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Screen for Leukotoxin Mutants in *Aggregatibacter actinomycetemcomitans*: Genes of the Phosphotransferase System Are Required for Leukotoxin Biosynthesis[▽]

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Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* is a pathogen that causes localized aggressive periodontitis and extraoral infections including infective endocarditis. Recently, we reported that *A. actinomycetemcomitans* is beta-hemolytic on certain growth media due to the production of leukotoxin (LtxA). Based on this observation and our ability to generate random transposon insertions in *A. actinomycetemcomitans*, we developed and carried out a rapid screen for LtxA mutants. Using PCR, we mapped several of the mutations to genes that are known or predicted to be required for LtxA production, including *ltxA*, *ltxB*, *ltxD*, and *tdeA*. In addition, we identified an insertion in a gene previously not recognized to be involved in LtxA biosynthesis, *ptsH*. *ptsH* encodes the protein HPr, a phosphocarrier protein that is part of the sugar phosphotransferase system. HPr results in the phosphorylation of other proteins and ultimately in the activation of adenylate cyclase and cyclic AMP (cAMP) production. The *ptsH* mutant showed only partial hemolysis on blood agar and did not produce LtxA. The phenotype was complemented by supplying wild-type *ptsH* in *trans*, and real-time PCR analysis showed that the *ptsH* mutant produced approximately 10-fold less *ltxA* mRNA than the wild-type strain. The levels of cAMP in the *ptsH* mutant were significantly lower than in the wild-type strain, and LtxA production could be restored by adding exogenous cAMP to the culture.

Aggregatibacter (formerly *Actinobacillus*) *actinomycetemcomitans* is a gram-negative capnophilic bacterium that colonizes the oral cavities of humans and Old World primates (13, 45, 53). *A. actinomycetemcomitans* has been associated with various diseases including localized aggressive periodontitis (45, 52) and subacute infective endocarditis (4, 6, 42). Localized aggressive periodontitis is an aggressive disease of the mouth that presents in adolescents and results in the loss of periodontal bone and ligament. When untreated, loss of teeth occurs. The bacterium expresses several putative virulence factors including a leukotoxin (LtxA) (30, 33). LtxA is a 113-kDa protein that has been reported to kill specifically white blood cells of humans and Old World primates (46–48). Production of LtxA by *A. actinomycetemcomitans* is believed to be one mechanism used by the bacterium to evade host immune responses (1). Clinical evidence suggests that LtxA is an important virulence factor for *A. actinomycetemcomitans* (21–23). In addition, we have recently shown that LtxA can act as a hemolysin that may be used during systemic infection for acquiring iron from the host (2). Indeed, iron, but not other metals, regulates LtxA secretion in *A. actinomycetemcomitans* (3).

LtxA belongs to the RTX (repeats in the toxin) family of toxins, which includes *Escherichia coli* α-hemolysin, *Bordetella pertussis* adenylate cyclase, *Mannheimia haemolytica* leukotoxin, *Vibrio cholerae* RTX toxin, and *Actinobacillus pleuropneumoniae* Apx toxins (16, 37, 51). The current model for the production, activation, and secretion of LtxA in *A. actinomycetemcomitans* is based primarily on work done with other RTX toxins, especially *E. coli* α-hemolysin (7, 17). Biosynthesis of LtxA is apparently dependent on an operon that consists of four genes, *ltxCABD*. *ltxC* encodes a protein presumed to be responsible for the acylation of the toxin, *ltxA* encodes the toxin, and *ltxB* and *ltxD* code for predicted components of the membrane transport system. In addition, we recently discovered a TolC-like protein, TdeA, which is required for both LtxA secretion and drug efflux (5). There is also evidence that LtxA biosynthesis is regulated by other genes outside the LtxA operon. Indeed, Fong et al. (15) showed that LtxA expression is at least partly regulated by LuxS (autoinducer II), while Inoue et al. (25) found that the levels of LtxA are affected by cyclic AMP (cAMP) concentration within the cell. However, the genes and mechanisms involved in regulation are largely unknown.

An effective way to identify novel genes involved in the regulation of *ltxA* in *A. actinomycetemcomitans* is to isolate nonleukotoxic mutants. To date, there is no practical way to screen for mutants of *A. actinomycetemcomitans* that are defective in LtxA production. We recently found that *A. actinomycetemcomitans* is beta-hemolytic on certain growth media and that this phenotype is due to the production of LtxA (2). Based on this novel finding, we have developed a rapid assay to screen for LtxA mutants of *A. actinomycetemcomitans*. Herein, we describe the genetic screen and present data that support its use as an effective method for identifying novel LtxA genes in *A. actinomycetemcomitans* and potentially related bacteria. In addition, we have used this screen to characterize a gene that is involved in the cAMP-dependent regulation of *ltxA*.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
AA1698	JP2N containing pVJT128	2
AA1704	JP2N *ltxA*::IS*903*φ*kan*	2
CU1000	Rough clinical isolate; serotype f	27
CU1000N	Spontaneous Nal^r variant of CU1000	11
JK1025	CU1000N *ptsI*::IS*903*φ*kan*	This study
JP2	Highly leukotoxic; serotype b	50
JP2N	Spontaneous Nal^r variant of JP2	2
MP046	JP2N *ptsH*::IS*903*φ*kan*	This study
Plasmids		
pJAK16	IncQ broad-host-range vector; *tacp* Cm^r	J. A. Kornacki
pJK617	pJAK16 containing *ptsI*	This study
pMP021	pJAK16 containing *ptsH*	This study
pVJT128	Plasmid containing inducible IS*903*φ*kan*	27, 49

TABLE 2. Primers used in this study

Gene	Primer	Sequence (5′→3′)
PCR target genes		
ltxA	*ltxA*up	GCCCGGGATGGCAACTACTA CACTGCTAAATAC
	*ltxA*down	GCCCGGGCAGTAGTTGCTAA CGAATTTGC
ltxB	*ltxB*up	CGCAAATTCGTTAGCAACTA CTGC
	*ltxB*down	CAAGTTTTCATTATCGTTCG TTCC
ltxC	*ltxC*up	AAAAACTATTGGAATACCAA GTAC
	*ltxC*down	GTAGTAGTTGCCATAATCTA TTCTC
ltxD	*ltxD*up	ATTACAAGTAAATTAAAGGA ACGAAC
	*ltxD*down	GAAAACCGGAATGTTATATT CTATC
tdeA	*tdeA*up	CGCCATGGGGCGTGCCGCA TGCG
	*tdeA*down	CCGATTACAGCGTTGGCG CAAG
ptsH	*ptsH*up	TCCTGAAATCATAGTAAC CTTC
	*ptsH*down	GGTTTGTGGCATCTGGAA GATG
ptsI	*ptsI*up	GCGGATCCCAAGGAGATTTA ATATGATTTCAGGAATTC CGGC
	*ptsI*down	CGCAAGCTTCTAATTTAATG CTTTTTCATAC
RT-PCR target genes[a]		
ltxA	*ltxA*-s	GTGCTAGGTAAACATCGGT AAAG
	ltxA-as	GACCACAGAGGCAATTAACC
glyA	*glyA*-s	CCCAATTCACCAACAAAT ATGC
	glyA-as	ATTCTTTCGCACGCTCA ATAG

[a] RT-PCR, real-time PCR.

