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Identification of *Actinobacillus suis* Genes Essential for the Colonization of the Upper Respiratory Tract of Swine

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Actinobacillus suis **has emerged as an important opportunistic pathogen of high-health-status swine. A colonization challenge method was developed, and using PCR-based signature-tagged transposon mutagenesis, 13 genes belonging to 9 different functional classes were identified that were necessary for *A. suis* colonization of the upper respiratory tract of swine.**

Over the last two decades, *Actinbacillus suis* has emerged as an important pathogen of high-health-status (HHS) swine (30, 38). Although *A. suis* can persist as a commensal in the upper respiratory tract (URT), under conditions which are poorly understood, it can metastasize and cause septicemia, enteritis, abortion, arthritis, mastitis, pericarditis, myocarditis, and erysipelas-like cutaneous lesions (30). Little is known about the virulence factors of *A. suis*, but it is hypothesized that homologs of those found in the close relative, *Actinobacillus pleuropneumoniae* (10, 30), including RTX toxins, urease, and iron-regulated outer membrane proteins might play a role in pathogenesis. Despite the fact that there seem to be many shared virulence determinants, the spectrum of diseases that *A. suis* can cause and the range of hosts it can affect are different from those of *A. pleuropneumoniae*, so there are clearly other factors involved (10, 24, 42). In order to begin to understand the mechanism of the switch from benign commensal to invasive pathogen, we set out to first identify the factors of *A. suis* that are required for colonization of upper respiratory tract of swine, using signature-tagged transposon mutagenesis (STM).

A spontaneous nalidixic acid-resistant (Nal^r) variant of the virulent O2/K2 *A. suis* strain H91-0380 was used in this study (48, 62). *Escherichia coli* SM10 λ*pir* (47) was used as the donor strain in the conjugation experiments to deliver the tagged transposons. The *A. suis* and *E. coli* strains were routinely grown on blood agar or brain-heart infusion (BHI) plates at 37°C in an atmosphere of 5% CO_2. As required, antibiotics were added to the following final concentrations: kanamycin (Kan), 50 μg/ml; Nal, 25 μg/ml; ampicillin (Amp), 100 μg/ml; bacitracin (Bac), 10 μg/ml; crystal violet (Cv), 1 μg/ml; and chloramphenicol (Chl), 5 μg/ml. For mating experiments, *A. suis* and *E. coli* strains were grown in BHI broth with the appropriate antibiotics.

For this work, a new PCR STM system was developed using pLOF/Km (28, 56). To add the oligonucleotide tags, pLOF/Km was partially digested with NotI, blunt-ended, and recircularized, and pLOF/KmdeltaNotI (in which the NotI site upstream of the MluI, SfiI, NotI polylinker was inactivated) was obtained. Twenty-two double-stranded tags (synthesized as 21-mers and their reverse complements; Table 1) were cloned into the KpnI site of pUC18/NotI that had been blunt ended. The NotI fragments of the different tags were ligated with NotI-restricted pLOF/KmdeltaNotI and electrotransformed into *E. coli* DH5α λ*pir*; the arrangement of all of the different tagged plasmids was confirmed by PCR. The 22 pLOF/Km NotI tags (designated pLOF/Tag1, -2, -3, etc.) were then electroporated into *E. coli* SM10 λ*pir* and maintained at −70°C in 30% glycerol. *E. coli* SM10 λ*pir* cells carrying the different pLOF/Tag plasmids were used as donors for the conjugal transposition into *A. suis* H91-0380Nal^r. Briefly, overnight cultures of the donor and recipient were mixed (200 μl and 600 μl or 100 μl and 700 μl, respectively), harvested by centrifugation, washed with 10 mM $MgSO_4$, centrifuged, suspended in 50 μl of 10 mM of $MgSO_4$, and applied as spots onto 0.22-μm filters (Millipore Corporation, Bedford, MA) that had been placed onto nonselective BHI plates with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). The plates were incubated at 37°C for 6 to 8 h in 5% CO_2. The mating mixtures were washed off the filters with 5 ml of PBS, pelleted, and suspended in 100 μl of PBS and plated on BHI-Nal/Kan plates. The transconjugants were then "gridded" on blood agar, BHI-Nal/Kan, and BHI-Amp (to identify pLOF/Km cointegrates) plates. Nal^r Kan^r Amp^s *A. suis* transconjugants were grown overnight in BHI-Nal/Kan broth, frozen in 30% glycerol, and stored in individual vials at −70°C. A total of ~1,100 transconjugants with 22 different tags were obtained from more than 40 independent conjugation experiments. For each screen, input pools of 18 different transconjugants that had been grown overnight, harvested, and suspended in sterile PBS (~5×10^9 CFU/ml) were assembled. Five hundred μl/nostril was delivered in drops during the inhalation phase to two 3- to 4-week-old piglets (both male and female, Yorkshire) from the high-health-status herd at the Arkell Swine Research Station, Ontario, Canada. In preliminary experiments, it was determined that *A. suis* could be recovered from nasal cavities in fairly large numbers, although occasionally the recovery was low. Greater numbers of *A. suis* cells were always detected from pharyngeal swabs as well as in tonsillar tissues. Forty-eight hours postinfection, the piglets were euthanized and the anterior nasal cavity and pharynx were swabbed. The snout was then cut off at the level of the 3rd

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TABLE 1. Tag oligonucleotides used in this study

Tag no.	Nucleotide sequence[a]
1	5′-GTACCGCGCTTAAA**CGTTCA**G-3′
2	5′-GTACCGCGCTTAAA**TAGCCT**G-3′
3	5′-GTACCGCGCTTAAA**AGTCTC**G-3′
4	5′-GTACCGCGCTTAA**TAACGTG**G-3′
5	5′-GTACCGCGCTTAAA**CTGGTA**G-3′
7	5′-GTACCGCGCTTAA**TGTAACC**G-3′
9	5′-GTACCGCGCTTAA**TAGGCAA**G-3′
10	5′-GTACCGCGCTTAA**CAATCGT**G-3′
11	5′-GTACCGCGCTTAA**CGCAAAA**G-3′
12	5′-GTACCGCGCTTAA**TCAAGAC**G-3′
13	5′-GTACCGCGCTTAA**CTAGTAG**G-3′
14	5′-GTACCGCGCTTAA**CAGCTTA**G-3′
15	5′-GTACCGCGCTTAA**CACTGAT**G-3′
16	5′-GTACCGCGCTTAA**GCACTAT**G-3′
17	5′-GTACCGCGCTTAA**TGCACAT**G-3′
18	5′-GTACCGCGCTTAA**GTCGAAT**G-3′
21	5′-GTACCGCGCTTAA**CTTGACA**G-3′
22	5′-GTACCGCGCTTAAA**GACTAG**G-3′
23	5′-GTACCGCGCTTAA**TCGATGA**G-3′
25	5′-GTACCGCGCTTAAA**CCTTGT**G-3′
26	5′-GTACCGCGCTTAA**GTACGAA**G-3′
28	5′-GTACCGCGCTTAA**TTATGGG**G-3′

