* (data not shown). A substitution mutation of serine 382 to threonine was selected based on the phenotypes of reduced SRP activity without any growth defects in *E. coli* (Tian & Beckwith, 2002). The *A. actinomycetemcomitans ffh* mutant did not display any growth defects, similar to the *E. coli* phenotype. A defect in SRP activity was inferred by the observed decrease in leukotoxin secretion, which is dependent on two predicted inner membrane proteins (LtxB and D). LtxB is an ABC transporter protein that facilitates leukotoxin translocation across the inner membrane by hydrolysis of ATP (Kachlany, 2010). LtxD trimers form part of the channel spanning the periplasm (Thanabalu *et al.*, 1998). TdeA (TolC) interacts with LtxD to complete the channel for secretion into the milieu (Thanabalu *et al.*, 1998). The recovery of leukotoxin from the culture medium of the mutant strain, following transformation of a plasmid expressing wild-type *ffh*, suggests that one of the proteins required for leukotoxin secretion is dependent on the SRP pathway. The absence of any effect on the amount

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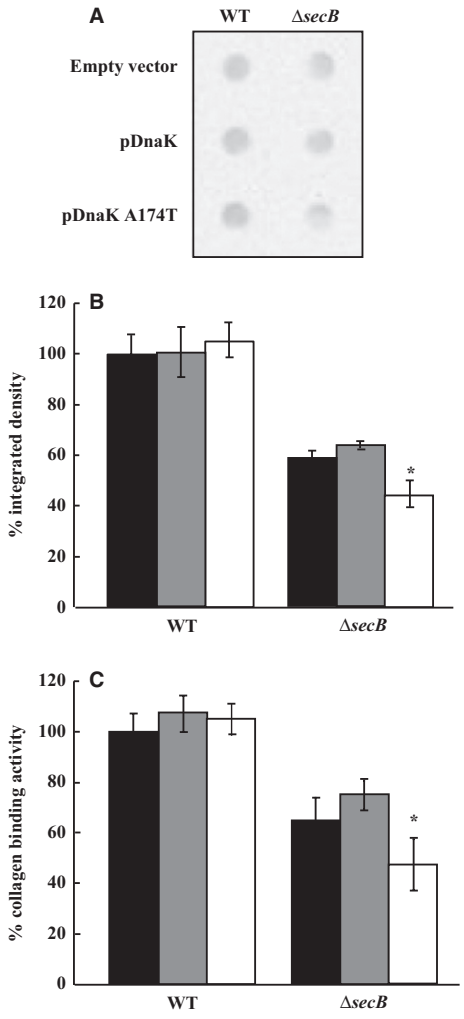

Figure 6 Determination of EmaA protein levels in different *Aggregatibacter actinomycetemcomitans* strains. (A) Analysis of membrane-associated EmaA as determined by immunodot blot. Membrane fractions were generated by differential centrifugation; equal protein concentrations were applied to the membrane and probed with an anti-EmaA monoclonal antibody. Plasmids were transformed into either the wild-type strain (WT) or the Δ*secB* mutant strain. Empty vector, strain transformed with empty vector; pDnaK, strain transformed with DnaK-expressing plasmid (pDnaK); pDnaK A174T: strain transformed with DnaK A174T-expressing plasmid (pDnaK A174T). (B) Relative integrated densities of the immunodot blot of DnaK expressed by the strains containing the constructs described above for (A). Empty vector (black bars), pDnaK (grey bars), and pDnaK A174T (white bars). (C) Collagen-binding activities of the strains in (A) and (B) as measured by enzyme-linked immunosorbent assay. Empty vector (black bars); pDnaK (grey bars); pDnaK A174T (white bars). Asterisk denotes a statistical significance at $P < 0.05$.

of EmaA in the membrane, surface structures, and the collagen-binding activity of this strain suggest that the SRP pathway is not involved in EmaA secretion.