MATERIALS AND METHODS

Bacterial strains and media. *A. actinomycetemcomitans* strain JP2N (a nalidixic acid-resistant variant of JP2) (50) was used for all experiments except where noted. AA1704 is an *ltxA* mutant of JP2N and has been previously described (2). Strain AA1698 is a derivative of JP2N that harbors the transposon mutagenesis plasmid, pVJT128 (27, 49) (Table 1). Nalidixic acid was used for counter-selection during conjugation experiments with *E. coli* to construct the *A. actinomycetemcomitans* strains used here.

A. actinomycetemcomitans was grown on AAGM plates (40 g of trypticase soy agar and 6 g of yeast extract per liter, 0.75% glucose, and 0.4% $NaHCO_3$; glucose and $NaHCO_3$ were added after the medium was autoclaved) (12). For liquid cultures, cells were grown in AAGM broth prepared by replacing trypticase soy agar with trypticase soy broth (30 g). Where indicated, medium for *A. actinomycetemcomitans* was supplemented with 2 μg/ml of chloramphenicol and 20 μg/ml of kanamycin. Plates were incubated at 37°C in a 10% CO_2 environment for 72 h, and liquid cultures were incubated for 24 to 48 h.

For examination of the hemolytic phenotype, Columbia agar (Accumedia, Lansing, MI) was supplemented with 5% sheep blood (PML Microbiologicals, Inc., Wilsonville, OR) after the medium cooled to 48°C. Plates were incubated as described above.

Mutagenesis of *A. actinomycetemcomitans*. Strain AA1698 was grown on chloramphenicol plates, and individual colonies were selected and grown overnight in AAGM broth containing chloramphenicol and 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). IPTG was used to induce the transposon system as previously described (27, 49). After incubation, dilutions were made from the suspensions and plated onto kanamycin plates. Individual colonies, representing potentially independent mutants, were picked and patched onto blood plates. Nonhemolytic mutants were characterized by the lack of a zone of clearing around the patch.

Strain JK1025 was constructed by mutagenizing strain CU1000N with pVJT128 and selecting mutants that produced rough-textured colonies on agar (29).

PCR. DNA was PCR amplified using *Taq* DNA polymerase (Qiagen, Valencia, CA). Colony PCR was performed by adding a small amount of a colony into the PCR tube with a sterile pipette tip. For all reactions, the denaturation step was carried out at 94°C for 1 min, and the primer extension step was carried out at 72°C for 1 min. For amplification of *ltxA*, an annealing temperature of 60°C was used for 3 min. For *ltxC*, *ltxB*, and *ltxD*, annealing temperatures of 55°C, 60°C, and 55°C, respectively, were used for 1 min. All PCR products were confirmed by DNA agarose gel electrophoresis. The DNA sequences of primers used are noted in Table 2.

Examination of LtxA. To confirm that mutants were defective in LtxA production, we isolated total protein from supernatants of cultures as previously described (26). Briefly, after cells were grown overnight in AAGM broth, cultures were centrifuged, and 500 μl of supernatant was precipitated with 1 ml of ice-cold ethanol. The pelleted protein was resuspended in sodium dodecyl sulfate (SDS) loading dye and run on an SDS-polyacrylamide gel electrophoresis (PAGE) gel. The gel was stained with Coomassie blue and visualized. For examination of cell-associated protein, the bacterial pellet was resuspended directly in SDS loading dye and run on an SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and processed for Western blot analysis probing with anti-LtxA antibody (9).

Inverse PCR. Inverse PCR on genomic DNA was performed as described previously (27). Briefly, 10 to 20 μg of genomic DNA was digested with EcoRI (which does not cleave IS*903*φ*kan*). Precipitation of the fragments was done by ethanol precipitation, followed by dilution and ligation to circularize the fragments. Resulting fragments were amplified for 30 cycles using primers directed outward from the ends or the transposon. Amplified fragments were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) for sequencing. Nucleotide sequence was determined by the University of Medicine and Dentistry of New Jersey Molecular Research Facility.

Genetic complementation. *ptsH* was amplified by PCR, cloned into pCR2.1, sequenced, and subcloned into pJAK16 (49). The derivative plasmid was mobilized by conjugation from an *E. coli* donor to strain MP046 as previously described (27, 49). IPTG at a final concentration of 1.0 mM was added to plates and liquid cultures for complementation assays.

Real-time PCR. Total bacterial RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA) and further purified by DNase I treatment, followed by passage through an RNeasy spin column (Qiagen, Valencia, CA). Reverse transcription-PCR was subsequently carried out using TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ). Quantitative real-time PCR was performed using the Sybr Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom). The DNA sequences of primers used are noted in Table 2. The threshold cycle

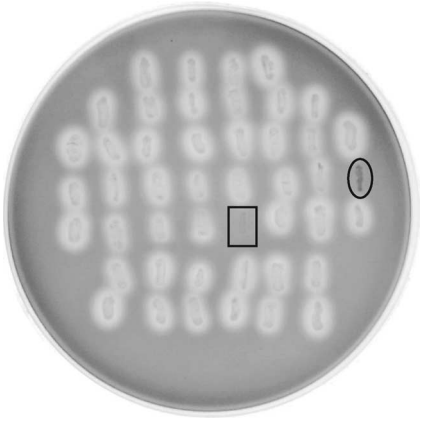

FIG. 1. Blood agar screen for non-beta-hemolytic mutants of *A. actinomycetemcomitans*. Random transposon mutants were patched onto blood agar and grown for 3 days. The squared patch represents a known *ltxA* mutant, and the circled patch is a non-beta-hemolytic mutant isolated with this screen.