[a] The consensus 5′ ends have higher Δ*G*s to optimize the PCRs. The variable 3′ ends of each of the oligonucleotide tags that permit amplification of the specific DNA sequences are indicated in bold.

premolar, and swabs of cranial nasal cavity and posterior nasal cavity were obtained. Finally, the lingual and palatine tonsils were dissected out. The swabs were streaked on "super-selective" BHI (SSBHI) plates containing nalidixic acid, kanamycin, bacitracin, and crystal violet. The tissues were finely minced and then homogenized in glass tissue grinders in 3 ml of PBS, and 100 μl of each homogenate was likewise plated. All animal procedures were approved by the University of Guelph Animal Care Committee and were in accordance with the guidelines of the Canadian Council for Animal Care.

The mutants recovered on SSBHI plates were harvested in PBS and pooled. Approximately 150 to 200 μl of each cell suspension was pelleted and suspended in 300 μl TE-PCR buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA), boiled for ~10 min, and centrifuged for 1 min, and 10 μl of each supernatant was used in 50-μl PCRs (28). The PCR mixtures contained a final concentration of 1× PCR buffer (Invitrogen, Life Technologies, Carlsbad, Calif.), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 1 μM of a unique Tag primer, 1 μM of the universal KanR1 primer, and 2.5 U of *Taq* DNA polymerase (Invitrogen, Life Technologies). The PCR conditions were as follows: 1 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension step of 7 min at 72°C, yielding a PCR product of 500 bp. The resultant fragments were visualized by ethidium bromide staining following electrophoresis in a 1.5% agarose gel.

Two piglets were used to screen each input pool. Mutants that were lost in the first screen were combined and tested in a second screen, again in two animals. Strains that were eliminated from the URT of both pigs in two independent screens (i.e., four animals) were analyzed further by direct sequencing of the inverse PCR products of the regions flanking the transposon as described previously (15), except primer SO69 (5′-TACTCCTGATGATGCATGG-3′) was used in place of TEF69. The sequences were compared to entries in the nonredundant public databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/BLAST) using the BlastX and BlastN programs (3) and assigned to cluster of orthologous groups of proteins (COG) functional categories published at http:/www.ncbi.nlm.nih.gov/COG/.

Putative colonization-defective mutants were further characterized by competing individual mutants with the wild-type strain, *A. suis* H91-0380 Nal^r carrying pFP12 (34). The competitive index (CI) was calculated as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria (61). For in vivo studies, the mutant and wild-type strains were grown separately overnight in 20 ml of BHI broth with appropriate antibiotics and the next day the bacteria were pelleted and suspended in PBS to the same optical density at 600 nm (OD_{600}) (~2.8). Two piglets were inoculated intranasally with 2× 500 μl of a 1:1 mixture of the mutant and wild type. In order to calculate the input ratio, 10-fold dilutions of each were plated to determine the exact number of CFU. The pigs were sacrificed 48 h postinfection, and samples were collected and processed as described above. The colonies recovered from lingual and palatine tonsils were used to calculate the output ratio. To determine the in vitro CI, mutant and wild type *A. suis* strains were first grown on blood agar and then harvested in sterile BHI broth and the suspensions were adjusted to the same OD_{600}. Ten-fold dilutions of the strains were plated to calculate the exact input ratio, and 0.2 ml of each strain was used to inoculate 20 ml of BHI/Nal broth. The cultures were grown for ~7 h (stationary phase), and then dilutions of the mixed culture were plated on BHI and BHI-Kan plates. The number of CFU obtained on BHI-Kan plates (mutant) was subtracted from the number obtained on BHI plates (mutant and wild type) to calculate the number of wild-type *A. suis* cells. The in vitro CI was calculated as described above.

Of 770 mutants screened for their ability to colonize the URT of piglets, 97 independent mutants (40 rRNA gene mutants and 57 independent mutants of 30 different genes) were found to be colonization deficient. This rate of loss of ST mutants was higher (12.6%) than has been reported in many other studies (2 to 5%) (e.g., references 26 and 45). It is speculated that competition with commensal organisms (streptococci, enterococci, and staphylococci were detected) together with a relatively small number of colonization sites available in tonsil might be a reason for this high loss.

Although transposition by Tn*10* is considered "random" (27), the consensus sequence 5′-N**GCT**N**AGC**N-3′ has been shown to be a preferred substrate sequence recognized by the Tn*10* transposase (8). In this STM screen, 40 independent colonization defective mutants were identified with Tn*10* insertions in rRNA genes (Table 2). The targets for most of these insertions in the 16S rRNA genes were 5′-A**GCTAAGC**A-3′ and 5′C**GC**AC**TG**TT-3′; in the 23S rRNA genes, Tn*10* integration was guided by the sequence 5′-CA**CTAAGC**T-3′. Fuller et al. (15) also found a large number of transposon insertions in the rRNA genes in the related organism, *A. pleuropneumoniae*. In the present study, multiple independent insertions were also seen in *cpxR* (2), *hns* (5), *pomA* (2), *aceE* (3), *lpdA* (2), *nifU* (3), *nrdA* (3), *guaA* (2), tRNA-Tyr (3), PM1911 (6), and PM1778 (7). Taken together, these data suggest that