The involvement of a chaperone-mediated secretion pathway was suggested by a decrease of secreted EmaA observed in the Δ*secB* strain. SecB is a conserved, well-characterized cytoplasmic chaperone facilitating the targeting of secretory proteins to the Sec-translocon (Watanabe & Blobel, 1989; van der Sluis & Driessen, 2006). A null mutant of *secB* in *E. coli* does not display any growth defects, which is similar to what is observed in the *A. actinomycetemcomitans* mutant (data not shown). In addition, the steady-state levels of most *E. coli* outer membrane proteins were not changed (Baars *et al.*, 2006). However, a small subset of proteins was observed to be affected by the mutation and was found to be decreased by 30–70% of wild-type levels (Baars *et al.*, 2006). The reduction of EmaA associated with the Δ*secB* strain (40%) is well within the levels found for affected *E. coli* membrane proteins. The partial reduction in the secretion of EmaA suggests that SecB is not unique for targeting the protein to the translocon and that less efficient, alternate chaperone(s) replace SecB activity, under specific conditions.

Inactivation of SecB results in an increase in the synthesis of DnaK, as well as other proteins associated with a 'heat-shock response', to mitigate the misfolding and aggregation of proteins in the cytoplasm under these conditions (Kerner *et al.*, 2005; Baars *et al.*, 2006). We have observed a similar increase in the amount of DnaK in the cytoplasm when *secB* was deleted in *A. actinomycetemcomitans*. This observation and reports that DnaK facilitates the export of some secretory proteins (Kusukawa *et al.*, 1989; Wild *et al.*, 1992a; Qi *et al.*, 2002; Janakiraman *et al.*, 2009) suggested a chaperone-like function for DnaK in the secretion of EmaA. The involvement of DnaK in EmaA secretion is supported by the finding that overexpression of the *dnaK* A174T, which appears to be a dominant–negative mutation in *A. actinomycetemcomitans*, reduces the amount of EmaA secretion. The finding that EmaA secretion was not completely ablated may be attributed to the expression of the wild-type copy of *dnaK* on the chromosome or other chaperones may be associated with secretion under these conditions.

A region within the extended signal peptide of EmaA, corresponding to amino acids 16–39 (Fig. 1C), was found to be necessary for the modulation of EmaA secretion in response to DnaK activity. This sequence is required for maximum secretion of

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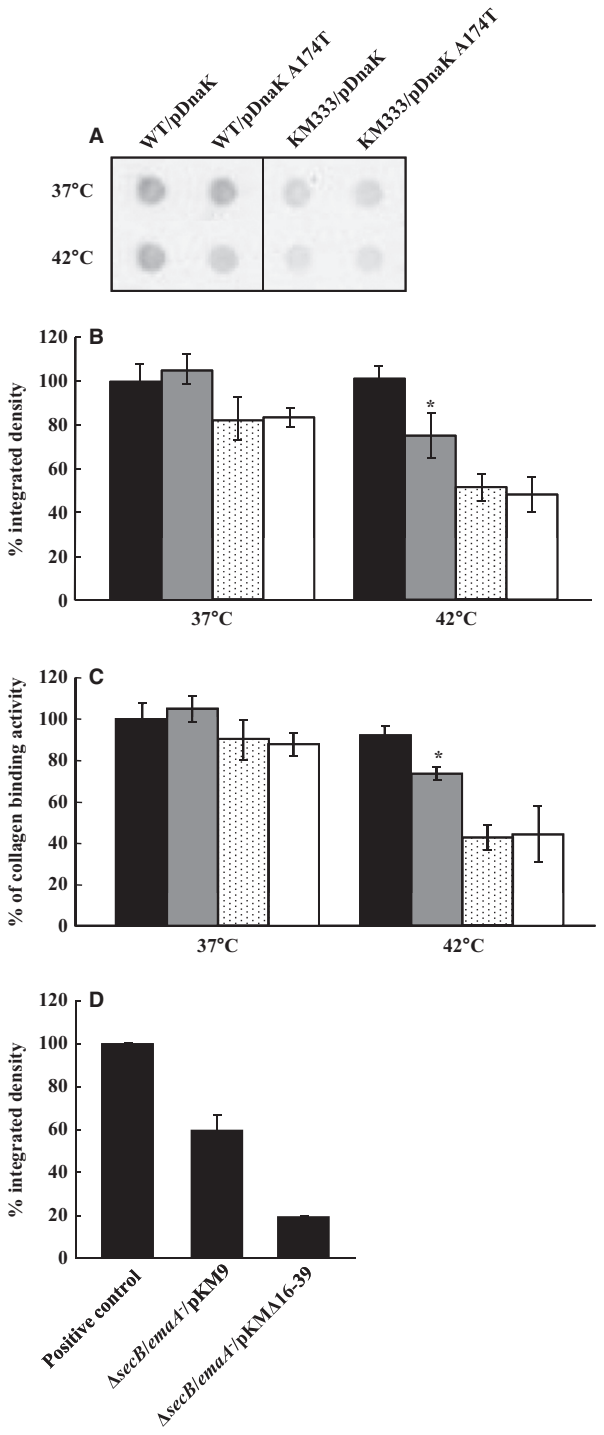