(C_T) of the internal control gene *glyA* was subtracted from the C_T of the target gene to obtain the ΔC_T. The normalized relative changes of the mRNA expression level of each target gene was expressed as the $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ is equal to the ΔC_T of the *ptsH* mutant minus ΔC_T of JP2. These experiments were performed in triplicate.

Determination of cAMP concentration. Intracellular cAMP levels were determined using a cAMP enzyme immunoassay system (GE Healthcare, Piscataway, NJ). Cells were collected at 24 h by centrifugation and resuspended in the lysis buffer provided by the manufacturer. Samples were boiled at 100°C for 15 min and centrifuged at 2,000 × *g* for 5 min at 4°C. The cAMP concentrations in supernatants were determined according to the manufacturer's instructions using standards provided in the kit. The average intracellular cAMP concentration (determined in duplicate) was expressed in femtomoles per milligram of total protein.

RESULTS

A screen for LtxA mutants. Knowledge of LtxA genetics in *A. actinomycetemcomitans* has been hampered by the lack of a rapid and efficient screen for mutants. We have previously used an assay based on killing of HL-60 cells by *A. actinomycetemcomitans* culture supernatants (M. P. Isaza and S. C. Kachlany, unpublished results). While effective, this bioassay is time-consuming and not easily amenable to high-throughput screening. Therefore, we sought to take advantage of our recent observation that LtxA is required for β-hemolysis on Columbia blood agar (2).

We began by generating random transposon mutants in *A. actinomycetemcomitans* as previously described (27, 28, 43). The transposon IS903φ*kan* creates nonpolar insertions with no apparent hot spots (27, 49). After transposon mutants were selected on kanamycin, the colonies were patched onto blood agar in a grid format. Figure 1 shows a plate from one typical experiment. The boxed patch represents AA1704, a known *ltxA* mutant strain (2), and the circled patch is a transposon mutant isolated with our screen.

Examination of LtxA mutants. After screening approximately 2,500 random transposon mutants on blood agar, we identified 15 that were partially or non-beta-hemolytic. We wished to further confirm the non-beta-hemolytic phenotype of several of these mutants (Fig. 2A). As expected, when streaked onto blood agar, wild-type JP2N was hemolytic. In contrast, the control *ltxA* mutant strain, AA1704, and others isolated here (MP007, MP028, MP040, and MP043) were all non-beta-hemolytic.

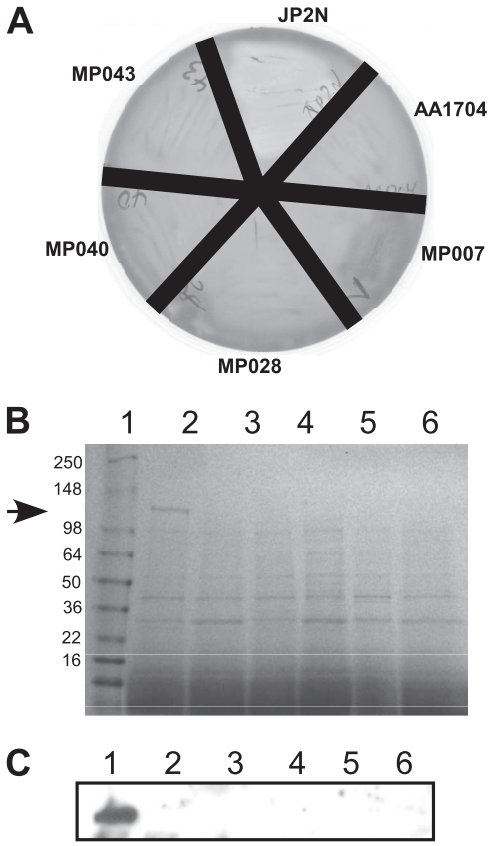

FIG. 2. Examination of non-beta-hemolytic mutants. (A) Several mutants were restreaked on blood agar. JP2N is the wild-type strain; AA1704 is an isogenic *ltxA* mutant of JP2N; and MP007, MP028, MP040, and MP043 are mutants isolated here. (B) Supernatants were isolated from bacterial cultures as described in Materials and Methods and subjected to SDS-PAGE analysis. The gel was stained with Coomassie blue. The arrow indicates the position of LtxA. Lane 1, JP2N; lane 2, AA1704; lane 3, MP007; lane 4, MP028; lane 5, MP040; lane 6, MP043. (C) Western blot analysis of cell-associated protein from the strains described above. Lanes are identical to those in panel B. The blot was probed with anti-LtxA antibody to detect cell-associated LtxA.

To determine if LtxA was being produced in these strains, we examined secreted and cell-associated protein from the wild-type and mutant strains (Fig. 2B and C). SDS-PAGE analysis of supernatant (secreted) protein from JP2N revealed a band at the expected size representing LtxA (Fig. 2B, arrow). In contrast, the supernatants from all other mutant strains lacked the LtxA band, indicating that any LtxA produced by these strains was below the limit of detection. We then examined cell-associated protein using Western blot analysis with anti-LtxA antibody (Fig. 2C). Similar to our results with supernatants, only JP2N produced LtxA. These results indicate that mutants unable to perform β-hemolysis on blood agar are also defective for LtxA production or secretion. This fact further confirms the correlation between β-hemolysis and LtxA production in *A. actinomycetemcomitans*.