TABLE 2. Putative colonization-essential genes of *Actinobacillus suis*

Mutant[a]	Nearest homolog species	% Similarity (span)[b]	Product	Putative function	$CI_{in\ vivo}$[c]	$CI_{in\ vitro}$[c]
Transcription (COG 1D K)						
2–43 (+1)[d]	*cpxR*, *Pasteurella multocida*	60 (166)	CpxR	Response regulator to stress, pilus biogenesis	0.28	0.83
3–39	*hupA*, *Pasteurella multocida*	79 (72)	HupA	Global transcriptional regulator, DNA-binding protein Huα	0.29	0.66
3–31 (+4)	*hns*, *Pasteurella multocida*	54 (106)	H-NS	DNA-binding, global regulator	0.18	4
15-8	YP0456, *Yersinia pestis*	63 (65)	Putative right-origin DNA-binding protein	Global regulator	0.35	0.37
Outer membrane, cell wall biogenesis (COG ID M)						
23–58 (+1)	*pomA*, *Mannheimia haemolytica*	77[f] (378)	Outer membrane protein A	Attachment, structural integrity	0.07	0.5
1–28	*pfhaB1*, *Pasteurella multocida*	96 (31 nt)	Putative filamentous hemagglutinin	Attachment	0.17	0.17
22–100	*lcbB*, *Neisseria meningitidis*	34 (64)	Putative bifunctional polymerase	Cell envelope biogenesis	0.66	0.66
Amino acid metabolism and transport (COG ID E)						
4–4	VCA0160, *Vibrio cholerae*	73 (38)	Tryptophan-specific transport protein	Channeling of tryptophan into cell membrane	1	1.5
4–21	*lmo0845* (~*yxjH*), *Listeria monocytogenes* (~*Bacillus subtilis*)	54 (211)	YxjH protein	Methionine biosynthesis	0.12	1.6
Energy production and conversion (COG ID C)						
2–50	*atpG*, *Haemophilus influenzae*	67 (227)	ATP synthase gamma chain	Pump H^+ ions, synthesize ATP, maintain electrophysiology of the cell, protect cells from oxidative and acidic stress	0.67	2.1
3–26	*atpC*, *Pasteurella multocida*	76 (30)	ATP synthase epsilon chain	Pump H^+ ions, synthesize ATP, maintain electrophysiology of the cell, protect cells from oxidative and acidic stress	NT[e]	0.31
18–18 (+2)[d]	*aceE*, *Haemophilus influenzae*	88 (118)	Pyruvate dehydrogenase, E1 component	Catalyze oxidative decarboxylation of pyruvate to acetyl coenzyme A	NT	0.02
18-15 (+1)[d]	*lpdA*, *Haemophilus influenzae*	95 (147)	Dihydrolipoamide dehydrogenase	Catalyze oxidative decarboxylation of pyruvate to acetyl coenzyme A	0.41	0.64
22–47 (+2)[d]	*nifU*/*iscU*, *Haemophilus influenzae*	87 (125)	IscU	Fe-S assembly, iron homeostasis in eukaryotes	0.001	0.5
Lipid metabolism (COG ID I)						
3–52	*est*, *Pasteurella multocida*	42 (124)	Esterase	Lipolytic enzyme, hydrolyzes lung surfactant	NT	0.66
Carbohydrate transport and metabolism (COG ID G)						
26–75	*ptsH*, *Pasteurella multocida*	89 (19)	PtsH	Phosphocarrier protein, transport of sugars	NT	0.48
Nucleotide metabolism (COG ID F)						
23–48	*thyA*, *Haemophilus influenzae*	74 (81)	Thymidylate synthetase	Thymine nucleotide metabolism	0.12	0.33

Continued on facing page

TABLE 2—*Continued*

Mutant[a]	Nearest homolog species	% Similarity (span)[b]	Product	Putative function	$CI_{in\ vivo}$[c]	$CI_{in\ vitro}$[c]
18–21 (+2)[d]	*nrdA*, *Rickettsia conorii*	35 (281)	NrdA, ribonucleoside-diphosphate reductase alpha chain	Catalyzes conversion of ribonucleotides to corresponding deoxyribonucleotides	NT	0.36
26–80 (+1)[d]	*guaA*, *Pasteurella multocida*	100 (81)	GMP synthase	Purine biosynthesis	0.3	1.6
Translation, ribosomal structure, and processing (COG ID J)						
18-8	tRNA-Gly3, *Haemophilus influenzae*	97 (175 nt)	tRNA-glycine	Addition of glycine amino acid to the polypeptide chain	0.02	1.2
23–27 (+2)[d]	tRNA-Tyr, *Haemophilus influenzae*	97 (175 nt)	tRNA-tyrosine	Addition of tyrosine amino acid during protein synthesis	0.15	0.33
22–29	*tufB*, *Haemophilus influenzae*	96 (175 nt)	Elongation factor TuB	Processing factors for protein synthesis	NT	0.28
16-2	*tufA*, *Pasteurella multocida*	75 (136)	Elongation factor TuA	Processing factors for protein synthesis	NT	0.45
15–26	*rpl9*, *Haemophilus influenzae*	49 (81)	50S ribosomal protein L9	Proper folding and configuration of rRNA	NT	1.7
23–37	*rpl32*, *Haemophilus influenzae*	94 (38)	50S ribosomal protein L32	Proper folding and configuration of rRNA	NT	0.34
3–44 (+18)	*HirrnC23S*, *Haemophilus influenzae*	83 (164 nt)	23S rRNA	Protein synthesis	NT	2.0
3–45 (+20)	*rrnA 16S*, *Actinobacillus equuli*	99 (261 nt)	16S rRNA	Protein synthesis	2.3	0.81
Function unknown COG ID S						
4-2 (+5)[d]	*PM1911*, *Pasteurella multocida*	63 (158)	Unknown	Conserved hypothetical (putative metal-binding protein)	0.01	0.93
3–54 (+6)[d]	*PM1778*, *Pasteurella multocida*	64 (73)	Unknown	Unknown	0.4	0.66
17–21	*PM1568*, *Pasteurella multocida*	75 (68)	Unknown	Unknown	1	1.1
3–17	*PM0682*, *Pasteurella multocida*	92 (28)	Unknown	Unknown	1	1.92
17–66	No significant match in the database		Unknown	Unknown	NT	1.3

[a] Mutants lost from both animals in two independent screens.
[b] Percent similarity determined using BLASTX (span in amino acids, except tRNA and rRNA genes indicated in bp).
[c] Calculated as (mutant CFU/wild-type CFU recovered)/(mutant CFU/wild-type CFU_{input}).
[d] Number of independent insertions in the same reading frame.
[e] NT, not tested.
[f] Based on sequence of entire gene (1,122 bp).

although Tn*10* has proven to be useful in the study of *Actinobacillus* spp. (10, 15), it alone cannot be used to do a saturating screen.