Figure 7 EmaA analysis in strains expressing different forms or levels of DnaK. (A) EmaA immunodot blot analysis. Bacterial lysates (corresponding to 4×10^7 colony-forming unit bacteria) were prepared and immobilized on nitrocellulose membranes. EmaA was detected using a monoclonal antibody specific for EmaA in cells grown at 37°C or 42°C. WT/pDnaK, wild-type strain transformed with a plasmid expressing the full-length gene under the control of the endogenous promoter; WT/pDnaK A174T, wild-type strain transformed with a plasmid expressing the alanine to threonine DnaK substitution mutant (A174T) under the control of the endogenous promoter; KM333/pDnaK, signal peptide chromosomal deletion of amino acids 16–39 strain transformed with a plasmid expressing the full-length *dnaK* gene; KM333/pDnaK A174T, the KM333 strain transformed with plasmid expressing the mutant form of *dnaK* (A174T). (B) Relative integrated densities of the immunodot blot of EmaA expressed by the strains containing the constructs described above for (A). WT/pDnaK (black bars); WT/pDnaK A174T (grey bars); KM333/pDnaK (dotted bars); KM333/pDnaKA174T (white bars). (C) Collagen-binding activities of the strains in (A) and (B) as measured by enzyme-linked immunosorbent assay. WT/pDnaK (black bars); WT/pDnaK A174T (grey bars); KM333/pDnaK (dotted bars); KM333/pDnaK A174T (white bars). (D) Determination of EmaA protein levels in strains expressing either full-length or EmaAΔ16-39. Positive control, the *emaA* mutant strain transformed with a plasmid expressing full-length EmaA; Δ*secB*/*emaA*$^-$/pKM9, Δ*secB*/*emaA*$^-$ double mutant strain transformed with the plasmid expressing full-length EmaA; Δ*secB*/*emaA*$^-$/pKMΔ16-39, Δ*secB*/*emaA*$^-$ double mutant strain transformed with the plasmid expressing EmaAΔ16-39. Asterisk denotes a statistical significance at $P < 0.05$.

EmaA during growth of the bacterium at 42°C, a condition where DnaK is upregulated (Jiang *et al.*, 2011). The sequences of some signal peptides have been found to contain DnaK-binding motifs, which include four or five hydrophobic amino acids flanked by basic amino acids (Rudiger *et al.*, 1997; Van Durme *et al.*, 2009). Analysis of the signal peptide using an algorithm to predict pentapeptide DnaK-binding sites in the protein sequence (Van Durme *et al.*, 2009) (http://limbo.switchlab.org) predicted three binding sites: 16–22 (WIAVSEL); 39–45 (KIFIAAA); and 47–53 (LLFLSFN). Two of the three sequences are completely or partially contained in the region of the peptide (amino acids 16–39); when the sequence is deleted, EmaA does not show the same response to DnaK activity as observed with the intact signal peptide. All of the data lend support for an interaction of DnaK with the extended signal peptide of EmaA under specified conditions.

Display of EmaA appendages, mediators of collagen binding of the periodontal pathogen *A. actinomycetemcomitans*, on the bacterial surface is the consequence of a chaperone-dependent secretion pathway of EmaA monomers. Interaction of the newly translated polypeptide with the dedicated SecB chaperone mediates secretion. Furthermore, general chaperones, e.g. DnaK, may associate with the extended

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region of the signal peptide to stabilize the polypeptide chain to ensure SecB interaction with the protein for membrane targeting and translocation. Alternatively, binding of DnaK to the signal peptide may occur only under conditions where the general chaperone is upregulated due to changes in the environment and physiology of the bacterium. The development of new genetic and biochemical techniques will be required to demonstrate a direct interaction between the EmaA signal peptide and DnaK in *A. actinomycetemcomitans*.

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