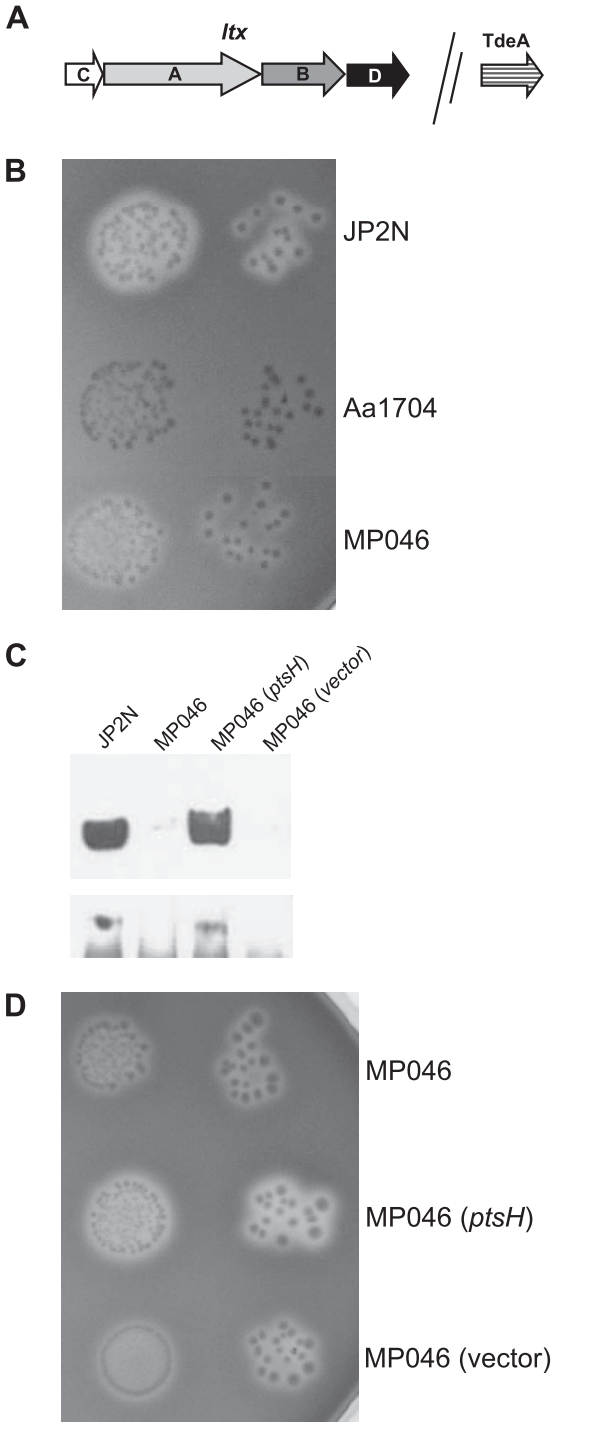

FIG. 3. Characterization of the *ptsH* mutant. (A) Map of the genes that constitute the LtxA operon in *A. actinomycetemcomitans*. (B) Partial hemolysis by MP046 on blood agar. JP2N is the wild-type strain, and AA1704 is an *ltxA* mutant. (C) Western blot analysis of MP046. The top panel represents supernatant (secreted) protein, and the bottom panel shows cell-associated protein. The last two lanes represent complementation experiments with MP046. The blot was probed with anti-LtxA antibody. (D) Complementation of hemolysis on blood agar.

TABLE 3. PCR mapping of LtxA mutants

Strain	Hemolysis	Relative product size after PCR amplification of the indicated gene[a]				
		ltxC	*ltxA*	*ltxB*	*ltxD*	*tdeA*
MP07	+/−	WT	WT	WT	WT	Mut
MP028	−	WT	WT	Mut	WT	WT
MP035	−	WT	Mut	WT	WT	WT
MP038	−	WT	Mut	WT	WT	WT
MP040	−	WT	WT	Mut	Mut	WT
MP041	+/−	WT	WT	WT	WT	Mut
MP042	−	WT	Mut	WT	WT	WT
MP043	−	WT	Mut	WT	WT	WT
MP046	+/−	WT	WT	WT	WT	WT
MP047	−	WT	WT	WT	WT	Mut

[a] WT, product size equal to that of the wild type; Mut, increase in the product size of approximately 1 kbp.

PCR mapping of transposon insertions. The LtxA operon contains four genes (Fig. 3A), all putatively required for LtxA production and activity, and an unlinked *tolC*-like gene, *tdeA*, which encodes a protein required for the export of LtxA (5). To identify the insertion sites of IS*903*ϕ*kan* in several of our mutants, we designed PCR primers to amplify each of four *ltx* genes and *tdeA* and performed PCR. Genes with a transposon insertion show a PCR product that is approximately 1 kb larger than the wild-type size (data not shown). Table 3 shows that we isolated *ltxA*, *ltxB*, *ltxD*, and *tdeA* mutants but not an *ltxC* mutant. However, we have recently isolated an *ltxC* mutant using a direct screening method, indicating that mutations in *ltxC* are not lethal (unpublished data). Furthermore, one of the mutants (MP046) apparently contained the transposon insertion outside of the known *ltx* genes.

Identification of a *ptsH* mutant. One of the mutants (MP046) that contained a transposon insertion outside of the known *ltx* genes displayed a partial hemolytic phenotype on blood agar compared to an *ltxA* mutant, AA1704 (Fig. 3B). LtxA was not detected in the supernatants or cell pellets of MP046 (Fig. 3C). Because of the unique phenotype of this mutant, we wished to further study the genetic basis of this observation. To map the sites where IS*903*ϕ*kan* inserted outside of the *ltx* operon, we performed inverse PCR on genomic DNA as described previously (27). The products were cloned into plasmid pCR-TOPO2.1 and sequenced using universal primers. The sequence indicated an insertion of the IS*903*ϕ*kan* transposon within the first codon of the open reading frame. This putative open reading frame, designated *ptsH*, would encode a protein with significant sequence similarity to histidine-containing protein, HPr (Fig. 4A). To further confirm the location of this mutation, we designed primers that amplify the region flanking the IS*903*ϕ*kan* insertion. Strain MP046 showed a PCR product that is approximately 1 kb larger than the wild-type size (data not shown), confirming that the site of insertion was within the *ptsH* gene.

HPr is part of phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), which is responsible for the transport and phosphorylation of sugars into the cell (8). Because the transposon inserted into one of the genes that controls sugar transport, we wished to determine if this mutation was affecting the overall growth of the strain. We performed