In this STM screen, nine mutants that carried mini-Tn*10* insertions in the genes encoding transcriptional regulatory proteins were lost. Among these, two independent mutants harbored Tn*10* integration in a homolog of *cpxR*, a gene which encodes the response regulator protein of the *cpxA-R* two-component regulatory system. The CpxR mutant, 2-43, grew slightly less well than the wild type in vitro ($CI_{in\ vitro}$ = 0.83) and was markedly attenuated in vivo ($CI_{in\ vivo}$ = 0.28). In uropathogenic *E. coli*, the CpxA-R system is involved in the P pilus biogenesis and is thought to regulate the expression of other virulence factors, including hemolysin and cytotoxic necrotizing factor (22). Attenuation of the *cpxR* ST mutant was likely due to effects on the expression of more than one gene, but further work will be required to identify the CpxR regulon in *A. suis*.

Global regulatory proteins, such as HU, integration host factor (IHF), and H-NS (histone-like proteins), and their functional equivalents in other genera are known to bind DNA and play an important role in gene regulation (36). Several *A. suis* mutants of *hns*, *hupA*, and a protein carrying a helix-turn-helix (HTH) sequence were colonization defective in initial screens. Although the *hns* mutant 3-31 appeared to grow much better in vitro ($CI_{in\ vitro}$ = 4.0), it was greatly attenuated in vivo ($CI_{in\ vivo}$ = 0.18). The reason(s) for the more robust growth in vitro is not immediately obvious; however, in *E. coli*, it is known that

although H-NS acts as a positive regulator in vivo, it behaves as a negative regulator in vitro (36). Several studies have concluded that this protein is a transcriptional repressor and a translation inhibitor (43, 52) and growth of the wild type in vitro might have been repressed with respect to the *A. suis hns* mutant. H-NS plays a vital role in cellular response to changes in the environment and controls the expression of many different genes in *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella* spp. (20, 63).

Another colonization-deficient mutant carried an insertion in a *hupA* gene homolog. In competition assays, it grew somewhat more slowly in vitro ($CI_{in\ vitro} = 0.66$) but was more clearly attenuated in vivo ($CI_{in\ vivo} = 0.29$). HupA mutants are asynchronous in DNA replication initiation and have been associated with attenuated colonization of *S. enterica* serovar Typhimurium in chicken alimentary tract (60).

A mutant with insertion in a gene whose product is 63% similar to a putative DNA-binding protein of *Yersinia pestis* with an HTH motif also was unable to colonize the URT. Many proteins with HTH motifs bind to the major groove of the DNA helix (36) and some, such as the VirB protein of *Shigella flexneri*, are key regulators of virulence gene expression (7). Given the low and comparable $CI_{in\ vitro}$ and $CI_{in\ vivo}$ (0.37 and 0.35), loss of this mutant may, however, have been a function of slow growth.

In this screen, mutants with insertions in three different genes related to the cell surface components (COG ID-M) were found to be colonization deficient. The first of these genes was an ortholog of the *pomA* gene of *Mannheimia hemolytica* (*ompA* gene in *E. coli*). Two independent colonization-deficient mutants carried mini-Tn*10* inserted at the 33rd bp of the $ompA_{A.s.}$ gene, immediately upstream of the sequence G**GCT**T**AGC**A. Growth of the *ompA* mutant, 23-58, was somewhat attenuated in vitro ($CI_{in\ vitro} = 0.5$) and more markedly attenuated in vivo ($CI_{in\ vivo} = 0.07$). The *ompA* gene of *A. suis* was cloned, and based on the entire gene sequence, $OmpA_{A.s.}$ was found to share 70% identity and 77% similarity with PomA of the bovine respiratory pathogen *M. hemolytica*. The OmpA protein of *E. coli*, which is the best characterized of these outer membrane proteins, is known to be vital for structural integrity of the cell membrane and for the generation of normal cell morphology. In addition, $OmpA_{E.c.}$ recognizes many ligands on eukaryotic target molecules, including glycoprotein motifs on human brain microvascular endothelial cells and is implicated in the invasion of these cells (39). The OmpA homolog of nontypeable *Haemophilus influenzae* (NTHi), OmpP5, plays an important role in the initiation of infection by mediating binding to the nasopharyngeal mucin-sialic acid oligosaccharides (41) and attachment to the epithelial cells of eustachian tubes (32). As well, attenuation due to a mutation in an OmpP5 homolog has also been observed in an STM screen of *A. pleuropneumoniae* (15). Further studies will be needed to determine whether the inability of the *A. suis ompA* mutant is due to a failure to bind to the tissues of the URT or an inability to survive the environment of the URT.

Another mutant that did not survive within porcine nasopharyngeal tissues had a mutation in a gene that shared 96% identity at the nucleotide level (but only 31 nucleotides) with the extreme 5′ end of the filamentous hemagglutinin (FHA) gene, *pfhaB1*, of *Pasteurella multocida*. The role of FHA in attachment of *Bordetella pertussis* to respiratory cilia has been extensively documented (58). In an STM screen of *P. multocida*, Fuller et al. (14) found an *fha* mutant that was markedly attenuated. In view of the fact that the region of homology was very short and there was no difference between the competitive indices in vitro and in vivo (0.17), caution is needed in speculating on its role in *A. suis* colonization at this point.

A mutant of *lcbB*, whose product might play a role in the biosynthesis of cell envelope carbohydrates, was also found to be colonization defective. In STM and other in vitro and in vivo infection studies (23, 46), mutants bearing transposon insertions in polysaccharide biosynthesis clusters have been reported to be unable to survive in the host. Alhough there have been several studies reporting the involvement of polysaccharides in adherence and colonization of members of the family *Pasteurellaceae* (23), given the limited homology, caution is also needed in interpreting the role of the *lcbB* mutant.

Two genes involved in amino acid transport and biosynthesis were identified in this screen. The first of these genes is a homolog of the *lmo0845* gene product of *Listeria monocytogenes*, which in turn, is a homolog of *yxjH* of *Bacillus subtilis*. In *B. subtilis*, this gene appears to function in sulfate assimilation and methionine biosynthesis and is a part of the S-box regulon (5). In this study, the growth of wild type *A. suis* was better in vivo compared to the *yxjH* mutant, while the mutant grew slightly better in vitro. A second mutant with an insertion in a gene encoding tryptophan-specific transport protein homolog grew equally well as the wild type when competed individually in vivo, so its role in colonization remains unclear.