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A.a MYSKDVEITAANGLHTRPAAQFVKEAKAFASDITVTSAGKSASAKSLFKLQTLALTQGTVITISAEGEDAEKAVEHLVALIPTLE 85
P.m MYSKDVEIIAPNGLHTRPAAQFVKEAKAFASDITVTSAGKSASAKSLFKLQTLALSQGTVITISAEGEDEQKAVEHLVALIPTLE 85
H.s MYSKDVEITAPNGLHTRPAAQFVKEAKAFVSDITVTSAGKSASAKSLFKLQTLGLTQGTVITISADGEDEQKAVEHLVALIPTLE 85
M.s MYSKDVEITAPNGLHTRPAAQFVKEAKAFASDVTVTSAGKSASAKSLFKLQTLGLTQGTVITISAEGEDEQNAVDHLVALIPTLE 85
A.s MYSKDVEITAAAGLHTRPAAQFVKEAKAFASDITVTSAGKSASAKSLFKLQTLGLTQGTVITISAEGEDEQTAVDHLVALMPTLE 85
H.i MYSKDVEIIASNGLHTRPAAQFVKEAKAFSSEITVTSGGKSASAKSLFK-QTLALTQGTILTISADGEDEQQAVEHLVALIPTLE 84
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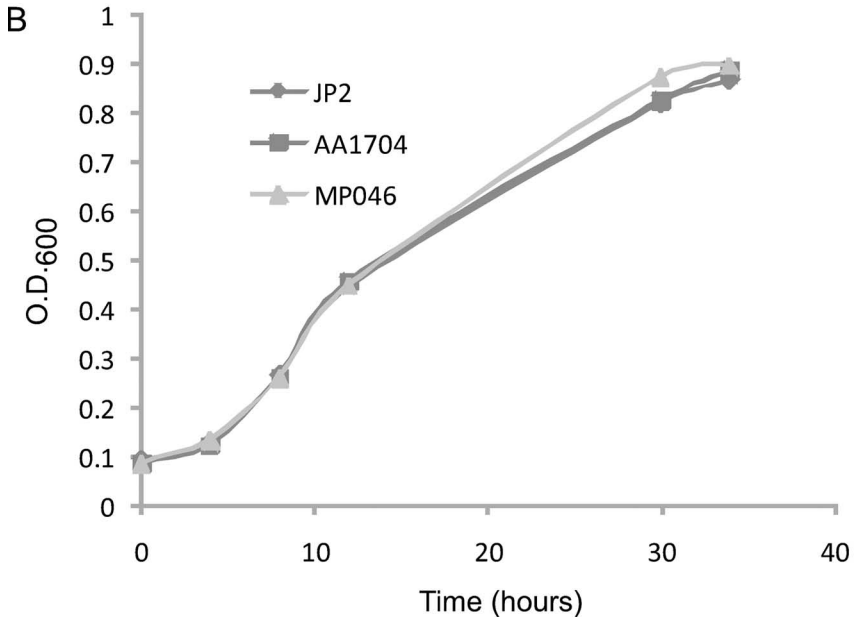

FIG. 4. (A) Amino acid sequence alignment HPr from related bacteria: *A. actinomycetemcomitans* (A.a), *Pasteurella multocida* (P.m; accession no. AE006128.1), *Haemophilus somnus* (H.s; accession no. NZ AAB002000004.1), *Mannheimia succiniciproducens* (M.s; accession no. AE016827.1), *Actinobacillus succinogenes* (A.s; accession no. NZ AAKC01000013.1), and *Haemophilus influenzae* (H.i; accession no. NZ AADP01000001.1). (B) Growth curve comparing MP046 to the wild-type strain (JP2) and the *ltxA* mutant, AA1704. The line for JP2 partially overlaps the strain AA1704 curve.

growth experiments with glucose and found that strain MP046 grew at the same rate as the parent strain in the complex medium used here (Fig. 4B).

To confirm that our insertion was not polar on downstream genes, we cloned and expressed wild-type *ptsH* in *trans* in strain MP046. The complemented mutant strain regained the hemolytic phenotype (Fig. 3D), and LtxA was detected in the cell supernatant and pellet (Fig. 3C). Thus, the insertion in *ptsH* is responsible for the observed defect in LtxA production.

Characterization of the *ptsH* mutant. We performed real-time PCR to determine if the phenotype observed in MP046 was the result of transcriptional regulation of *ltxA*. Our results indicate that, relative to the expression of the housekeeping gene *glyA* (39), the level of *ltxA* transcription in strain MP046 was approximately 1/10 of the wild-type level (Fig. 5A). Thus, *ptsH* apparently plays a role in the transcription of *ltxA*. While a decrease in mRNA stability could also explain these results, MP046 expressed *glyA* at the same level as the wild-type strain, indicating that a general effect on mRNA stability did not occur.

Inoue et al. (25) have shown that cAMP was involved in the regulation of *ltxA* since the levels of LtxA and cAMP decreased as fermentable sugars were added to the culture. They proposed a catabolite repression mechanism to explain their results. To determine if cAMP levels were altered in the *ptsH* mutant, we examined intracellular levels of cAMP in the MP046 and the wild-type strains. Our results indicate that the cAMP levels in the *ptsH* mutant are decreased by approximately half of the wild-type levels (Fig. 5B). Furthermore, when exogenous cAMP (1.0 mM) was added to the culture, LtxA was detected in the supernatant of the *ptsH* mutant (Fig. 5C). Thus, the partial hemolysis and absence of LtxA may be attributed to the diminished levels of intracellular cAMP due to a defect in the PTS machinery.

Another PTS gene, *ptsI*, is required for LtxA production. We wished to determine if the observed effects on LtxA production are specific for HPr or whether other PTS genes are involved. Enzyme I (EI; encoded by *ptsI*) is the protein that acts just upstream of HPr in the PTS pathway and is responsible for transferring a phosphate group to HPr (Fig. 6A) (8). We tested a *ptsI* mutant (JK1025) for hemolysis and found that it was also a non-hemolytic mutant whose phenotype could be complemented by supplying wild-type *ptsI* in *trans* but not with the empty vector (Fig. 6B). Thus, EI is also involved in LtxA biosynthesis.

DISCUSSION

We report here an efficient genetic screen for the identification of genes that play a role in LtxA production in *A.*

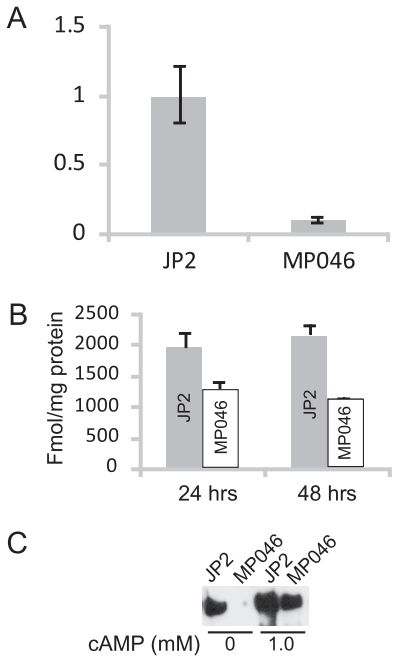

FIG. 5. (A) Real-time PCR analysis of *ltxA* transcription in JP2 and MP046. The *y* axis represents relative levels of mRNA. The *glyA* housekeeping gene was used to standardize values as described in Materials and Methods. (B) Levels of cAMP in fmol/mg protein from JP2 and MP046 at 24 and 48 h. (C) Western blot analysis of supernatant protein of MP046 grown in the presence of 1.0 mM cAMP for 24 h. The blot was probed with anti-LtxA antibody.

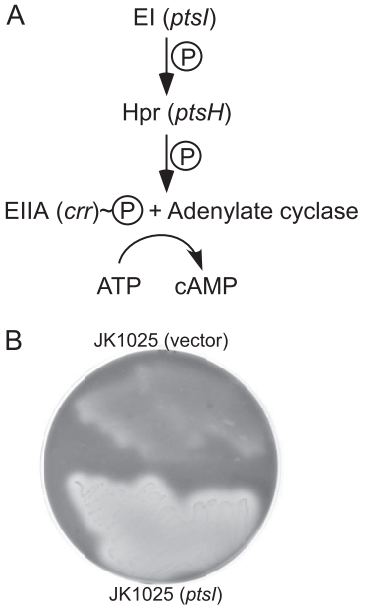

FIG. 6. (A) The pathway for activation of adenylate cyclase by EI and HPr. The model is based on the *E. coli* PTS system. (B) Hemolysis of a *ptsI* mutant on blood agar. JK1025 is a *ptsI* transposon mutant derived from strain CU1000 (11, 27).

actinomycetemcomitans. This is the first report of an approach to screen random mutants of *A. actinomycetemcomitans* for loss of the leukotoxic phenotype. Using this screen, we have confirmed the importance of several genes known or expected to be involved in LtxA biosynthesis, and we identified and characterized a gene that may play a novel role in *ltxA* expression. Importantly, we have also demonstrated the strong correlation between LtxA production and hemolysis. All of our mutants affected LtxA biosynthesis, confirming that this toxin is apparently the only hemolysin that *A. actinomycetemcomitans* produces. Thus, hemolysis proves to be a useful assay in which to study LtxA biology.

The *ltx* operon consists of four genes, *ltxCABD*. In addition, *tdeA* has been shown to be required for LtxA secretion (5) and serves as the TolC-like outer membrane channel (31). We found that the phenotypes of the *ltxB* and *ltxD* mutants (MP028 and MP040, respectively) on blood agar were essentially indistinguishable from *ltxA* mutants. As suggested by sequence similarities to other RTX secretion genes, it is likely that LtxB and LtxD are involved in LtxA secretion (18, 20, 34). Thus, one prediction for *ltxB* and *ltxD* mutants is that they would produce LtxA but not secrete it. However, we found that these mutants neither secreted LtxA nor retained it in the cell pellet (Fig. 2B and C). The fact that LtxA was not produced in these mutants is consistent with the findings of Guthmiller et al. (19), who reported that, while expression of *ltxA* was unaffected in *ltxB* and *ltxD* mutants, the amount of intracellular LtxA decreased significantly. A likely explanation for this observation is that nonsecreted LtxA may be proteolyzed inside the cell. Indeed, studies on other RTX toxins suggest that the low or absent intracellular levels of RTX toxins in certain mutants can be attributed to the instability of the protein retained in the cytoplasm due to the lack of a complete transport system (41).

Using the hemolysis screen, we identified a gene that was previously not implicated in LtxA regulation. HPr is a small protein (~90 residues) encoded by *ptsH* and is part of the PTS. In both gram-negative and gram-positive bacteria, HPr participates in the regulation of various cellular processes, including transport and phosphorylation of sugars and amino acids and catabolite repression. In addition, in some bacteria, HPr can regulate non-PTS proteins, including transcriptional activators (14, 24, 36). The PTS has two general cytoplasmic proteins, EI and HPr, that can be paired with a number of enzymes with sugar specificity (EII). The overall reaction of sugar transport can be described as a cascade that starts with the removal of a phosphoryl group from phosphoenolpyruvate by EI and then phosphorylation of HPr at His-15 with HPr transferring its phosphoryl group to EII (8). $EIIA^{Glc}$, the glucose-specific enzyme, has been shown to regulate levels of cAMP by activating adenylate cyclase (Fig. 6A) (32). Intracellular levels of cAMP are highest when $EIIA^{Glc}$ is in the phosphorylated state. Therefore, PTS mutants express low levels of cAMP (10, 35, 38). Interestingly, we found that the *ptsH* mutant grew at the same rate as the wild-type strain, even though this mutant presumably has a defect in sugar transport. However, this result is consistent with reports for other bacterial *ptsH* mutants (40). One explanation for this observation is that *A. actinomycetemcomitans* possesses HPr-like proteins, such as diphosphoryltransfer protein, that can partially substitute for HPr when grown in certain media (44). In addition, the bacteria may be utilizing other carbon sources in the complex medium. To date, no minimal medium exists for *A. actinomycetemcomitans*, and

so we are unable to determine if there is a defect in sugar transport. Nonetheless, under our experimental conditions, the mutant strain grows as well as the wild-type strain.

We have shown here that a defect in HPr in *A. actinomycetemcomitans* results in low levels of *ltxA* expression, and we correlate this observation to decreased production of cAMP. This finding is consistent with previous studies in *E. coli* that demonstrated low adenylate cyclase activity in *ptsH* mutants (10, 35, 38) and with the study of Inoue et al. (25), who reported that low intracellular levels of cAMP correlate with low levels of LtxA. The low, albeit detectable, levels of cAMP may explain why the mutant is partially hemolytic. Production of cAMP over time may allow for some *ltxA* to be transcribed and eventual accumulation of LtxA. cAMP can regulate several operons through binding of cyclic AMP receptor protein (CRP) to specific sites on the DNA. Inoue et al. (25) noted that the CRP binding site consensus sequence is not present within the *ltx* operon; however, *A. actinomycetemcomitans* CRP is 84% identical to *E. coli* CRP and therefore could recognize a slightly different sequence motif.

We found that another PTS gene, *ptsI*, is also required for LtxA-mediated hemolysis. This observation is not surprising, given that EI phosphorylates HPr and would result in a nonfunctional HPr, similar to the result when *ptsH* is inactivated. Thus, our current model proposes that HPr regulates levels of *ltxA* through its interaction with other PTS proteins, which affects the levels of cAMP. In turn, cAMP regulates levels of *ltxA* mRNA through an unknown mechanism. While HPr plays a significant role in transcriptional control in many gram-positive bacteria (14, 24, 36), a similar bifunctional mechanism has not yet been established for HPr in gram-negative organisms. Examining the effects of other PTS mutants and protein interactions with HPr will shed more light on our model.