Ten mutants with mutations in energy production and conversions (COG ID-C) were identified in the preliminary screens. Five transconjugants were lost during in vivo selection due to disruption of the pyruvate dehydrogenase (PDH) enzyme operon. In these strains, there were insertions in sequences that had high homology with the pyruvate dehydrogenase subunits, E1 (*aceE*) and E3 (*lpdA*) genes of *H. influenzae* and *P. multocida* respectively. The PDH cluster catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl coenzyme A, linking glycolysis with the tricarboxylic acid cycle (16). Its role in pathogenesis has been demonstrated in a number of species. For example, *lpdA* mutants of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* are avirulent in mice and rats, respectively (13, 50). In *S. enterica* serovar Typhimurium, the expression of *lpdA* is induced in response to the bactericidal proteins of neutrophils (40). In *A. pleuropneumoniae*, *lpdA* has also been identified as a virulence associated gene (15). Given the low and comparable $CI_{in\ vitro}$ and $CI_{in\ vivo}$ of the *lpdA* mutant (0.64 and 0.41) and very low $CI_{in\ vitro}$ of the *aceE* mutant (0.02), it is possible that mutation of PDH genes may have caused a global effect on the cell physiology, leading to the clearance of *A. suis* ST strains.

Disruption of the *atp* operon also gave rise to colonization-defective *A. suis* mutants. In strain 3-26, Tn*10* inserted in a gene homolog of *atpC*, while another attenuated mutant harbored the transposon in *atpG*, whose product is the gamma chain of ATP synthase. In in vivo competition assays, the wild-type *A. suis* was recovered in 15-fold-greater numbers than the *atpG* mutant, while in vitro, the mutant cells outnumbered the wild type ($CI_{in\ vitro} = 2.1$). ATP synthase gene products function in energy production and are crucial for main-

taining the electrical potential of the cell membrane and resistance to acid stress (44). Their role in virulence and survival has been shown in *S. enterica* serovar Typhimurium (17, 59), *Listeria monoctogenes* (44), *P. multocida* (14), and *A. pleuropneumoniae* (15). We speculate that the *atp* mutants of *A. suis* could not maintain cytoplasmic buffering in the face of oxidative and osmotic stresses in the respiratory tissue environments, though the reason for the robust growth of the *atpG* mutant and somewhat weaker growth of the *atpC* mutant in vitro is not obvious.

Three colonization-deficient mutants had Tn*10* insertions in the *iscU* gene, which is involved in iron-sulfur (Fe-S) assembly, an essential constituent of many apo-enzymes. As well, the *isc* operon has a critical role in sensing intracellular iron and oxidant levels (57). Agar and coworkers have shown that the IscU protein provides a scaffold for IscS-directed assembly of clusters, which are then used for the maturation of apo-Fe-S proteins (1). In the current study, an *iscU* mutant was found to be severely attenuated in vivo (CI = 0.001).

The *est* and *ptsH* gene products are known to have a role in virulence (6, 18, 64). In this study, *A. suis* mutants carrying Tn*10* within the *est* and *ptsH* genes were eliminated; however, their attenuation was not verified individually and their in vitro CIs were less than 1.

Mutants with mutations of three nucleotide metabolism genes (COG ID-F) were eliminated from the URT in initial screens. A 10-fold reduction in growth of a *guaA* mutant was seen in the in vivo competition assay, while in BHI medium, the mutant outcompeted the parent strain. The enzyme encoded by *guaA*, GMP synthase, is involved in the terminal steps of the purine biosynthetic pathway, where it converts XMP to GMP (33). Attenuation due to mutation in the *guaAB* operon has been reported in members of *Enterobacteriaceae* (33), as well as in *P. multocida* and *A. pleuropneumoniae* (14, 46), and a number of these auxotrophic mutants have been proposed for use as live attenuated vaccines.

A *thyA* mutant was also found to be colonization deficient, but its growth in vitro was also much less than that of the parental strain. *thyA*-deficient strains are unable to produce dTTP for DNA synthesis and can grow on minimal medium only in the presence of thymine or thymidine (2). The *thyA* gene is reported to be critical for the survival under these conditions, while deficiency of many other metabolic genes may only cause biostasis (2). In *Shigella flexneri*, mutation of the *thyA* gene leads to greatly reduced virulence (11).

Three independent *nrdA* mutants were colonization defective. The *nrdA* gene encodes ribonucleotide reductase, which catalyzes conversion of ribonucleotides to the corresponding deoxyribonucleotides for DNA synthesis and repair (9) and is important for survival under oxidative stress (49). In BHI medium, the mutant grew less well than the wild type ($CI_{in\ vitro}$ = 0.36) and since its in vivo survival was not compared with the wild type, its role in colonization of *A. suis* has not been confirmed.

Gene products related to translation processing (COG ID-J) are fundamental for growth and stability of an organism and are needed in high concentrations (25). As rapid growth is a prerequisite for colonization (31, 55), proteins must be synthesized at a high rate. In addition, there are reports of further functions of tRNA genes in peptidoglycan synthesis (53), post-translational addition of amino acids to proteins (29), and interaction with the host (21). In this study, a number of colonization-deficient mutants had Tn*10* insertions in genes related to protein synthesis, including those for tRNA-glycine 3 and tRNA-tyrosine 1. In competition assays, the tRNA-Gly3 mutant was recovered in much lower numbers from the host ($CI_{in\ vivo}$ = 0.02), while it grew equally well as the wild type in vitro ($CI_{in\ vitro}$ = 1.2). In contrast, the tRNA-Tyr1 mutant was equally attenuated in both in vivo and in vitro competition assays. In an STM study of *A. pleuropneumoniae*, mutants of tRNA genes, tRNA-Leu and tRNA-Glu were found to be attenuated (15). The elongation factors (TuA and TuB) and ribosomal protein genes were also identified in this screen; however, their attenuation was not confirmed separately in vivo.