In summary, we have developed and utilized a method to isolate *A. actinomycetemcomitans* mutants that are defective in LtxA production based on β-hemolysis on blood agar. This rapid screen may allow for the identification of new target proteins for the development of therapeutics that interfere with LtxA activity and *A. actinomycetemcomitans* pathogenesis.

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REFERENCES

1. **Baehni, P. C., C. C. Tsai, W. P. McArthur, B. F. Hammond, B. J. Shenker, and N. S. Taichman.** 1981. Leukotoxic activity in different strains of the bacterium *Actinobacillus actinomycetemcomitans* isolated from juvenile periodontitis in man. Arch. Oral Biol. **26:**671–676.
2. **Balashova, N. V., J. A. Crosby, L. Al Ghofaily, and S. C. Kachlany.** 2006. Leukotoxin confers beta-hemolytic activity to *Actinobacillus actinomycetemcomitans*. Infect. Immun. **74:**2015–2021.
3. **Balashova, N. V., R. Diaz, S. V. Balashov, J. A. Crosby, and S. C. Kachlany.** 2006. Regulation of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* leukotoxin secretion by iron. J. Bacteriol. **188:**8658–8661.
4. **Berbari, E. F., F. R. Cockerill III, and J. M. Steckelberg.** 1997. Infective endocarditis due to unusual or fastidious microorganisms. Mayo Clin. Proc. **72:**532–542.
5. **Crosby, J. A., and S. C. Kachlany.** 2007. TdeA, a TolC-like protein required for toxin and drug export in *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*. Gene **388:**83–92.
6. **Das, M., A. D. Badley, F. R. Cockerill, J. M. Steckelberg, and W. R. Wilson.** 1997. Infective endocarditis caused by HACEK microorganisms. Annu. Rev. Med. **48:**25–33.
7. **Delepelaire, P.** 2004. Type I secretion in gram-negative bacteria. Biochim. Biophys. Acta **1694:**149–161.
8. **Deutscher, J., C. Francke, and P. W. Postma.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. **70:**939–1031.
9. **Diaz, R., L. A. Ghofaily, J. Patel, N. V. Balashova, A. C. Freitas, I. Labib, and S. C. Kachlany.** 2006. Characterization of leukotoxin from a clinical strain of *Actinobacillus actinomycetemcomitans*. Microb. Pathog. **40:**48–55.
10. **Feucht, B. U., and M. H. Saier, Jr.** 1980. Fine control of adenylate cyclase by the phosphoenolpyruvate:sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **141:**603–610.
11. **Fine, D. H., D. Furgang, J. Kaplan, J. Charlesworth, and D. H. Figurski.** 1999. Tenacious adhesion of *Actinobacillus actinomycetemcomitans* strain CU1000 to salivary-coated hydroxyapatite. Arch. Oral Biol. **44:**1063–1076.
12. **Fine, D. H., D. Furgang, H. C. Schreiner, P. Goncharoff, J. Charlesworth, G. Ghazwan, P. Fitzgerald-Bocarsly, and D. H. Figurski.** 1999. Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: implications for virulence. Microbiology **145:**1335–1347.
13. **Fine, D. H., J. B. Kaplan, S. C. Kachlany, and H. C. Schreiner.** 2006. How we got attached to *Actinobacillus actinomycetemcomitans*: a model for infectious diseases. Periodontol. 2000 **42:**114–157.
14. **Flores, N., R. de Anda, S. Flores, A. Escalante, G. Hernandez, A. Martinez, O. T. Ramirez, G. Gosset, and F. Bolivar.** 2004. Role of pyruvate oxidase in *Escherichia coli* strains lacking the phosphoenolpyruvate:carbohydrate phosphotransferase system. J. Mol. Microbiol. Biotechnol. **8:**209–221.
15. **Fong, K. P., W. O. Chung, R. J. Lamont, and D. R. Demuth.** 2001. Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. Infect. Immun. **69:**7625–7634.
16. **Frey, J., and P. Kuhnert.** 2002. RTX toxins in *Pasteurellaceae*. Int. J. Med. Microbiol. **292:**149–158.
17. **Gentschev, I., G. Dietrich, and W. Goebel.** 2002. The *E. coli* alpha-hemolysin secretion system and its use in vaccine development. Trends Microbiol. **10:**39–45.
18. **Guthmiller, J. M., D. Kolodrubetz, M. P. Cagle, and E. Kraig.** 1990. Sequence of the lktB gene from *Actinobacillus actinomycetemcomitans*. Nucleic Acids Res. **18:**5291.
19. **Guthmiller, J. M., D. Kolodrubetz, and E. Kraig.** 1995. Mutational analysis of the putative leukotoxin transport genes in *Actinobacillus actinomycetemcomitans*. Microb. Pathog **18:**307–321.
20. **Guthmiller, J. M., E. Kraig, M. P. Cagle, and D. Kolodrubetz.** 1990. Sequence of the *lktD* gene from *Actinobacillus actinomycetemcomitans*. Nucleic Acids Res. **18:**5292.
21. **Haraszthy, V. I., G. Hariharan, E. M. Tinoco, J. R. Cortelli, E. T. Lally, E. Davis, and J. J. Zambon.** 2000. Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J. Periodontol. **71:**912–922.
22. **Haubek, D., J. M. Dirienzo, E. M. Tinoco, J. Westergaard, N. J. Lopez, C. P. Chung, K. Poulsen, and M. Kilian.** 1997. Racial tropism of a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. J. Clin. Microbiol. **35:**3037–3042.
23. **Haubek, D., O. K. Ennibi, K. Poulsen, S. Poulsen, N. Benzarti, and M. Kilian.** 2001. Early-onset periodontitis in Morocco is associated with the highly leukotoxic clone of *Actinobacillus actinomycetemcomitans*. J. Dent. Res. **80:**1580–1583.
24. **Horstmann, N., G. Seidel, L. M. Aung-Hilbrich, and W. Hillen.** 2007. Residues His-15 and Arg-17 of HPr participate differently in catabolite signal processing via CcpA. J. Biol. Chem. **282:**1175–1182.
25. **Inoue, T., I. Tanimoto, T. Tada, T. Ohashi, K. Fukui, and H. Ohta.** 2001. Fermentable-sugar-level-dependent regulation of leukotoxin synthesis in a variably toxic strain of *Actinobacillus actinomycetemcomitans*. Microbiology **147:**2749–2756.
26. **Kachlany, S. C., D. H. Fine, and D. H. Figurski.** 2000. Secretion of RTX leukotoxin by *Actinobacillus actinomycetemcomitans*. Infect. Immun. **68:**6094–6100.
27. **Kachlany, S. C., P. J. Planet, M. K. Bhattacharjee, E. Kollia, R. DeSalle, D. H. Fine, and D. H. Figurski.** 2000. Nonspecific adherence by *Actinobacillus actinomycetemcomitans* requires genes widespread in bacteria and archaea. J. Bacteriol. **182:**6169–6176.
28. **Kachlany, S. C., P. J. Planet, R. Desalle, D. H. Fine, D. H. Figurski, and J. B. Kaplan.** 2001. *flp-1*, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. Mol. Microbiol. **40:**542–554.
29. **Kaplan, J. B., M. B. Perry, L. L. MacLean, D. Furgang, M. E. Wilson, and D. H. Fine.** 2001. Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. Infect. Immun. **69:**5375–5384.
30. **Kolodrubetz, D., T. Dailey, J. Ebersole, and E. Kraig.** 1989. Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. Infect. Immun. **57:**1465–1469.