In these experiments, a large number of ST mutants with insertions in the 16S and 23S rRNA genes appeared to be colonization deficient. The *E. coli* genome carries seven rRNA (*rrn*) operons, *P. multocida* has six, and *A. suis* possesses at least four 16S rRNA operons (based on Southern hybridization using a 16S rRNA gene probe; data not shown). Asai and coworkers showed that *E. coli* requires all of the operons for optimal adaptation to changing physiological conditions (4). These operons are under the control of growth rate-dependent promoters, and expression of RPs and translation factors are linked to the cellular concentration of rRNA (55). These reports indicate a selective advantage for the growing organism, and deletion of rRNA gene might have adverse effect, especially under in vivo conditions. However, when the 16S rRNA mutant 3-45 was competed with the wild type, it was recovered at a higher rate from the host than the wild type ($CI_{in\ vivo}$ = 2.3), despite the fact that its $CI_{in\ vitro}$ was somewhat less than 1. Although it is hard to reconcile this finding, when CIs are done with single mutants such results are not uncommon (e.g., references 19 and 46). Also surprisingly, the $CI_{in\ vitro}$ of the 23S rRNA mutant 3-44 was 2.0. The loss of a very large number of *A. suis* rRNA mutants with inserts in several different sites suggests the need for further analysis of the role of ribosomal genes in pathogenesis.

Although the genome sequencing of many pathogens has been completed, the functions of a large fraction (~ 40%) of the predicted genes are unknown (COG ID-S). In STM screens, one or more hypothetical genes are often reported as having a role in virulence (37). In the current study, mutants of four hypothetical genes were identified as colonization defective (Table 2). Of these four mutants, one (PM1911) was markedly attenuated in vivo ($CI_{in\ vivo}$ = 0.01). Although the $CI_{in\ vivo}$ of mutant 17-66, which did not share any similarity with any known gene was not determined, its $CI_{in\ vitro}$ was greater than 1.

In this study, four of the mutants grew as well as or better than the wild type when competed individually in vivo, these mutants do not appear to be essential for *A. suis* colonization (i.e., those bearing Tn*10* insertions in the tryptophan transport gene homolog, two unknowns, and in a 16SrRNA gene); and three had similar in vivo and in vitro CIs (i.e., *lcbB*, *pfhaB1*, and a global regulator gene) (Table 2). These mutants were, however, eliminated in both animals in two screens (i.e., in four animals) and, except for the unknowns, their role in the pathogenesis is known in other pathogens (7, 23). The fact that

$CI_{in\ vivo}$ values do not reflect the results of the initial screen is reported in many STM studies (e.g., references 19 and 46), which points to a weakness of the STM method.

In the present study, mutants carrying insertions in the metabolic genes *estA*, *ptsH*, and *nrdA* genes and the mutants with Tn*10* in the genes associated in the translation process, such as elongation factor genes *tufA* and *tufB*, and ribosomal protein genes *rpl9* and *rpl32*, were also lost. These are all known to be highly expressed genes that play an important role in the survival and virulence of several different pathogens (12, 35, 51, 65), but they were not competed with the wild type individually, so the nature of their colonization defect remains to be confirmed. Although the $CI_{in\ vitro}$ of *tufA*, *tufB*, and *rpl32* was less than 1, the *rpl9* mutant was clearly not attenuated in vitro.

To our knowledge, this is the first report of transposon mutagenesis of *A. suis*. For these experiments, 22 uniquely tagged Tn*10* transposons that could be amplified by PCR were constructed. Although the problem of "hot spots" is acknowledged, these transposons should be useful for the study of other *Pasteurellaceae*. As well, a nasopharyngeal colonization challenge system was developed that, again, should be useful for the study of a variety of organisms. Using this challenge system, we were able to demonstrate for the first time that the principal colonization sites of *A. suis* in swine were likely the nasopharynx and lingual and palatine tonsils.

In the current STM screen, 28 genes belonging to 9 different COGs were identified that appeared to be essential for *A. suis* colonization; 13 were later confirmed to be colonization defective in CI experiments. The homologs of many of these genes, e.g., genes of the *atp* and PTS complexes, *ompA*, *pfhaB1*, *lcbB*, *hupA*, *hns*, *tufA*, tRNA genes, *lpdA*, and *guaA* have also been identified in genome-wide screens of other mucosal organisms, and some of these have been well established as virulence genes of important pathogens (14, 15, 22, 46, 54). The response regulator *cpxR* was identified for the first time in an STM screen. In addition to *cpxR*, *nrdA*, and *nifU*, four unknown genes and the ribosomal genes *rpl9* and *rpl32* were identified for the first time in a genome-wide virulence screen. Experiments to identify the receptors for, and/or the precise function of, some of these colonization factors are currently under way.

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REFERENCES

1. **Agar, J. N., C. Krebs, J. Frazzon, B. H. Huynh, D. R. Dean, and M. K. Johnson.** 2000. IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] cluster in IscU. Biochemistry **39:**7856–7862.
2. **Ahmad, S. I., S. H. Kirk, and A. Eisenstark.** 1998. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annu. Rev. Microbiol. **52:**591–625.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **15:**403–410.
4. **Asai, T., C. Condon, J. Voulgaris, D. Zaporojets, B. Shen, M. Al-Omar, C. Squires, and C. L. Squires.** 1999. Construction and initial characterization of *Escherichia coli* strains with few or no intact chromosomal rRNA operons. J. Bacteriol. **181:**3803–3809.
5. **Auger, S., A. Danchin, and I. Martin-Verstraete.** 2002. Global expression profile of *Bacillus subtilis* growth in the presence of sulfate or methionine. J. Bacteriol. **184:**5179–5186.
6. **Behari, J., and P. Youngman.** 1998. Regulation of *hly* expression in *Listeria monocytogenes* by carbon sources and pH occurs through separate mechanisms mediated by PrfA. Infect. Immun. **66:**3635–3642.
7. **Beloin, C., S. McKenna, and C. J. Dorman.** 2002. Molecular dissection of VirB, a key regulator of the virulence cascade of *Shigella flexneri*. J. Biol. Chem. **277:**15333–15344.
8. **Bender, J., and N. Kleckner.** 1992. Tn*10* insertion specificity is strongly dependent upon sequences immediately adjacent to the target-site consensus sequence. Proc. Natl. Acad. Sci. USA **89:**7996–8000.
9. **Borovok, I., R. Kreisberg-Zakarin, M. Yanko, R. Schreiber, G. Cohen, and Y. Aharonowitz.** 2002. *Streptomyces* spp. contain Ia and class II ribonucleotide reductases: expression analysis of the genes in vegetative growth. Microbiology **148:**391–404.
10. **Bossé, J. T., and J. I. MacInnes.** 1997. Genetic and biochemical analyses of *Actinobacillus pleuropneumoniae* urease. Infect. Immun. **65:**4389–4394.
11. **Cersini, A., A. M. Salvia, and M. L. Bernardini.** 1998. Intracellular multiplication and virulence of *Shigella flexneri* auxotrophic mutants. Infect. Immun. **66:**549–557.
12. **Cordeiro-da Silva, A., M. C. Borges, E. Guilvard, and A. Ouaissi.** 2001. Dual role of the *Leishmania major* ribosomal protein S3a homologue in regulation of T- and B-cell activation. Infect. Immun. **69:**6588–6596.
13. **Dacheux, D., O. Epaulard, A. de Groot, B. Guery, R. Leberre, I. Attree, B. Polack, and B. Toussaint.** 2002. Activation of the *Pseudomonas aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase *aceAB* operon. Infect. Immun. **70:**3973–3977.
14. **Fuller, T. E., M. J. Kennedy, and D. E. Lowery.** 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb. Pathog. **29:**25–38.
15. **Fuller, T. E., S. Martin, J. F. Teel, G. R. Alaniz, M. J. Kennedy, and D. E. Lowery.** 2000. Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. Microb. Pathog. **29:**39–51.
16. **Gao, H., X. Jiang, K. Pogliano, and A. I. Aronson.** 2002. The E1β and E2 subunits of the *Bacillus subtilis* pyruvate dehydrogenase complex are involved in regulation of sporulation. J. Bacteriol. **184:**2780–2788.
17. **Garcia del Portillo, F., J. W. Foster, and B. B. Finlay.** 1993. Role of acid tolerance response genes in *Salmonella typhimurium* virulence. Infect. Immun. **61:**4489–4492.
18. **Groisman, E. A., and M. H. J. Saier.** 1990. *Salmonella* virulence: new clues to intramacrophage survival. Trends Biochem. Sci. **15:**30–33.
19. **Harper, M., J. D. Boyce, I. W. Wilkie, and B. Adler.** 2003. Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. Infect. Immun. **71:**5440–5446.
20. **Harrison, J. A., D. Pikard, C. F. Higgins, A. Khan, S. N. Chatfield, T. Ali, C. J. Dorman, C. E. Hormaeche, and G. Dougan.** 1994. Role of *hns* in the virulence phenotype of pathogenic salmonellae. Mol. Microbiol. **13:**133–140.
21. **Hromockyj, A. E., S. C. Tucker, and A. T. Maurelli.** 1992. Temperature regulation of *Shigella* virulence: identification of the repressor gene *virR*, an analogue of *hns*, and partial complementation by tyrosyl transfer RNA (tRNA1 (Tyr)). Mol. Microbiol. **6:**2113–2124.
22. **Hung, D. L., T. L. Raivio, C. H. Jones, T. J. Silhavy, and S. J. Hultgren.** 2001. Cpx signalling pathway monitors biogenesis and affects assembly and expression of P pili. EMBO J. **20:**1508–1518.
23. **Jacques, M., and S. E. Paradis.** 1998. Adhesin-receptor interactions in *Pasteurellaceae*. FEMS Microbiol. Rev. **22:**45–59.
24. **Jeannotte, M.-E., D. Slavic, J. Frey, P. Kuhnert, and J. I. MacInnes.** 2002. Analysis of non-porcine strains of *Actinobacillus suis*. Vet. Microbiol. **85:**83–93.
25. **Karlin, S., and J. Mrazek.** 2000. Predicted highly expressed genes of diverse prokaryotic genomes. J. Bacteriol. **182:**5238–5250.
26. **Karlyshev, A. V., P. C. F. Oyston, K. Williams, G. C. Clark, R. W. Titball, E. A. Winzeler, and B. W. Wren.** 2001. Application of high-density array-based signature-tagged mutagenesis to discover novel *Yersinia* virulence-associated genes. Infect. Immun. **69:**7810–7819.
27. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
28. **Lehoux, D. E., F. Sanschagrin, and R. C. Levesque.** 1999. Defined oligonucleotide tag pools and PCR screening in signature-tagged mutagenesis of essential genes from bacteria. BioTechniques **26:**473–480.
29. **Leibowitz, M. J., and R. L. Soffer.** 1969. A soluble enzyme from *Escherichia coli* which catalyzes the transfer of leucine and phenylalanine from tRNA to acceptor proteins. Biochem. Biophys. Res. Commun. **36:**47–53.
30. **MacInnes, J. I., and E. T. Lally.** 2004. The genus *Actinobacillus. In* W. Dworkin et al. (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed. Springer-Verlag, New York, N.Y. [Online.] http://link.springer-ny.com/link/service/books/10125/.