31. **Koronakis, V.** 2003. TolC—the bacterial exit duct for proteins and drugs. FEBS Lett. **555:**66–71.
32. **Krin, E., O. Sismeiro, A. Danchin, and P. N. Bertin.** 2002. The regulation of Enzyme IIA(Glc) expression controls adenylate cyclase activity in *Escherichia coli*. Microbiology **148:**1553–1559.
33. **Lally, E. T., E. E. Golub, I. R. Kieba, N. S. Taichman, J. Rosenbloom, J. C. Rosenbloom, C. W. Gibson, and D. R. Demuth.** 1989. Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. J. Biol. Chem. **264:**15451–15456.
34. **Lally, E. T., I. R. Kieba, N. S. Taichman, J. Rosenbloom, C. W. Gibson, D. R. Demuth, G. Harrison, and E. E. Golub.** 1991. *Actinobacillus actinomycetemcomitans* leukotoxin is a calcium-binding protein. J. Periodontal Res. **26:**268–271.
35. **Levy, S., G. Q. Zeng, and A. Danchin.** 1990. Cyclic AMP synthesis in *Escherichia coli* strains bearing known deletions in the *pts* phosphotransferase operon. Gene **86:**27–33.
36. **Muller, W., N. Horstmann, W. Hillen, and H. Sticht.** 2006. The transcription regulator RbsR represents a novel interaction partner of the phosphoprotein HPr-Ser46-P in *Bacillus subtilis*. FEBS J. **273:**1251–1261.
37. **Narayanan, S. K., T. G. Nagaraja, M. M. Chengappa, and G. C. Stewart.** 2002. Leukotoxins of gram-negative bacteria. Vet. Microbiol. **84:**337–356.
38. **Nelson, S. O., B. J. Scholte, and P. W. Postma.** 1982. Phosphoenolpyruvate: sugar phosphotransferase system-mediated regulation of carbohydrate metabolism in *Salmonella typhimurium*. J. Bacteriol. **150:**604–615.
39. **Nielsen, K. K., and M. Boye.** 2005. Real-time quantitative reverse transcription-PCR analysis of expression stability of *Actinobacillus pleuropneumoniae* housekeeping genes during in vitro growth under iron-depleted conditions. Appl. Environ. Microbiol. **71:**2949–2954.
40. **Nothaft, H., S. Parche, A. Kamionka, and F. Titgemeyer.** 2003. In vivo analysis of HPr reveals a fructose-specific phosphotransferase system that confers high-affinity uptake in *Streptomyces coelicolor*. J. Bacteriol. **185:**929–937.
41. **Oropeza-Wekerle, R. L., E. Muller, P. Kern, R. Meyermann, and W. Goebel.** 1989. Synthesis, inactivation, and localization of extracellular and intracellular *Escherichia coli* hemolysins. J. Bacteriol. **171:**2783–2788.
42. **Paturel, L., J. P. Casalta, G. Habib, M. Nezri, and D. Raoult.** 2004. *Actinobacillus actinomycetemcomitans* endocarditis. Clin. Microbiol. Infect. **10:**98–118.
43. **Planet, P. J., S. C. Kachlany, D. H. Fine, R. DeSalle, and D. H. Figurski.** 2003. The widespread colonization island of *Actinobacillus actinomycetemcomitans*. Nat. Genet. **34:**193–198.
44. **Reichenbach, B., D. A. Breustedt, J. Stulke, B. Rak, and B. Gorke.** 2007. Genetic dissection of specificity determinants in the interaction of HPr with enzymes II of the bacterial phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*. J. Bacteriol. **189:**4603–4613.
45. **Slots, J., and M. Ting.** 1999. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol. 2000 **20:**82–121.
46. **Taichman, N. S., R. T. Dean, and C. J. Sanderson.** 1980. Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from *Actinobacillus actinomycetemcomitans*. Infect. Immun. **28:**258–268.
47. **Taichman, N. S., D. L. Simpson, S. Sakurada, M. Cranfield, J. DiRienzo, and J. Slots.** 1987. Comparative studies on the biology of *Actinobacillus actinomycetemcomitans* leukotoxin in primates. Oral Microbiol. Immunol. **2:**97–104.
48. **Taichman, N. S., and J. M. Wilton.** 1981. Leukotoxicity of an extract from *Actinobacillus actinomycetemcomitans* for human gingival polymorphonuclear leukocytes. Inflammation **5:**1–12.
49. **Thomson, V. J., M. K. Bhattacharjee, D. H. Fine, K. M. Derbyshire, and D. H. Figurski.** 1999. Direct selection of IS*903* transposon insertions by use of a broad-host-range vector: isolation of catalase-deficient mutants of *Actinobacillus actinomycetemcomitans*. J. Bacteriol. **181:**7298–7307.
50. **Tsai, C. C., B. J. Shenker, J. M. DiRienzo, D. Malamud, and N. S. Taichman.** 1984. Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. Infect. Immun. **43:**700–705.
51. **Welch, R. A.** 2001. RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. Curr. Top. Microbiol. Immunol. **257:**85–111.
52. **Zambon, J. J.** 1985. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J. Clin. Periodontol. **12:**1–20.
53. **Zambon, J. J.** 1996. Periodontal diseases: microbial factors. Ann. Periodontol. **1:**879–925.

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