31. **Marcotte, H., and M. C. Lavoie.** 1998. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol. Mol. Biol. Rev. **62:**71–109.
32. **Miyamoto, N., and L. O. Bakaltez.** 1996. Selective adherence of non-typeable *Haemophilus influenzae* (NTHi) to mucus or epithelial cells in chinchilla eustachian tube and middle ear. Microb. Pathog. **21:**343–356.
33. **Noriega, F. R., G. Losonsky, C. Lauderbaugh, F. M. Liao, J. Y. Wang, and M. M. Levine.** 1996. Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a strain CVD 1205: construction, safety, immunogenicity, and potential efficacy as a mucosal vaccine. Infect. Immun. **64:**3055–3061.
34. **Pagotto, F. J., H. Salimnia, P. A. Totten, and J. R. Dillon.** 2000. Stable shuttle vectors for *Neisseria gonorrhoeae*, *Haemophilus* spp. and other bacteria based on a single origin of replication. Gene **244:**13–19.
35. **Paustian, M. L., B. J. May, and V. Kapur.** 2002. Transcriptional response of *Pasteurella multocida* to nutrient limitation. J. Bacteriol. **184:**3734–3739.
36. **Perez-Martin, J., and Victor de Lorenzo.** 1997. Clues and consequences of DNA bending in transcription. Annu. Rev. Microbiol. **51:**593–628.
37. **Perry, R. D.** 1999. Signature-tagged mutagenesis and the hunt for virulence factors. Trends Microbiol. **7:**385–388.
38. **Phillips, J. E.** 1984. Genus III. *Actinobacillus* Brumpt 1910, 849^{AL}, p. 570–575. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. I. Williams and Wilkins, Baltimore, Md.
39. **Prasadarao, N. V.** 2002. Identification of *Escherichia coli* outer membrane protein A receptor on human brain microvascular endothelial cells. Infect. Immun. **70:**4556–4563.
40. **Qi, S. Y., Y. Li, A. Szyroki, I. G. Giles, A. Moir, and C. D. O'Connor.** 1995. Salmonella typhimurium responses to a bactericidal protein from human neutrophils. Mol. Microbiol. **17:**523–531.
41. **Reddy, M. S., J. M. Bernstein, T. F. Murphy, and H. S. Faden.** 1996. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. Infect. Immun. **64:**1477–1479.
42. **Rycroft, A. N., and L. H. Garside.** 2000. *Actinobacillus* species and their role in animal disease. Vet. J. **159:**18–36.
43. **Schroder, O., and R. Wagner.** 2000. The bacterial DNA-binding protein H-NS represses ribosomal RNA transcription by trapping RNA polymerase in the initiation complex. J. Mol. Biol. **298:**737–748.
44. **Shabala, L., B. Budde, T. Ross, H. Siegumfeldt, M. Jakobsen, and T. McMeekin.** 2002. Responses of *Listeria monocytogenes* to acid stress and glucose availability revealed by a novel combination of fluorescence microscopy and microelectrode ion-selective techniques. Appl. Environ. Microbiol. **68:**1794–1802.
45. **Shea, J. E., J. D. Santangelo, and R. G. Feldman.** 2000. Signature-tagged mutagenesis in the identification of virulence genes in pathogens. Curr. Opin. Microbiol. **3:**451–458.
46. **Sheehan, B. J., J. T. Bossé, A. J. Beddek, A. N. Rycroft, J. S. Kroll, and P. L. Langford.** 2003. Identification of *Actinobacillus pleuropneumoniae* genes important for survival during infection in its natural host. Infect. Immun. **71:**3960–3970.
47. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilisation system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
48. **Slavic, D., J. DeLay, M. A. Hayes, and J. I. MacInnes.** 2000. Comparative pathogenicity of different *Actinobacillus suis* O/K serotypes. Can. J. Vet. Res. **64:**81–87.
49. **Smalley, D., E. R. Rocha, and C. J. Smith.** 2002. Aerobic-type ribonucleotide reductase in the anaerobe *Bacteroides fragilis*. J. Bacteriol. **184:**895–903.
50. **Smith, A. W., H. Roche, M. Trombe, D. E. Briles, and A. Hakansson.** 2002. Characterization of the dihydrolipoamide dehydrogenase from *Streptococcus pneumoniae* and its role in pneumococcal infection. Mol. Microbiol. **44:**431–448.
51. **Spence, J. M., and V. L. Clark.** 2000. Role of ribosomal protein L12 in gonococcal invasion of Hec1B. Cells **68:**5002–5010.
52. **Spurio, R., M. Durrenberger, M. Falconi, A. La Teana, C. L. Pon, and C. O. Gualerzi.** 1992. Lethal overproduction of the *Escherichia coli* nucleoid protein H-NS: ultramicroscopic and molecular autopsy. Mol. Gen. Genet. **231:**201–211.
53. **Stewart, T. S., R. J. Roberts, and J. L. Strominger.** 1971. Novel species of tRNA. Nature **230:**36–38.
54. **Struve, C., C. Forestier, and K. A. Krogfelt.** 2003. Application of a novel multi-screening signature-tagged mutagenesis assay for identification of *Klebsiella pneumoniae* genes essential in colonization and infection. Microbiology **149:**167–176.
55. **Tao, H., C. Bausch, C. Richmond, F. R. Blattner, and T. Conway.** 1999. Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. J. Bacteriol. **181:**6425–6440.
56. **Tascon, R. I., E. F. Rodriguez-Ferri, C. B. Gutierrez-Martin, I. Rodriguez-Barbosa, P. Berche, and J. A. Vazquez-Boland.** 1993. Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn*10* derivative. J. Bacteriol. **175:**5717–5722.
57. **Tong, W.-H., and T. Rouault.** 2000. Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. **19:**5692–5700.
58. **Toumanen, E., A. Weiss, R. Rich, F. Zak, and O. Zak.** 1986. Filamentous hemagglutinin and pertussis toxin promote adherence of *Bordetella pertussis* to cilia. Dev. Biol. Stand. **61:**197–204.
59. **Turner, A. K., L. Z. Barber, P. Wigley, S. Muhammad, M. A. Jones, M. A. Lovell, S. Hulme, and P. A. Barrow.** 2003. Contribution of proton-translocating proteins to the virulence of *Salmonella enterica* serovars Typhimurium, Gallinarum, and Dublin in chickens and mice. Infect. Immun. **71:**3392–3401.
60. **Turner, A. K., M. A. Lovell, S. D. Hulme, L. Zhang-Barber, and P. A. Barrow.** 1998. Identification of *Salmonella typhimurium* genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. **66:**2099–2106.
61. **Unsworth, K. E., and D. W. Holden.** 2000. Identification and analysis of bacterial virulence genes in vivo. Phil. Trans. R. Soc. Lond. **355:**613–622.
62. **van Ostaaijen, J., J. Frey, S. Rosendal, and J. I. MacInnes.** 1997. *Actinobacillus suis* strains isolated from healthy and diseased swine are clonal and carry *apxICABD*$_{\text{var. suis}}$ and *apxIICA*$_{\text{var. suis}}$ toxin genes. J. Clin. Microbiol. **35:**1131–1137.
63. **White-Ziegler, C. A., A. Villapakkam, K. Ronaszeki, and S. Young.** 2000. H-NS controls *pap* and *daa* fimbrial transcription in *Escherichia coli* in response to multiple environmental cues. J. Bacteriol. **182:**6391–6400.
64. **Wilhelm, S., J. Tommassen, and K.-E. Jaeger.** 1999. A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. J. Bacteriol. **181:**6977–6986.
65. **Wool, I. G.** 1996. Extraribosomal functions of ribosomal proteins. Trends Biochem. Sci. **21:**164–165.

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